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Improved Pyrogallol Autoxidation Method: A Reliable and Cheap Superoxide-Scavenging Assay Suitable for All Antioxidants

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Supporting Information

ABSTRACT: The original pyrogallol (1,2,3-trihydroxybenzene) method, which was developed specifically for superoxide dismutase, is now widely used for measuring superoxide-scavenging of other antioxidants. However, the strong pH effect has been ignored. In this study, the influencing factors have been systematically investigated for the first time, and a number of experiments have proved that the pH is of major importance. As major antioxidants contain carboxylic acid, ester, or lactone groups, pH 8.2 should be modified to physiological pH 7.4. The improved procedure is as follows. A pyrogallol solution (in 1 M HCl) is thoroughly mixed with pH 7.4 Tris-HCl buffer; $A_{325\ nm}$ is measured every 30 s for 5 min at 37 °C. As the $\Delta A_{325\ nm,\ control}$ value reflects the initial concentration of substrate $^{\bullet}O_2^-$, it should be well controlled to guarantee the accuracy of the method. The improved pyrogallol method is a reliable and cheap superoxide-scavenging assay suitable for all types of antioxidants.

KEYWORDS: *pyrogallol autoxidation, antioxidant, superoxide-scavenging, $^{\bullet}O_2^-$, Marklund method*

INTRODUCTION

There are several methods for determining the superoxide-scavenging activities of foods, including cytochrome *c* reduction,[1] nitrotetrazolium blue chloride (NBT),[2] electron spin resonance (ESR),[1] chemiluminescence,[1] fluorescence,[1] and high-performance liquid chromatography.[3] All of these methods require special and expensive instruments or biological agents. The pyrogallol (1,2,3-trihydroxybenzene) autoxidation method is relatively cheap. It was originally designed by Marklund specifically for superoxide dismutase (SOD), not for other antioxidants.[4] In recent decades, because of its convenience, it has also been used in the determination of other antioxidants such as polyphenols,[5] phenolic acids,[6] tannins,[7] flavonoids,[8] anthocyanins,[9] anthraquinones,[10] polysaccharides,[11,12] and even various nutritional additives[13,14] and extracts.[15]

Most of these antioxidants contain carboxylic acid (−COOH), ester, or lactone groups, which are sensitive to alkaline solutions. The applicability of the original pyrogallol method for such antioxidants is therefore seriously questioned in this study.

In this paper, the factors (especially the pH) affecting the pyrogallol method are investigated and then an improved method is described.

MATERIALS AND METHODS

Chemicals. Pyrogallol (98%), Trolox (97%), melatonin (98%), epigallocatechin gallate (EGCG, 97%), 2,6-di-*tert*-butyl-4-methylphenol (BHT, 99%), (+)-catechin, (±)-α-tocopherol (96%), protocatechuic acid (97%), *p*-coumaric acid (97%), sinapic acid (97%), isoferulic acid (97%), and L-methionine (98%) were obtained from the Sigma-Aldrich Shanghai Trading Co. (Shanghai, China); GSH (glutathione, 98%), β-carotene (98%), *p*-nitroblue tetrazolium chloride (NBT, 98%), and riboflavin were obtained from Amresco Inc. (Solon, OH, USA); laminarin was obtained from the Aladdin Chemistry Co. (Shanghai, China); chlorogenic (98%), ferulic (98%), vanillic (98%), and caffeic acids (98%), rhein (98%), emodin (98%), aloeemodin (98%), resveratrol (98%), and magnolol (98%) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); SOD freeze-dried powder was kindly donated by the Gaungzhou Qibao Biological Co. (Gaungzhou, China), and the 8-hydroxyquinoline derivative was kindly donated by Prof. H. P. Zeng (South China University of Technology); ethyl acetate extract from *Garcinia mangostana* hulls (EAGM) was extracted using a Soxhlet extractor with ethyl acetate in our laboratory. All other reagents were of analytical grade.

Effects of Monitoring Wavelength. Tris-HCl buffer (0.05 M, pH 8.2; 2950 μL) containing 1 mM Na_2EDTA was placed in a quartz cuvette. Then 50 μL of pyrogallol solution (12 mM in 1 mM HCl) was added to the buffer. Immediately after the mixture was shaken vigorously, the reaction mixture was repeatedly scanned (the intervals varied from 1 to 2 min), using a UV−visible spectrophotometer (Techcomp, Shanghai, China), from 200 to 700 nm at room temperature (23.8 °C). The measurement was repeated in pH 7.4 Tris-HCl buffer using a 60 mM pyrogallol solution (in 1 mM HCl).

Effects of pH Value. The reaction mixture was prepared as described above. Immediately, the reaction mixture was monitored, using a UV−visible spectrophotometer, at 325 nm for 12 h. This procedure was repeated in different Tris-HCl buffers, with pH values of 7.0, 7.4, 7.8, 8.6, and 9.0, to investigate the effects of the pH.

Effects of Temperature. At pH 7.4, 2950 μL of Tris-HCl buffer (0.05 M, pH 7.4, 17 °C) containing 1 mM Na_2EDTA was placed in a quartz cuvette. Then 50 μL of pyrogallol solution (60 mM in 1 mM HCl, 17 °C) was added to the buffer. Immediately after the mixture was vigorously shaken, the absorbance of the mixture was measured at 325 nm every 30 s for 5 min at 17 °C. This experimental procedure was repeated at 27, 37, and 47 °C to observe the effects of temperature at pH 7.4.

The procedure was repeated, using a 12 mM pyrogallol solution, to observe the effects of temperature at pH 8.2.

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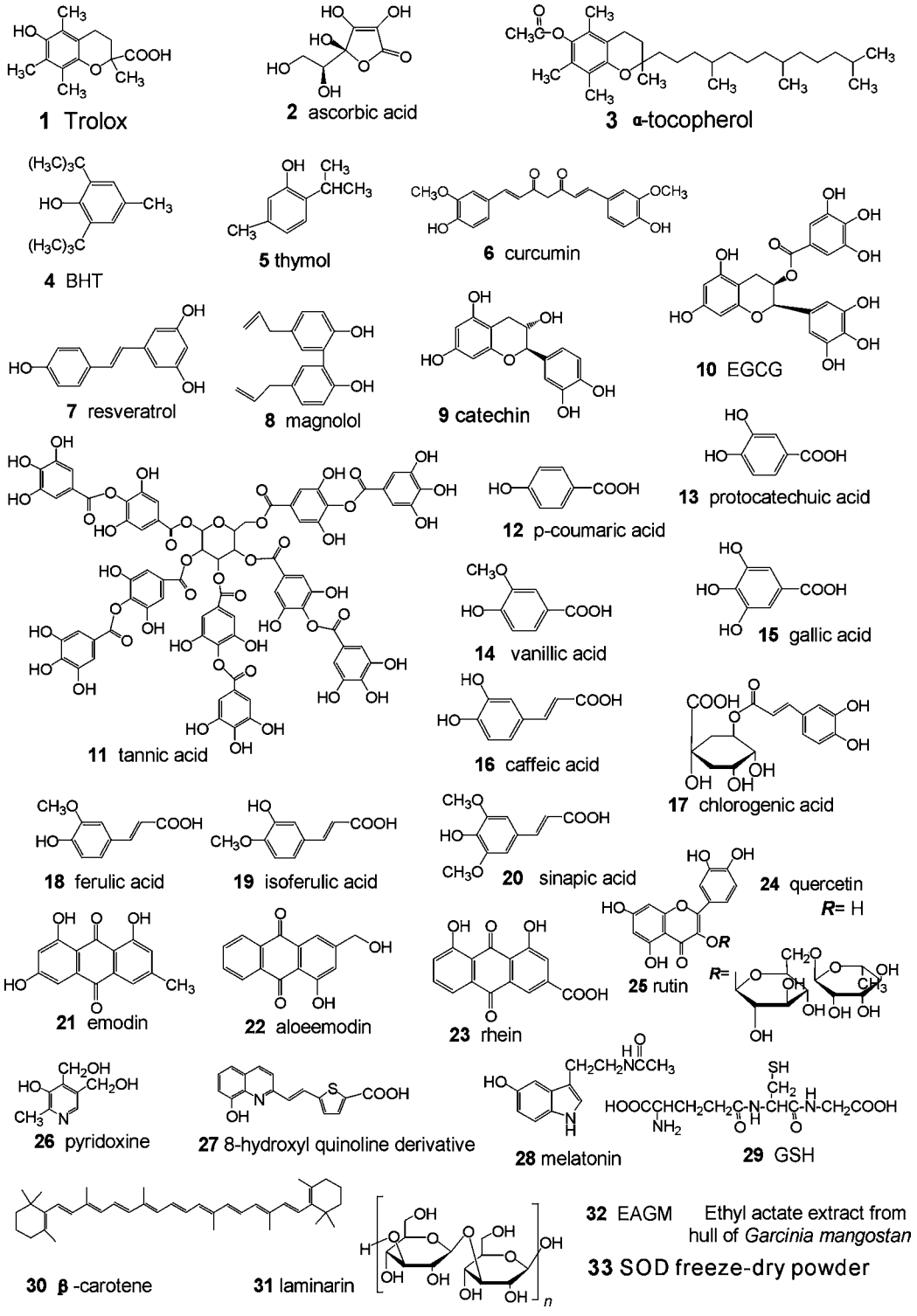

Figure 1. Structures of 33 selected antioxidants.

Effects of Pyrogallol Concentration. After x μL of pyrogallol solution (60 mM pyrogallol in 1 mM HCl; x = 20, 40, 60, 80, 100, 120, and 240) had been thoroughly mixed with $(3000 - x)$ μL of Tris-HCl buffer (0.05 M, pH 7.4) containing 1 mM Na_2EDTA, the absorbance of the mixture at 325 nm was measured every 30 s for 5 min at 37 °C to observe the effects of pyrogallol concentration at pH 7.4.

The procedure was repeated at pH 8.2, using 12 mM pyrogallol, to observe the effects of pyrogallol concentration at this pH.

Comparison of $^{\bullet}O_2^-$ Radical-Scavenging Activities of 33 Selected Antioxidants, at pH 7.4 and pH 8.2. To further investigate the effects of pH, the $^{\bullet}O_2^-$ radical-scavenging activities at pH 7.4 and 8.2 of 33 selected antioxidants, including SOD (Figure 1), were measured.

In the experiments, the Tris-HCl buffer for analyzing BHT, thymol, curcumin, and β-carotene was prepared using n-butanol-saturated water; the others were prepared using distilled water (Figure 1).

The detailed experimental protocol was as follows. At pH 7.4, x μL of sample solution was mixed with $(2950 - x)$ μL of Tris-HCl buffer (0.05 M, pH 7.4, 37 °C) containing 1 mM Na_2EDTA and 50 μL of pyrogallol (60 mM in 1 mM HCl, 37 °C) and then rapidly shaken by hand at 37 °C.

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The value of $A_{325\,nm}$ was measured against the Tris-HCl buffer every 30 s for 5 min. The $^{\bullet}O_2^-$-scavenging ability was calculated as

$$\left(\frac{\Delta A_{325nm,\,control}}{T} - \frac{\Delta A_{325nm,\,sample}}{T}\right) \Big/ \frac{\Delta A_{325nm,\,control}}{T} \times 100\%$$

Here, $\Delta A_{325\,nm,\,control}$ is the increase in $A_{325\,nm}$ of the mixture without the sample and $\Delta A_{325\,nm,\,sample}$ is that for the mixture with the sample; $T = 5$ min.

The experimental protocol was repeated at pH 8.2, using a pyrogallol concentration of 12 mM.

In the comparative measurements, the value of $\Delta A_{325\,nm,\,control}/T$ at pH 8.2 was equal to that at pH 7.4.

Validation of the Improved Method (Comparison with the Original Method and NBT Method). Validation of the improved pyrogallol method was carried out according to FDA guidelines,[16] with regard to linearity, sensitivity, precision, and reproducibility. Validation of the original method and the NBT method was also carried out for comparison. In these tests, Trolox was used as the reference compound.

The improved pyrogallol method was performed as described above. Briefly, at pH 7.4, x μL of Trolox solution was mixed with $(2950 - x)$ μL of pH 7.4 Tris-HCl buffer (0.05 M, 37 °C) containing Na_2EDTA (1 mM) and 50 μL of pyrogallol (60 mM in 1 mM HCl, 37 °C) and then shaken rapidly at 37 °C. Then $\Delta A_{325\,nm}$ was determined, and the $^{\bullet}O_2^-$-scavenging ability was calculated as described above.

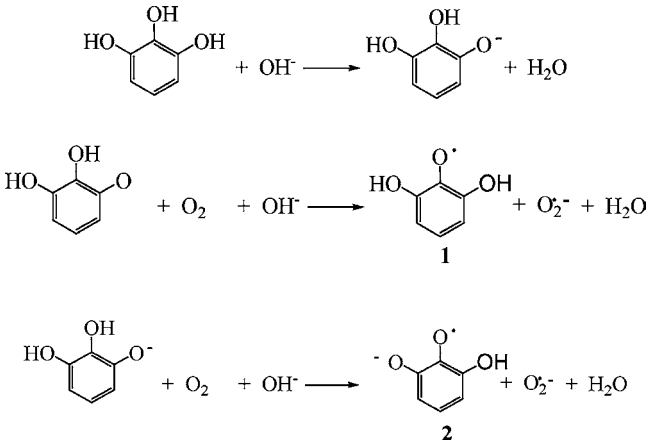
The original pyrogallol method was performed as described by Marklund.[4] However, the pH value was adjusted to 8.2, the temperature was room temperature, and the monitoring wavelength was 420 nm.

The NBT method we used was described by Siddhuraju.[2] Trolox solution was prepared in 0.05 M KH_2PO_4–KOH buffer (pH 7.4). The photoinduced reactions were performed using 26 W screwed fluorescent lamps. The final concentration of Trolox in the reaction mixture was 0, 125, 250, 500, 750, 1000, or 1250 μg/mL. The total volume of the reaction mixture was 1 mL, and the final concentrations of riboflavin, methionine, and NBT were 3.0×10^{-3}, 6.0, and 0.1 mM, respectively. The reactant was illuminated for 8 min, and then its absorbance was measured at 560 nm. An unilluminated reaction mixture was used as a blank. The degree of scavenging was calculated using the following equation: scavenging (%) = $[(A_0 - A)/A_0] \times 100$. A_0 was the absorbance of the reaction mixture without Trolox, and A was that with Trolox.

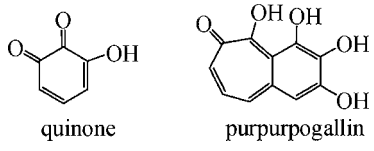
Statistical Analysis. When the pyrogallol antioxidant assay was used for the 33 selected antioxidants, the experiments were performed in triplicate, and the data were recorded as the mean ± SD. The IC_{50} value was defined as the concentration for 50% superoxide free radical inhibition and was calculated by linear regression and analyzed using Origin 6.0 professional software (OriginLab Corp., Northampton, MA, USA). Determination of significant differences between the mean IC_{50} values at pH 7.4 and 8.2 was performed by using the t test ($p < 0.05$). The analysis was performed by using SPSS software (v. 12, SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Pyrogallol can autoxidize in alkaline solutions to produce $^{\bullet}O_2^-$ anion radicals; the complicated mechanism can be briefly described as follows:[17−22]



The semiquinones **1** and **2** could react to form purpurogallin via quinone:



Purpurogallin, which has extensive $\pi-\pi$ conjugation, is easily detected by a spectrophotometer, so the absorbance reflects the generation of both purpurogallin and superoxide radicals ($^{\bullet}O_2^-$). Obviously, a lower absorbance indicates higher inhibition of $^{\bullet}O_2^-$. This is the principle of the pyrogallol autoxidation method, which was originally designed by Marklund, specifically for SOD.[4]

In earlier studies,[4,23,24] 420 nm was selected as the monitoring wavelength for $^{\bullet}O_2^-$ generation. However, the UV spectra at pH 7.4 and 8.2 proved that 325 nm was much more sensitive than 420 nm (Figure 2). In this study, 325 nm was therefore regarded as the best monitoring wavelength for detecting concentrations of $^{\bullet}O_2^-$.

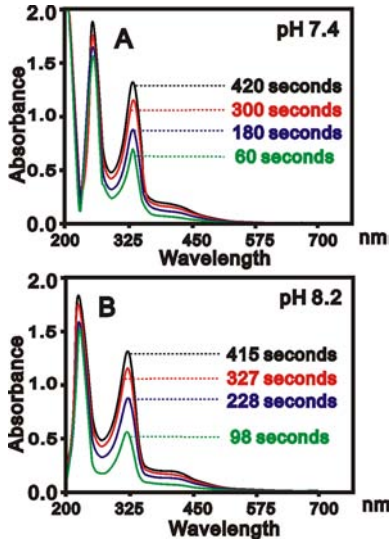


Figure 2. UV spectra of pyrogallol autoxidation at different reaction times (23.8 °C): (A) at pH 7.4, the pyrogallol concentration was 1.00 mM; (B) at pH 8.2, the pyrogallol concentration was 0.20 mM.

Under monitoring at 325 nm, it was observed that $^{\bullet}O_2^-$ generation always rapidly increased in the initial stage, decreased after a maximum, and then reached a steady state. However, at different pH values, the initial increasing stage varied from 12 min to 7 h (Figure 3A). Of course, only a linear ($R \geq 0.998$) increase is suitable for determining $^{\bullet}O_2^-$ radical-scavenging activity. At physiological pH 7.4, the linear ($R \geq 0.998$) increase occurred within 0−30 min, whereas it occurred within 0−8 min at pH 8.2 (Figure 3B). Therefore, physiological pH 7.4 is more suitable and flexible than pH 8.2, which was previously used for determining $^{\bullet}O_2^-$ radical-scavenging activity.

Investigation of temperature effects (see Supporting Information, file 1) indicated that the $\Delta A_{325\,nm}$ values for pyrogallol autoxidation were different at 17, 27, 37, and 47 °C. In general, at lower temperatures (17 and 27 °C), pyrogallol autoxidation

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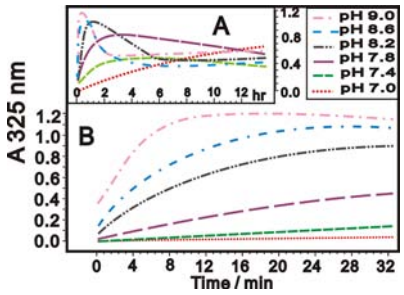

Figure 3. Kinetics curve of 0.20 mM pyrogallol autoxidation at different pH values (32 °C).

accelerated with increasing temperature, so the $\Delta A_{325\ nm}$ value increased. However, at higher temperatures (37 and 47 °C), an increase in temperature had an extreme effect: pyrogallol autoxidation was nearly complete within the first few seconds. In addition, the inherent changes in the pK_a values of the buffer resulting from higher temperatures might lead to the decrease in $\Delta A_{325\ nm}$ values. Therefore, the $\Delta A_{325\ nm}$ values decreased at 37 and 47 °C. Nevertheless, the physiological temperature of 37 °C was considered to be the best temperature for the assay.

In terms of chemical kinetics, the reaction rate always increases with reactant concentration, but not necessarily linearly. Our data (see Supporting Information, file 2) demonstrated that there were good linear relationships (R = 0.970−0.999) between the $A_{325\ nm}$ value and reaction time for pyrogallol concentrations of 0.077−0.92 mM at pH 8.2. At pH 7.4, quite good linear relationships (R = 0.995−1.000) were observed within the pyrogallol concentration range 0.40−4.80 mM. This means that there is scope for use of a wider concentration range for measurements at pH 7.4. Of course, the practical pyrogallol concentration range could be adjusted according to the individual spectrophotometer.

To quantitatively analyze the pH effects, the $^{\bullet}O_2^-$ radical-scavenging activities of 33 selected antioxidants (including SOD) were measured at pH 7.4 and 8.2 and compared (see Supporting Information, file 3). The activity ratios, defined as $IC_{50,7.4}/IC_{50,8.2}$ are listed in Table 1. The ratios indicate the extent of the pH effect at pH 8.2 compared with that at pH 7.4. The lower the ratio

Table 1. Comparison of IC_{50} Values of 33 Selected Antioxidants between pH 7.4 and 8.2[a]

antioxidant	resource and characterization	pK_a	IC_{50} value (μg/mL) pH 7.4	IC_{50} value (μg/mL) pH 8.2	ratio value
Trolox	exogenous, synthetic, vitamin analogue	3.89 [26]	603.00 ± 5.77 a	2416.67 ± 11.55 b	0.25
ascorbic acid	exogenous, natural, vitamin, lactone, phenolic	4.1 [27]	7.26 ± 0.06 a	11.63 ± 0.55 b	0.62
α-tocopherol	exogenous, natural, vitamin		69.38 ± 0.48 a	106.04 ± 3.87 b	0.65
BHT	exogenous, synthetic, phenol, industrial antioxidant	12.23 [28]	8.77 ± 0.39 b	5.53 ± 0.80 a	1.58
thymol	exogenous, natural, phenol	8.81 [29]	154.14 ± 8.28 a	155.29 ± 4.97 a	0.99
curcumin	exogenous, natural, phenol	9.12 [30]	33.33 ± 2.50 a	30.35 ± 1.26 a	1.10
resveratrol	exogenous, natural, phenolic stilbene	9.4 [31]	6.73 ± 0.079 a	6.34 ± 0.19 a	1.06
magnolol	exogenous, natural, phenolic phenylpropanoid	7.01 [32]	31.19 ± 1.78 a	30.32 ± 0.73 a	1.03
catechin	exogenous, natural, polyphenol	7.8 [33]	1045.57 ± 39.80 a	1171.57 ± 51.19 b	0.89
EGCG	exogenous, natural, polyphenol	7.6 [34]	25.99 ± 0.63 a	46.45 ± 0.42 b	0.56
tannic acid	exogenous, natural, tannin	7.0 [35]	8.43 ± 0.34 a	15.35 ± 0.39 b	0.55
p-coumaric acid	exogenous, natural, phenolic acid	4.36 [36]	2.32 ± 0.75 a	7.94 ± 0.32 b	0.29
protocatechuic acid	exogenous, natural, phenolic acid	4.35 [36]	309.50 ± 10.50 a	599.69 ± 31.77 b	0.52
vallinic acid	exogenous, natural, phenolic acid	4.16 [36]	372.22 ± 1.99 a	1851.18 ± 235 b	0.20
gallic acid	exogenous, natural, phenolic acid	4.27 [36]	267.41 ± 7.73 a	532.69 ± 29.46 b	0.50
caffeic acid	exogenous, natural, phenolic acid	4.47 [36]	12.13 ± 0.24 a	13.10 ± 0.28 b	0.92
chlorogenic acid	exogenous, natural, phenolic acid ester	3.58 [37]	15.35 ± 0.18 a	19.12 ± 0.45 b	0.80
ferulic acid	exogenous, natural, phenolic acid	4.52 [36]	13.94 ± 0.31 a	15.87 ± 0.09 b	0.88
isoferulic acid	exogenous, natural, phenolic acid	4.52	73.79 ± 7.54 a	83.93 ± 12.46 b	0.88
sinapic acid	exogenous, natural, phenolic acid	4.47 [36]	3.29 ± 0.080 a	3.96 ± 0.47 b	0.83
emodin	exogenous, natural, anthraquinone	5.70 [38]	22.67 ± 0.43 b	21.51 ± 0.11 a	1.05
aloeemodin	exogenous, natural, anthraquinone	8.49 [38]	90.74 ± 4.60 b	70.43 ± 2.15 a	1.29
rhein	exogenous, natural, anthraquinone	4.51 [38]	123.46 ± 2.96 a	219.33 ± 5.85 b	0.56
quercetin	exogenous, natural, flavonoid	6.62 [39]	28.71 ± 0.52 b	24.53 ± 0.49 a	1.17
rutin	exogenous, natural, flavonoid glycoside	7.4 [40]	42.03 ± 0.45 b	36.93 ± 0.26 a	1.14
pyridoxine	exogenous, natural, heterocyclic phenol, vitamin	9.8 [41]	11.04 ± 0.12 a	10.69 ± 0.16 a	1.03
8-hydroxyl Quin Deriv.	exogenous, synthetic, heterocyclic phenol	3.5 [42]	11.93 ± 1.20 a	16.37 ± 0.98 b	0.73
melatonin	endogenous, heterocyclic phenol		712.37 ± 19.60 a	2224.5 ± 288.61 b	0.32
GSH	endogenous, polypeptide	5.96 [43]	33.31 ± 1.84 a	42.17 ± 1.34 b	0.79
β-carotene	exogenous, natural, polyene, terpene		172.17 ± 1.48 a	171.23 ± 4.38 a	1.00
laminarin	exogenous, natural, polysaccharide		9232.4 ± 1794.98	nd	
EAGM	exogenous, natural, extract		12.03 ± 0.59 a	12.43 ± 0.29 a	0.97
SOD powder	endogenous, natural, superoxide dismutase		821.85 ± 6.05 b	201.50 ± 8.44 a	4.08

[a] IC_{50} value is defined as the concentration of 50% superoxide radical inhibition and calculated by linear regression analysis and expressed as the mean ± SD (n = 3). The linear regression was analyzed by Origin 6.0 professional software. Means with different letters in the same row are significantly different ($p < 0.05$), whereas the same superscripts are not significantly different ($p < 0.05$). nd, cannot be detected. Ratio was defined as $IC_{50,\ 7.4}/IC_{50,\ 8.2}$. Their dose response curves at pH 7.4 and 8.2 are shown in the Supporting Information, file 3.

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value is, the greater the pH effect. A high ratio value (≈1 or >1) suggests there is only a weak or no pH effect in the pH 8.2 solution.

A correlation graph of the pK_a values and the ratio values was plotted (Figure 4). On the basis of this graph, the correlation

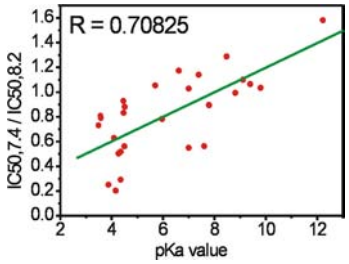
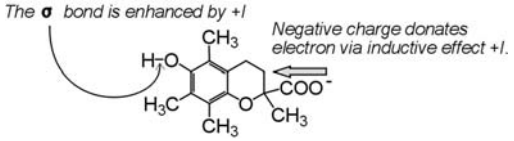


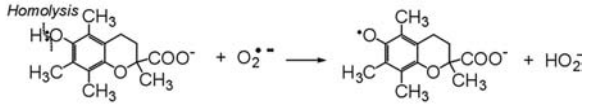
Figure 4. Correlation graph between pK_a values and the value of $IC_{50,\ 7.4}/IC_{50,\ 8.2}$.

coefficient (R value) was calculated by linear regression analysis. The R value clearly demonstrated that the ratio was associated with the pK_a value of the antioxidant. In other words, the pH effect could be attributed to the acidity of the antioxidant and, ultimately, to acidic groups such as −COOH, ester, lactone, and multiphenolic −OH groups. Trolox, a standard antioxidant, is a typical example.

Trolox contains −COOH groups, and we assumed that −COOH might be converted to $-COO^-$ anions at pH 8.2. The negative charge can donate an electron to the O−H σ-bond via an inductive effect (+I), enhancing the O−H σ-bond. The generation of H from homolysis of ArO−H therefore becomes more difficult.



On the other hand, it has been reported that $^{\bullet}O_2^-$ can be scavenged via a "donating hydrogen ($^{\bullet}H$)" approach.[25] Therefore, the proposed mechanism of $^{\bullet}O_2^-$ radical scavenging by Trolox can be explained by the following reaction:



In this case, less generation of $^{\bullet}H$ caused a weaker antioxidant capacity, and hence we found that its IC_{50} value at pH 8.2 was significantly ($p < 0.05$) higher than that at pH 7.4, and the ratio was 0.25 (Figure 5 and Table 1).

Other antioxidants, as well as Trolox, contain acidic groups, including p-coumaric acid, protocatechuic acid, vanillic acid, gallic acid, caffeic acid, chlorogenic acid, ferulic acid, isoferulic acid, sinapic acid, rhein, 8-hydroxyquinoline derivatives, melatonin, and GSH. Although tannic acid, α-tocopherol, ascorbic acid, and EGCG have no −COOH groups, their ester or lactone groups can be hydrolyzed at pH 8.2 to generate −COOH. Like Trolox, these antioxidants displayed low ratio values. This means that they were greatly affected by alkaline solutions (pH 8.2).

Antioxidants without −COOH, ester, or lactone groups, including BHT, thymol, curcumin, resveratrol, magnolol,catechin, rutin, quercetin, emodin, aloe-emodin, pyridoxine, β-carotene, laminarin, and EAGM (ethyl acetate extract from *G. mangostan* hulls, the total phenolic content was 14.35 ± 0.42 mg catechin/g) are hardly affected by the pH 8.2 solution (Table 1). They exhibited higher ratio values (≈1 or >1).

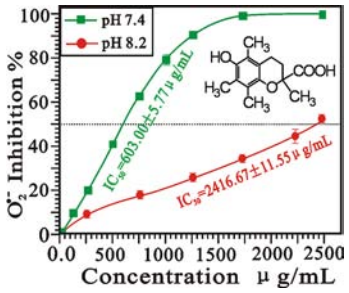


Figure 5. Comparison on $^{\bullet}O_2^-$ radical-scavenging activity of Trolox between pH 7.4 and 8.2. Each value is expressed as the mean ± standard deviation, $n = 3$.

Finally, it must be emphasized that the IC_{50} of SOD at pH 8.2 was much lower ($p < 0.05$) than that at pH 7.4. This means that the original pyrogallol assay at pH 8.2 actually significantly promoted SOD activity.

Therefore, as most antioxidants contain −COOH, ester, or lactone groups, which are sensitive to alkali, the assay was greatly distorted at pH 8.2, and of the four factors affecting the assay, the pH value was the major factor.

As shown in Figure 1 and Table 1, the 33 selected antioxidants covered almost all types of antioxidants, such as pure compounds and extracts; exogenous and endogenous; natural and synthetic; water-soluble and lipid-soluble; vitamins and nonvitamins; phenols, polyphenols, phenolic acids, flavonoids, anthraquinones, tannins, polysaccharides, polypeptides, polyenes, terpenes, phenylpropanoids, stilbene, heterocyclic phenols, and SOD itself. As all of these could be successfully measured in our experiments, the improved method was proved to be suitable for all types of antioxidants (including SOD).

The recommended procedure is as follows. First, a certain amount of pyrogallol solution (60 mM in 1 mM HCl, 37 °C) was thoroughly mixed with pH 7.4 Tris-HCl buffer (0.05 M, 37 °C) containing 1 mM Na_2EDTA (to remove metal ions, which may catalyze the reaction); the total volume was adjusted to 3000 μL using the buffer. The $A_{325\ nm}$ value of the mixture without a sample was measured every 30 s for 5 min at 37 °C. Second, an amount of pyrogallol solution equal to that used in the first step was added to a mixture with a sample, and the total volume was adjusted to 3000 μL using the buffer. The $A_{325\ nm}$ value of this mixture was also measured every 30 s for 5 min. The superoxide anion ($^{\bullet}O_2^-$)-scavenging ability was calculated as absorbance increments:

$$\left(\frac{\Delta A_{325\text{nm, control}}}{T} - \frac{\Delta A_{325\text{nm, sample}}}{T}\right) \Big/ \frac{\Delta A_{325\text{nm, control}}}{T} \times 100\%$$

Here, $\Delta A_{325\ nm,\ control}$ is the increment in $A_{325\ nm}$ of the mixture without a sample and $\Delta A_{325\ nm,\ sample}$ is that with a sample; $T = 5$ min. It is worth mentioning the following. (1) As a rule, we used distilled water to prepare the experimental buffer. However, in the assays, some of the lipid-soluble antioxidants (e.g., BHT, thymol, curcumin, and β-carotene) did not dissolve well in water. In these cases, we used n-butanol-saturated water rather than distilled water to prepare the Tris-HCl buffer. (2) Although the physiological temperature, 37 °C, was considered to be the best

temperature for the measurements, for laboratories that cannot maintain this temperature, 15−37 °C can be used. However, when the room temperature is lower than 15 °C, $\Delta A_{325\ nm}/T$ will be too low. (3) If the determining temperature cannot be strictly controlled at 37 °C, a variable room temperature will readily affect the pyrogallol autoxidation. For measurements that last for several days, even the same volume of pyrogallol solution may produce different $^{\bullet}O_2^-$ substrate concentrations. In this case, we can adjust the volume of pyrogallol solution appropriately to ensure that the system can yield the same value of $\Delta A_{325\ nm,\ control}/T$ (i.e., the same initial concentration of substrate $^{\bullet}O_2^-$). This procedure guarantees that the improved method is precise and reliable. This is especially important for comparative measurements. Of course, the value of $\Delta A_{325\ nm,\ control}/T$ can be specifically designed (usually 0.06−0.2 min^{-1}), according to the individual spectrophotometer. (4) The pyrogallol stock solution in 1 mM HCl remains stable for 48 h at room temperature.

To evaluate the validity of the improved pyrogallol method, the linearity, sensitivity, precision, and reproducibility were measured, along with those of the original pyrogallol and NBT methods.

The linearity graphs of the three methods were constructed by plotting the mean values of triplicate analyses and final concentrations. Our data (see Supporting Information, file 4) suggested that the improved method possessed a higher R value than the other methods did. In other words, its dose response was better than those of the original pyrogallol and NBT methods.

The sensitivity of the method was evaluated using the IC_{50} values. The IC_{50} values of Trolox were calculated to be 603.00 ± 5.77, 2416.67 ± 11.55, and 692.88 ± 88.98 μg/mL by the improved pyrogallol method, original pyrogallol method, and NBT method, respectively. Hence, the improved method appeared to be more sensitive than the original method and NBT method. This could be partly attributed to use of the more sensitive determining wavelength of 325 nm.

In our study, the precision of the method was estimated by measuring the value of the relative standard deviation (RSD, n = 3). The data (Supporting Information, file 4) indicated that the improved method was more precise ($p < 0.05$) than the original method and met the demands for bioanalytical assays (RSD < 10%).[16] The lower precision of the original method may result from the determining wavelength (420 nm) and using pH 8.2. Because the NBT method was based on determining formazon, which does not dissolve well in the buffer, the $A_{560\ nm}$ readings were variable and the method was less precise.

The reproducibility, however, represents the precision of the method under the same operating conditions over days and was checked by repeating the above "precision assay" procedure for 3 days. The RSD% for the IC_{50} values in the improved method was 6.09%, whereas the original method and the NBT method gave 35.38 and 14.72%, respectively. Hence, the improved method was much more ($p < 0.05$) reproducible than the other methods.

In conclusion, the improved method has higher linearity, sensitivity, precision, and reproducibility than the original method and the NBT method. The improved method requires only a spectrophotometer, a pH-meter, an electric balance, and some cheap chemical reagents. It is therefore considered to be a reliable and cheap superoxide-scavenging assay, suitable for all types of antioxidants (including SOD itself).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

File 1, effect of temperature; file 2, effect of pyrogallol concentration; file 3, dose response curves of 33 selected antioxidants at pH 7.4 and 8.2; file 4, validation of methods; file 5, preparation and determination of EAGM. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ESR, electron spin resonance; NBT, nitroblue tetrazolium chloride; EGCG, (−)-epigallocatechin gallate; BHT, 2,6-di-*tert*-butyl-4-methylphenol; GSH, glutathione; SOD, superoxide dismutase; Tris, tris(hydroxymethyl)aminomethane; RSD, relative standard deviation.

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