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Determination of Trace Hydroxyl Radicals by Flow Injection Spectrofluorometry and Its Analytical Application

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On the basis of the fluorescence increase of the reaction of ninhydrin with hydroxyl radicals, a new method for the determination of trace amounts of hydroxyl radicals by flow injection spectrofluorometry is presented. The introduction of flow injection analysis brought better reproducibility and avoided the effect of oxygen and other substances in the environment on the reaction of ninhydrin with hydroxyl radicals. Under optimum experimental conditions, the hydroxylated product of ninhydrin had excitation and emission maxima at 300 and 406 nm, respectively. The linear range was 2.60×10^{-7} to 4.00×10^{-5} M, and the limit of detection was 7.91×10^{-8} M. A high analysis rate of 22 samples per hour was obtained. The proposed method has been applied successfully to the determination of scavenging effects of thiourea and vitamin C on hydroxyl radicals as well as to the evaluation of antioxidant capacities of some natural food.

KEYWORDS: Hydroxyl radicals; Fenton-like reaction; ninhydrin; flow injection spectrofluorometry.

1. INTRODUCTION

Reactive oxygen radicals (ROS) have been linked to ischemic and reperfusion injury on a variety of tissues. Hydroxyl radicals ($HO^{\bullet}$), the strongest oxidants to be well-known, are thought to be generated within cells and tissues in the "oxidative stress" process, where they can attack proteins, lipids, and DNA. More importantly, $HO^{\bullet}$ can initiate secondary radicals that may result in irreparable damage (*1*−*3*).

Therefore, hydroxyl radicals have been deemed to play a critical role in many pathological processes. In order to demonstrate the role of $HO^{\bullet}$ in toxicology and in human diseases, it is necessary to monitor them accurately at real time.

The reported methods used for detecting $HO^{\bullet}$ include electron spin resonance (ESR) (*4*), high-performance liquid chromatography (HPLC) (*5*−*10*), chemiluminescence (CL) (*11*), electrochemistry (*12*, *13*), and spectrofluorometry (*14*, *15*). Compared with the above methods, spectrofluorometry is superior in many aspects: the equipment needed is inexpensive and easy to operate, the selectivity and sensitivity of spectrofluorometry are high, and reproducibility is good. The flow injection (FI) analysis has better reproducibility and rapid analysis, which can achieve real time, online, and automatic analysis of samples. So the combination of FI with spectrofluorometry to determine $HO^{\bullet}$ not only has high sensitivity but also can make the analytical operation automatic. At present, the combinations of FI technology with spectrofluorometry (*16*) used to determine free radicals have begun to receive more and more attention. So far, there are few papers addressing the determination of $HO^{\bullet}$ by FI-spectrofluorometry (*17*). In our work, $HO^{\bullet}$ generated from H_2O_2 and Co^{2+} was determined using ninhydrin—an inexpensive stable common reagent—as the trapping agent by FI-spectrofluorometry. The proposed method with a higher sampling rate (*17*) has been successfully applied to determine the scavenging effects of thiourea and vitamin C on hydroxyl radicals as well as to evaluate the antioxidant capacity of aqueous extracts of some natural food.

2. MATERIALS AND METHODS

2.1. Reagents. Ninhydrin was provided by Shanghai Sanaisi Chemical Group Co., Ltd. (99.0%). A 2.25×10^{-2} M ninhydrin stock solution was prepared with water. A stock solution of $CoSO_4 \cdot 7H_2O$ (4.00×10^{-2} M) was diluted with water to make a working solution of 4.00×10^{-5} M. A 5.00×10^{-4} M solution of H_2O_2 was prepared with water and standardized by titration with potassium permanganate. An NH_4Ac−NaOH buffer solution (0.20 M, pH 7.27) was used as the carrier. Vitamin C (L-ascorbic acid) and thiourea were purchased from Tianjin Bodi Chemical Co., Ltd. All chemicals used were of analytical reagent grade or highest purity available. Double- distilled water was used throughout.

2.2. Apparatus. Fluorometric spectra were obtained with a Cary Eclipse spectofluorometer with a xenon lamp and an 18-μL quartz flow-through cell (Varian, Australia). The flow injection apparatus equipped with an eight-channel actuated injection valve, two computer-controlled peristaltic pumps (pumps A and B, each pump with six channels), and an operational keyboard to set up parameters (FIA-3100, Beijing Wantuo, China) was used. All pH measurements were made with a pH-3C digital pH meter (Shanghai Lei Ci Device Works, China).

2.3. Experimental Procedure. In order to achieve the best compromise between peak height and shape of fluorometric spectra, the assembly in **Figure 1** was selected.

The procedure was as follows. First, set up the operation program (**Table 1**). After actuating the pump (P, **Figure 1**), 100 μL of H_2O_2

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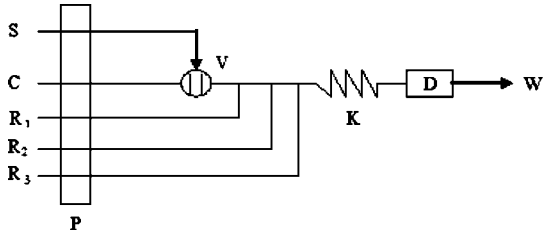


Figure 1. Schematic diagram of the instrumental setup. P: pump A or B; V: valve; K: single bead string reactor (SBSR, i.d. = 0.8 mm); D: fluorescence detector; W: waste. S: 5.00×10^{-4} M H_2O_2 or double-distilled water; C: carrier (pH 7.27, 0.20 M NH_4Ac–NaOH buffer solution); R_1: 4.00×10^{-5} M Co^{2+} or double-distilled water; R_2: double-distilled water or scavenger or sample; R_3: 2.25×10^{-5} M ninhydrin.

Table 1. Operation Program of Flow Injection

process[a]	valve location	time (s)	pump (round min^{-1})
1	S	17	35
2	I	12	35
3	I	120	0

[a] The circulations were five times, S, sampling; I, injection.

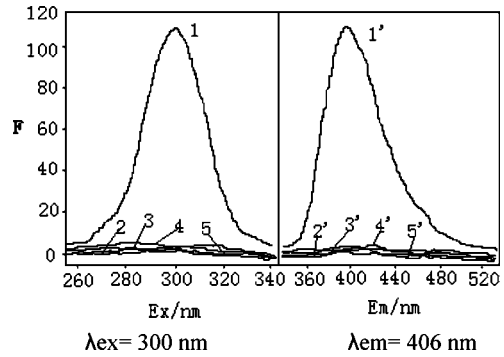


Figure 2. Emission and excitation spectra. 1,1′: $H_2O_2 + Co^{2+}$ + ninhydrin + buffer solution. 2,2′: H_2O_2 + ninhydrin + buffer solution. 3,3′: Co^{2+} + ninhydrin + buffer solution. 4,4′: $H_2O_2 + Co^{2+}$ + buffer solution. 5,5′: ninhydrin + buffer solution. H_2O_2 (5.00×10^{-4} M), Co^{2+} (4.00×10^{-5} M), ninhydrin (2.25×10^{-5} M), NH_4Ac–NaOH buffer solution (0.20 M, pH 7.27).

was sampled into the sampling loop when the valve (V, **Figure 1**) located sampling. Then, H_2O_2 sampled in the loop was injected into the single bead string reactor (K, **Figure 1**) by the carrier stream when the valve located injection. Other reagents (R_1, R_2, and R_3, **Figure 1**) were directly injected into K with the actuated pump. So H_2O_2 was mixed with Co^{2+} and HO• was obtained; then ninhydrin trapped HO• immediately. The mixture then passed the detecting cell of the spectrofluorometer where the fluorescence intensity was measured at 406 nm with excitation at 300 nm.

3. RESULTS AND DISCUSSION

3.1. Excitation and Emission Spectra. Fluorescence excitation and emission spectra of the reaction product were recorded at 300 and 406 nm, respectively. The blank was measured at the same conditions (**Figure 2**; 5,5′). Some comparison experiments were performed to ensure that the enhancement of the fluorescence intensity was indeed due to the reaction of HO• with ninhydrin. The experimental results demonstrated that the fluorescence intensity of the reaction mixture showed no significant increase when H_2O_2, Co^{2+}, and ninhydrin were added separately or when the two of them were added into the system (**Figure 2**; 2−4, 2′−4′). Only when H_2O_2, Co^{2+}, and ninhydrin

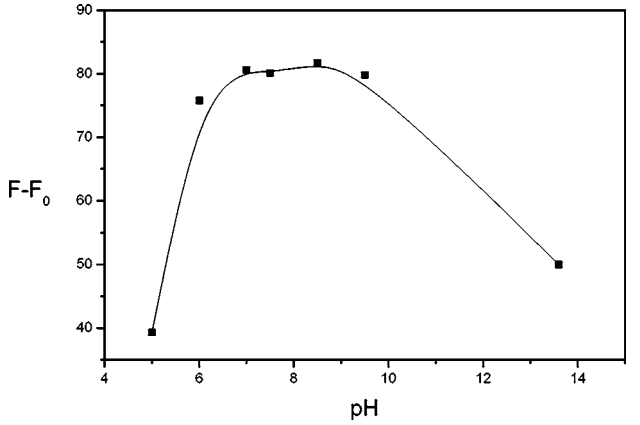


Figure 3. Effect of pH. H_2O_2 (5.00×10^{-4} M), Co^{2+} (4.00×10^{-5} M), ninhydrin (2.25×10^{-5} M).

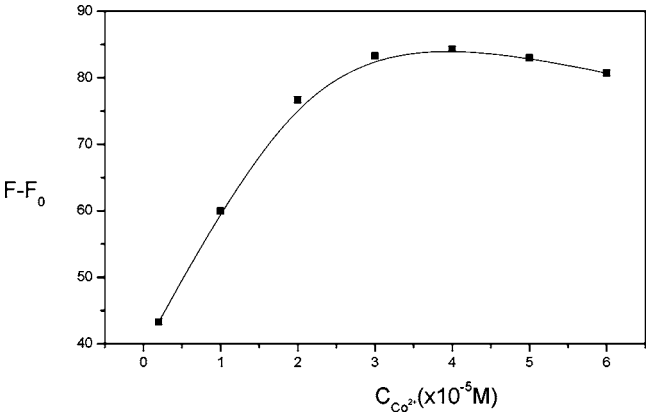


Figure 4. Effect of Co^{2+} concentration. H_2O_2 (5.00×10^{-4} M), ninhydrin (2.25×10^{-5} M), NH_4Ac–NaOH buffer (0.20 M, pH 7.27).

were added simultaneously, was a remarkable increase in fluorescence intensity found due to the formation of hydroxylated ninhydrin (**Figure 2**; 1,1′). These results proved that it was HO• that reacted with ninhydrin to form a high-fluorescent product.

3.2. Influence of Flow-System Variables. The variables studied for optimizations of the manifold parameters were sampling, injection, and stop-flow time; injection volume; and flow rate.

The sampling and injection time of H_2O_2 carried by buffer solution to the single bead string reactor (K, **Figure 1**) had definite effects on analytic sensitivity. The results showed that relative fluorescence intensity was the highest and the peak shape of fluorometric spectra was the best when the sampling and injection times were 17 and 12 s, respectively.

The stop-flow time in the reactor was another factor that affected the reaction extent because the Fenton-like reaction had a small reaction constant and needed enough time to generate HO•. Experiments showed that relative fluorescence intensity was higher with longer stop-flow time. However, the sample throughput was decreased and the vertical diffusion increased with longer time. To optimize the sensitivity and sample throughput simultaneously, 2 min of stop-flow time was chosen. The proposed method allowed a sample throughput of 22 samples per hour.

Injection volume had a significant effect on sensitivity. The volume of H_2O_2 solution injected varied from 50 to 300 μL using different specifications of sampling loops. The results showed that the relative fluorescence intensity increased with the increase of injection volume, while the increase began to

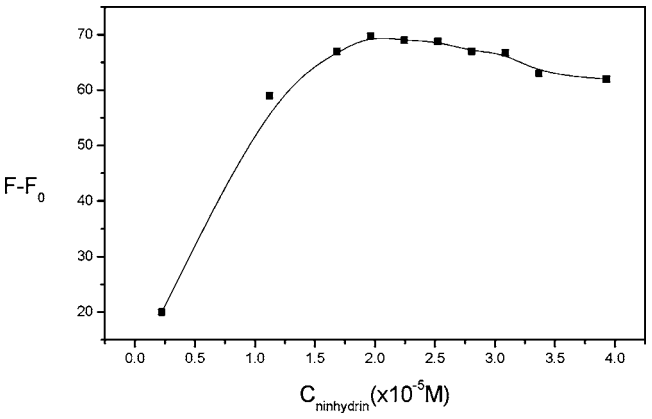


Figure 5. Effect of ninhydrin concentration. H_2O_2 (5.00×10^{-4} M), Co^{2+} (4.00×10^{-5} M), NH_4Ac–NaOH buffer solution (0.20 M, pH 7.27).

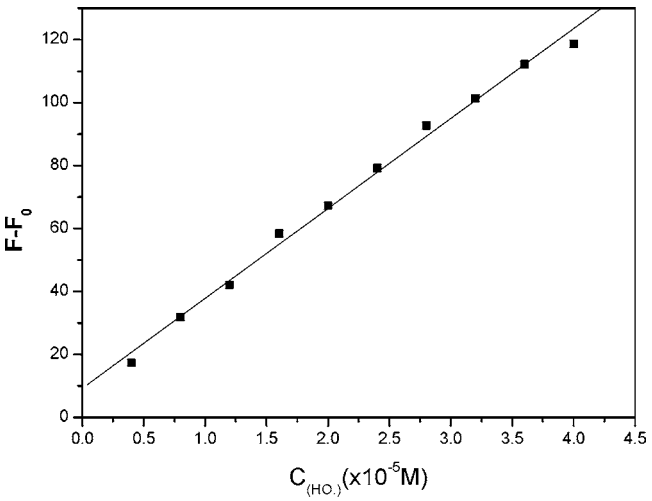
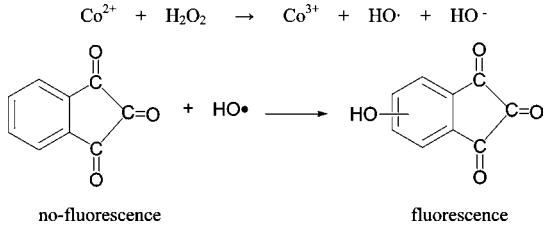


Figure 6. Linear correlation between relative fluorescence intensity and concentrations of hydroxyl radical ninhydrin (2.25×10^{-5} M), NH_4Ac–NaOH buffer solution (0.20 M, pH 7.27).

Scheme 1. Mechanism of the Reaction



be slight from 100 to 200 μL. At the same time, more injection decreased sample throughput. In order to optimize both the sensitivity and sample throughput, a 100-μL injection using a 100-μL sampling loop (20-cm-long, i.d. = 0.8 mm) was chosen.

The flow rate was an important factor to sample throughput. In this experiment, the single bead string reactor (SBSR, **Figure 1**) was a piece of PTFE tube (90-cm long, i.d. = 0.80 mm). The flow rate was adjusted by changing the rotation speed of the pump. The results showed that relative fluorescence intensity was high and stable at the rotation speed of 30−40 rpm and the baseline was stable; the peak shape was good. So, the rotation speed was chosen to be 35 rpm and the flow rates of the carrier, Co^{2+}, sample, and reagent (ninhydrin) solution were 2.80, 1.75, 1.75, and 1.75 mL/min, respectively.

3.3. Effect of pH. The pH value of the medium had a great effect on fluorescence intensity. In order to obtain the lowest fluorescence intensity of the reagent blank and the highest signal-to-noise ratio, the results showed that the optimum range was 6.80−8.40 (**Figure 3**). Therefore, a pH of 7.27 (0.20 M NH_4Ac−NaOH buffer solution) was chosen as the carrier.

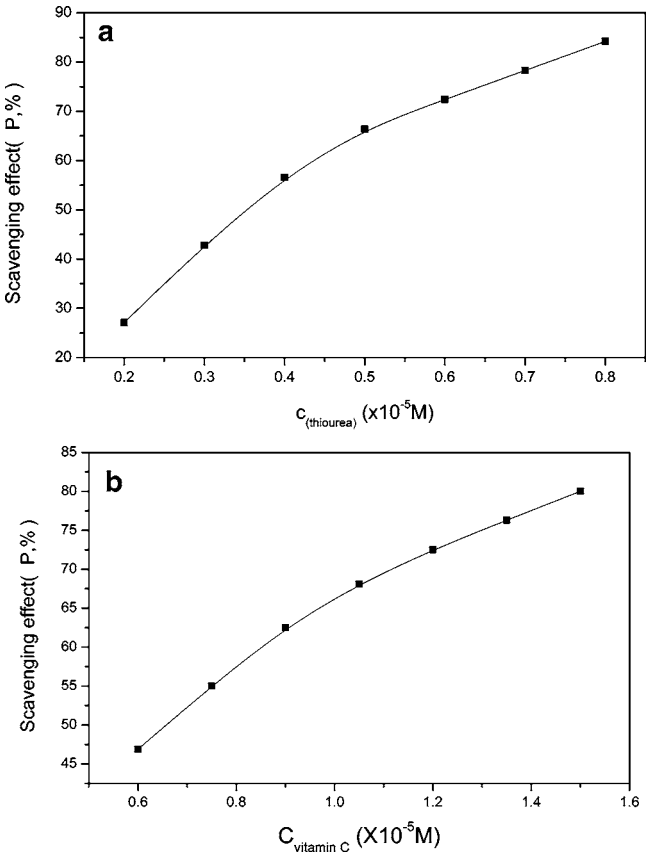


Figure 7. Relation between scavenging effect and scavenger concentration for (**a**) thiourea and (**b**) vitamin C.

3.4. Effect of Co^{2+} Concentration. The effect of Co^{2+} concentration was tested in the range from 0.20×10^{-5} to 6.00×10^{-5} M. The peak heights increased with Co^{2+} concentration increasing up to 3.00×10^{-5} M and then remained stable (**Figure 4**). Therefore, a 4.00×10^{-5} M Co^{2+} solution was chosen.

3.5. Effect of Ninhydrin Concentration. Ninhydrin was the trapping agent of HO•, whose concentration was the key parameter for the analytic sensitivity. The fluorescence intensity increased with the trapping agent concentration increasing in the range from 1.00×10^{-6} to 2.00×10^{-5} M, then the increasing tendency became slight (**Figure 5**). A suitable concentration of trapping agent was advantageous to guarantee HO• to be trapped completely, while superfluous trapping agent could lead to collision quench of fluorescence. Therefore, the concentration of ninhydrin solution injected was 2.25×10^{-5} M.

3.6. Effect of Interference. To examine the possible interference by foreign ions and some complexing agents, the concentration of H_2O_2 was fixed at 5.00×10^{-4} M. An error of ±5.0% in the relative fluorescence intensity was considered tolerable. No interference was encountered from (tolerance ratio in mol) Na^+, K^+ (1500); NH_4^+ (1000); Ca^{2+}, Zn^{2+} (500); Cd^{2+} (100); Fe^{2+}, Ni^{2+} (50); Cu^{2+} (10); Fe^{3+} (100); F^-, Cl^-, NO_3^- (1500); Br^-, I^-, Ac^-, O_2^- (1000); NO_2^- (500); glucose, sucrose (1000); sorbierite (500); DL-phenyalanine (100); DL-tyrosine, DL-tryptophan (50). The interference experiment proved that most components in biological materials had no or little effect on the determination of HO•, which also indicated that the selectivity of the proposed method was satisfied.

3.7. Analytical Characteristics. Under the optimum experimental conditions, there was a good linear correlation ($R = 0.9970$) between the relative fluorescence intensity and the concentration of hydroxyl radical in the range of 2.60×10^{-7} to 4.00×10^{-5} M (**Figure 6**). The regression equation was $\Delta F = 9.255C(\times 10^{-5}\ \text{M}) + 28.57$. The limit of detection was determined to be 7.91×10^{-8} M according to IUPAC (*18*) definition. The standard deviation of the peak height was 0.78, and the relative standard deviation (RSD) of the method was 0.99% obtained from a series of 11 standards, each containing 5.00×10^{-4} M H_2O_2 solution and 4.00×10^{-5} M Co^{2+} solution, which showed that the reproducibility of the proposed method was satisfied.

3.8. Reaction Mechanism. The hydroxyl radical was obtained by the reaction of Co^{2+} with H_2O_2 (Fenton-like reaction). The $HO^{\bullet}$ yield is higher than that of the Fenton reaction (*19*), which allows higher determination sensitivity. In our study, ninhydrin had no fluorescence at 406 nm, but when attacked by $HO^{\bullet}$, a product of aromatic hydroxylation was obtained which had strong fluorescence (**Scheme 1**). The hydroxyl was the electron donor, and carbonyl was the electron acceptor, which might effectively induce intramolecular charge transfer and showed strong fluorescence (*20*). The amount of $HO^{\bullet}$ could be determined directly by measuring the changes in the fluorescence intensity. The mechanism of the method was the following:

$$Co^{2+} + H_2O_2 \rightarrow Co^{3+} + HO^{\bullet} + HO^{-}$$

3.9. Verification of the Efficiency of the Method. Thiourea and vitamin C are known to be effective scavengers of $HO^{\bullet}$. Their scavenging effects on $HO^{\bullet}$ could be used to verify the efficiency of the proposed method and reflect their scavenging capacity of $HO^{\bullet}$, because they had no fluorescence at the measuring wavelength. The scavenging percentage (P) was calculated as $P\ (\%) = (F - F_s)/(F - F_0) \times 100$, where F was the fluorescence intensity with no scavenger (see **Figure 1**: S, H_2O_2 solution; R_1, Co^{2+} solution; R_2, double-distilled water), F_s was the fluorescence intensity with the scavenger (S, H_2O_2 solution; R_1, Co^{2+} solution; R_2, scavenger), and F_0 was the fluorescence intensity with only ninhydrin and buffer solution (S, R_1, R_2 were all double-distilled water). The results indicated that there was a correlation between the scavenging effect and scavenger concentration, which proved that the proposed method was feasible (**Figure 7**).

3.10. Study on the Antioxidant Capacities of Aqueous Extracts of Natural Food. Some natural food extracts were shown to possess high hydroxyl radical scavenging activity (*21*, *22*). We selected eight kinds of natural food purchased from local supermarkets, cleaned them, and let them dry naturally. A 1.500-g amount of each kind was weighed, and then the foods were marinated for 5.0 h in 20 mL of water after being crushed by grinding with a mortar and pestle separately. After that, the mixtures were centrifuged at 4000 rpm for 30 min, and the supernatant was filtered and centrifuged for 30 min once again. The newly prepared supernatant was used as the antioxidant in the experiment. The proposed method was applied to the determination of the scavenging effect on $HO^{\bullet}$ of the aqueous extracts of eight kinds of natural food (**Figure 8**). The scavenging percentage (P) was calculated as $P\ (\%) = (F - F_s)/(F - F_0) \times 100$ (see section 3.9)(F_s: with R_2 were samples of food extracts instead). The results showed that among the selected food, walnut, sunflower seed, and black sesame had strong scavenging capacities of $HO^{\bullet}$, which was in accordance with the work of Zhang (*23*) based on spectrofluorometric detection.

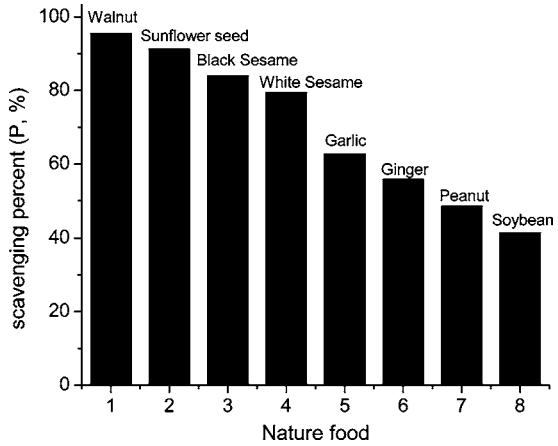


Figure 8. Comparison of scavenging capacity of eight food extracts for $HO^{\bullet}$.

In conclusion, a new determination method was presented using ninhydrin as the trapping reagent of $HO^{\bullet}$ by flow injection spectrofluorometry. The linear range of this method was 2.60×10^{-7} to 4.00×10^{-5} M with the RSD of 0.99%. The limit of detection was 7.91×10^{-8} M. The proposed method was simple, sensitive, automatic, and with a high analysis rate for the determination of $HO^{\bullet}$. It has been successfully applied to determine the scavenging effects of thiourea and vitamin C on $HO^{\bullet}$ and to evaluate antioxidant capacities of some natural food, which might serve as an important tool in sieving antioxidant compounds with applications in food and medical fields.

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