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Determination of the antioxidant capacity of different food natural products with a new developed flow injection spectrofluorimetry detecting hydroxyl radicals

Bo Tang[a,*], Li Zhang[a], Yue Geng[b]

[a] *College of Chemistry, Chemical Engineering and Materials Science, Shandong Normal University, Jinan 250014, China*
[b] *College of Life Science, Shandong Normal University, Jinan 250014, China*

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Abstract

This paper presents an automatic spectrofluorimetric method (flow injection analysis spectrofluorimetry) for detecting hydroxyl radicals. Based on H_2O_2 catalyzed by Co^{2+} yielding $HO^{\bullet}$, the method utilized sodium terephthalate to trap hydroxyl radicals and obtained sodium 2-hydroxyterephthalate by aromatic hydroxylation, which resulted in an increase in the fluorescence intensity. The relative fluorescence intensity was proportional to the concentration of hydroxyl radicals. Hydroxyl radicals could be determined indirectly, based on the increase of fluorescence. It was a simple, rapid, precise, sensitive and automatic technique for the determination of $HO^{\bullet}$. The relative standard deviation of 11 determinations was 0.57%. The method can be used to sieve antioxidant medicines and will have theoretical and practical guidance on the mechanism of hydroxyl radicals-damaging biology.

Keywords: Hydroxyl radicals; Flow injection spectrofluorimetry; Sodium terephthalate; Maize pollen polysaccharide

1. Introduction

Oxygen-derived free radicals have been linked to ischemic and reperfusion injury on a variety of tissues including heart. The hydroxyl radicals, the strongest oxidants to be well known, are thought to be generated within cells and tissues, where it can attack proteins, lipids and DNA. More important, it can initiate secondary radicals that can produce irreparable damages [1,2]. Hydroxyl radicals have been suggested to play a critical role in many pathological processes. Being highly reactive and short-lived, it is rather difficult to monitor its concentration in vivo, so a more detailed understanding of $HO^{\bullet}$ initiating cellular injury has not been attained due to the lack of highly sensitive and quantitative methods for its determination in complex biological systems. In order to establish the role of $HO^{\bullet}$ in toxicology and in human disease, it is necessary to monitor them accurately in vivo and at real time. Determination of antioxidant capacity and filtration of anti-caducity medicines also need new rapid analytical method.

Commonly used methods for detecting $HO^{\bullet}$, include electron spin resonance (ESR) [3,4], high-performance liquid chromatography (HPLC) [5,6], chemiluminescence (CL) [7,8], electrochemistry [9–11], spectrofluorimetry [12], and spectrophotometry. However, ESR technique needs expensive instruments, HPLC is complicated in operation, CL is poor in selectivity, electrochemistry is unsatisfying in reproducibility and spectrophotometry has low sensitivity [13,14]. Compared with above methods, spectrofluorimetry is more simple, sensitive and selective. The flow injection (FI) analysis has better reproducibility and rapid analysis, which can achieve real-time, online and automatic analysis of samples. The combination of FI with spectrofluorimetry to determine

* Corresponding author. Tel.: +86 531 6180010; fax: +86 531 6180017.
E-mail address: tangb@sdnu.edu.cn (B. Tang).

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$HO^{\bullet}$ not only has high sensitivity but also can make the analytical operation automatic. At present, the combinations of FI with spectrophotometry [15,16] and chemiluminescence [17,18] used to determine free radicals have begun to get more attention, while FI–spectrofluorimetry was never used to determine $HO^{\bullet}$ before; thus, in the future, such methods will find wide application.

In this work, $HO^{\bullet}$ was determined by FI–spectrofluorimetry with sodium terephthalate as the trapper. The proposed method shows high sensitivity and rapid speed in which sample throughput is 16 samples h^{-1} and the product is single without any isomers. The method was applied to the determination of antioxidant capacity of natural food products and maize pollen polysaccharide.

2. Experiment

2.1. Apparatus and reagents

2.1.1. Apparatus

The fluorescence spectra and fluorescence intensity were measured on Cary Eclipse spectrofluorimeter with a xenon lamp and 18 μl quartz flow-through cell (Varian, Australia). The flow injection apparatus equipped with an eight-channel actuated injection valve and two peristaltic pumps (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).

2.1.2. Reagents

4-Trifluormethylsalicylic acid was provided by Shandong Jinyimeng Chemical Group Co., Ltd. (99.5%). A stock water solution of $CoSO_4$ (0.10 mol l^{-1}) was diluted with water to prepare working solution of 4.0×10^{-3} mol l^{-1}. Sodium terephthalate stock solution was prepared from terephthalic acid through neutralization with NaOH and diluted to prepare a working solution of 1.0×10^{-3} mol l^{-1}. The solution of H_2O_2 was 0.3% (0.1009 mol l^{-1} standardized by titration with potassium permanganate) at the pH of 4.48. A 0.10 M borate buffer solution of pH = 7.40 was used as the carrier. All chemicals used were of analytical reagent or of high purity. Doubly distilled water was used throughout.

Table 1
The operation program of flow injection

Process[a]	Valve location	Time	Pump (round min^{-1})
1	S	30 s	30
2	I	10 s	30
3	S	3 min	0

[a] The circulations were five times.

2.2. Flow injection analysis assembly

Preliminary tests were carried out with the aid of different assemblies of flow manifold to optimize the apparatus. The assembly in Fig. 1 was selected as the one producing the best compromise between peak height and shape. The operation program was shown in Table 1.

2.3. Experimental procedure

2.3.1. Determination of $HO^{\bullet}$

Actuating the peristaltic (P, Fig. 1), H_2O_2 is sampled into the injection loop when the valve locates sampling. Then, H_2O_2 sampled in the loop is injected into the single bead string reactor (K, Fig. 1) by the carrier stream when the valve locates injection. Other reagents are injected into K directly with the actuated pump. H_2O_2 is mixed with Co^{2+} and $HO^{\bullet}$ is obtained. Immediately sodium terephthalate trapes $HO^{\bullet}$ and sodium 2-hydroxyterephthalate is produced. Then, the mixture passes into the detector cell of the spectrofluorimeter, where the fluorescence intensity is measured at 431 nm with excitation at 313 nm. The relative fluorescence intensity is proportional to the amount of $HO^{\bullet}$.

2.3.2. Determination of scavenging effect of $HO^{\bullet}$

The scavenging percentage (P) is calculated as P (%) = $(F - F_s)/(F - F_0) \times 100$, where F_s is the fluorescence intensity with the scavenger in S-stream, F the fluorescence intensity with just water in the S-stream and F_0 the fluorescence intensity in the absence of H_2O_2 and scavengers.

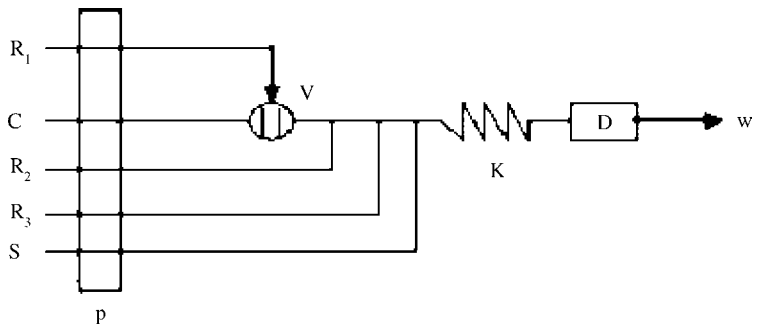
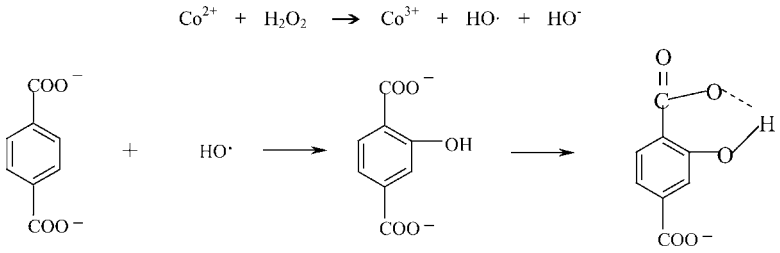


Fig. 1. A schematic diagram of instrumental set-up. P: pump; V: valve; K: single bead string reactor (SBSR, i.d. = 0.8 mm); D: detector; W: waste; R_1: H_2O_2; R_2: solution of sodium terephthalate (1.0×10^{-3} mol l^{-1}); R_3: Co^{2+} (4.0×10^{-3} mol l^{-1}); S: scavengers or samples; C: carrier (pH = 7.40, 0.10 mol l^{-1} of borate buffer solution).

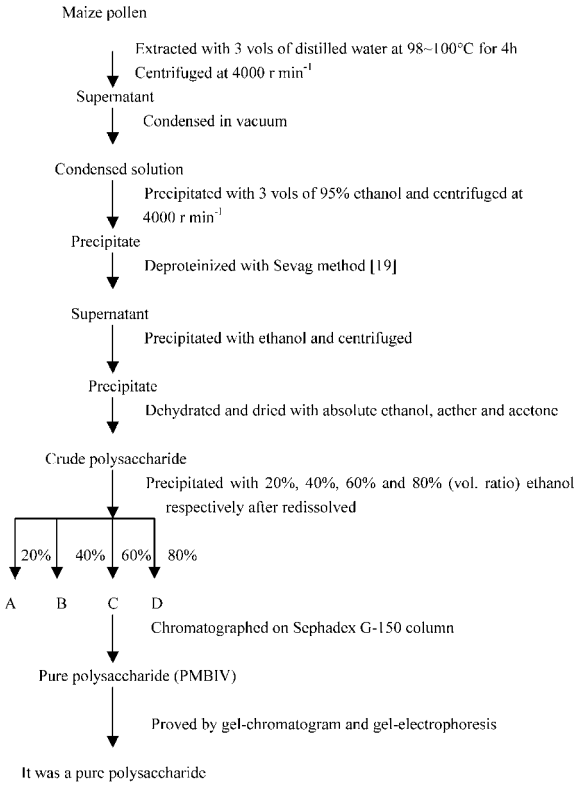


Scheme 1. Mechanism of the determination.

2.3.3. Determination of the antioxidant capacity of samples

2.3.3.1. Determination of the scavenging capacities of the water extracts of natural food products. 1.500 g of natural food was marinated for 10 h in 15 ml of water after being crushed. Then, the mixtures were centrifuged at 4000 rpm for 30 min. The supernatant was used in the experiment.

2.3.3.2. Determination of the antioxidant capacity of maize pollen polysaccharide. The treatment procedure for maize pollen polysaccharide was as follows. Different-grade polysaccharide (A, B, C, D) and PMBIV were determined by the proposed method.



The determination of polysaccharose samples by spectrophotometry [18,19]

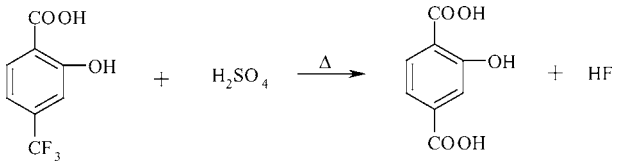
One millilitre of phosphate buffer solution (pH = 7.40, 0.15 m mol l^{-1}), 1.00 ml of crocus (40 μg ml^{-1}), 1.00 ml of EDTA–Fe^{3+} (0.945 mmol l^{-1}), 1.00 ml of H_2O_2 (3.0%) and 0.50 ml of samples in turn were added into a 10 ml of color comparison tube. The mixture was diluted to volume with doubly distilled water. It was incubated in water bath at 37 °C for 30 min. A blank was prepared by replacing the sample and the EDTA–Fe^{3+} volume with 2.0 ml of water. The absorbance was measured at 520 nm against a reagent blank. Then, the scavenging percentage (P) was calculated as P (%) = ($A_{sample} - A_{blank}$)/($A_{comparison} - A_{blank}$) × 100.

3. Results and discussion

3.1. Reactions and kinetics involved in the proposed method

Hydroxyl radicals were obtained by Co^{2+} catalyzing H_2O_2, in a reaction similar to Fenton. And the HO• yield is higher than Fenton reaction [20]. So, the sensitivity could increase with the HO• source. With two electron-withdrawing functional groups, sodium terephthalate has no fluorescence. After HO• attacking the trap compound, a product of aromatic hydroxylation was obtained and presumed as sodium 2-hydroxyterephthalate, which has strong fluorescence. The amount of HO• could be determined indirectly by measuring the changes in fluorescence intensity. Compared with sodium benzoate as the trapper, the product of sodium terephthalate trapping HO• is the only product without isomers formed. Benzoate acid, a common trap-compound of HO•, gives the isomer products of *o*, *p*, *m*- hydroxybenzoate acid. Terephthalate acid gives similar products, but because of the functional groups, the observed isomers are identical. So, the mechanism of the method was followed (Scheme 1). In order to validate the structure of the hydroxylation product, the standard 2-hydroxyterephthalate acid, whose structure was known, was synthesized through other reaction system [21,22]. The reaction was as follows (Scheme 2).

4-Trifluormethylsalicylic acid (0.80 g) was heated in a test tube on a Bunsen flame with 6.00 ml of concentrated sulfuric acid. The solution turned deep brown and hydrogen flu-



Scheme 2. The synthesis of standard 2-hydroxyterephthalate acid.

oride fumes were liberated. After complete removal of HF, the solution was poured into a mixture of ice and water. The precipitate was recrystallized from ethanol and water, with a yield of 0.60 g of 2-hydroxyterephthalate acid with m.p. 327–328 °C. Excitation and emission spectra of the product in borate (pH = 7.40) buffer solution were obtained. The spectra showed that the excitation and emission wavelengths were the same as those of the assumed compound. This was taken as evidence that the reaction product was the assumed one.

3.2. Excitation and emission spectra

Fluorescence excitation and emission spectra of the reaction mixture were recorded; the blank was measured at the same time. Some preliminary experiments were performed to ensure that the enhancement of the fluorescence intensity was indeed due to the reaction of $HO^{\bullet}$ with sodium terephthalate. The experimental results demonstrated that the fluorescence intensity of the reaction mixture showed no significant increase when Co^{2+}, H_2O_2 and sodium terephthalate were added separately, or when the two of them were added in combination to the system (as shown from the spectra in Fig. 2). Only when Co^{2+}, H_2O_2 and sodium terephthalate were added simultaneously a remarkable increase in fluorescence intensity due to the formation of sodium 2-hydroxyterephthalate was found. These results proved that it was $HO^{\bullet}$ that reacted with sodium terephthalate to form the product with strong fluorescence.

3.3. Optimization of manifold parameters

The variables studied for optimization of the manifold parameters were injection, sampling and stop-flow time, injection volume and flow rate. The reagent concentrations used in these experiments were as follows: 4.0×10^{-3} mol l^{-1} of Co^{2+} stream, 1.0×10^{-3} mol l^{-1} of sodium terephthalate and 0.10 M borate buffer solution of pH = 7.40 as the carrier.

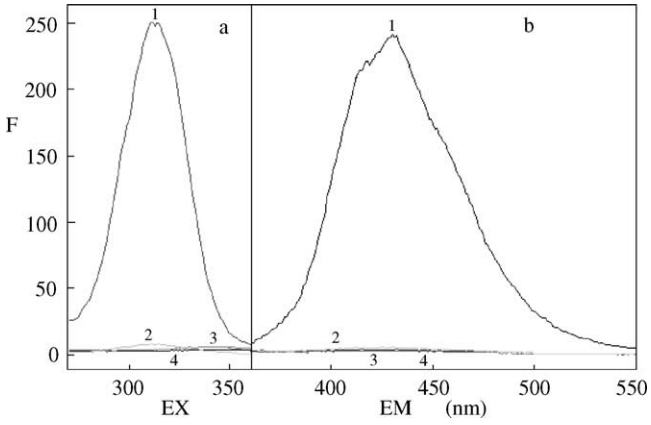


Fig. 2. Mechanism of the determination. 1: H_2O_2 + Co^{2+} + sodium terephthalate + buffer solution; 2: H_2O_2 + sodium terephthalate + buffer solution; 3: Co^{2+} + sodium terephthalate + buffer solution; 4: H_2O_2 + Co^{2+} + buffer solution H_2O_2 (0.3%); Co^{2+} (4.00×10^{-3} mol l^{-1}); sodium terephthalate (1.00×10^{-3} mol l^{-1}); buffer solution: pH = 7.40, 0.10 mol l^{-1} of borate.

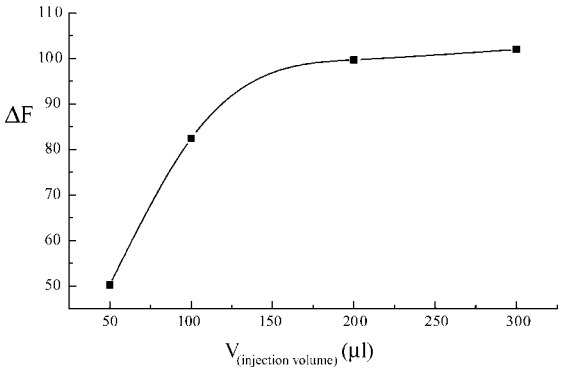


Fig. 3. Effect of volume injected.

3.4. Effect of injection volume

The amount of H_2O_2 decides the yield of $HO^{\bullet}$ and, therefore, has a significant effect on sensitivity. The volume of H_2O_2 solution injected was varied between 50 and 300 μl. The results show that the relative fluorescence intensity increases with the increase of injection volume; above 200 upto 300 μl the increase of ΔF is pretty small (Fig. 3). At the same time, bigger injection volume meant lower sample throughput. In order to optimize both the sensitivity and the sample throughput, a 200 μl was chosen.

3.5. Effect of flow rate

The reactor was a piece of PTFE tube (90 cm long and 0.80 mm i.d.). The flow rate was an important factor to sample throughput. In this experiment, the flow rate was adjusted by changing rotation speed of the pump. The results showed that ΔF was high and stable at the rotation speed of 25∼30 round min^{-1} and the baseline was stable, the peak shape was good when it was 30 round min^{-1}. So, the rotation speed chosen was 30 round min^{-1} and the flow rates of carrier, sample and reagents were 2.60, 2.60, 1.38, 2.60 ml min^{-1}, respectively.

3.6. Effect of sampling and injection time

Different sampling and injection time was tested on the sensitivity and peak shape. The results showed that ΔF was highest and the peak shape was better when they were 30 and 10 s, respectively (Table 2). Because of the small kinetic rate constant of the Fenton reaction, the stop-flow time had important effect on the reaction degree. The experiment showed that the relative fluorescence intensity was higher with longer stop-flow time and more $HO^{\bullet}$ was produced. But the sample throughput was lower and the vertical diffusion increased with longer time. In order to optimize the sensitivity and sample throughput simultaneously, 3 min of stop-flow was

Table 2
Sampling and injection time

Sampling time (s)	Injection time (s)				
	ΔF 8	ΔF 9	ΔF 10	ΔF 11	ΔF 12
20			87.3		
25			90.0		
30	80.1	83.5	95.2	96.1	97.8
35			96.0		

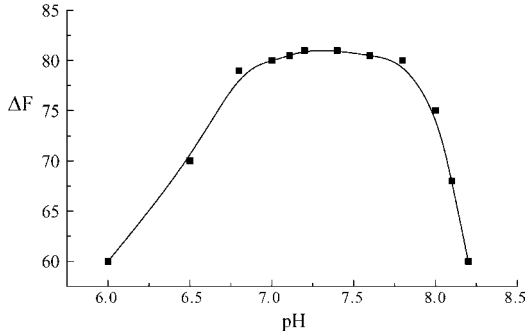


Fig. 4. Effect of pH.

chosen. The proposed method allowed a sample throughput of 16 samples h^{-1}.

3.7. Effect of pH

The pH of carrier stream had a great effect on fluorescence intensity by influencing the yield of HO•. The results showed that the optimum pH range was 6.80–7.90 (Fig. 4). The borate buffer was more sensitive in comparison to acetate, ammoniacal, phosphate and Tris–buffer solutions. Therefore, borate buffer solution (pH = 7.40, 0.10 mol l^{-1}) was chosen as the carrier.

3.8. Optimization of reagent concentrations

The effect of Co^{2+} concentration was tested in the range from 1.0×10^{-3} to 1.0×10^{-2} mol l^{-1}. The peak heights increased with Co^{2+} concentration increasing upto 4.0×10^{-3} mol l^{-1} and kept stable since then (Fig. 5). Therefore, the concentration of Co^{2+} solution injected was 4.0×10^{-3} mol l^{-1}.

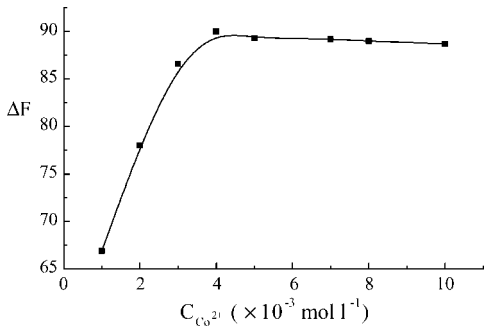


Fig. 5. Effect of Co^{2+} concentration.

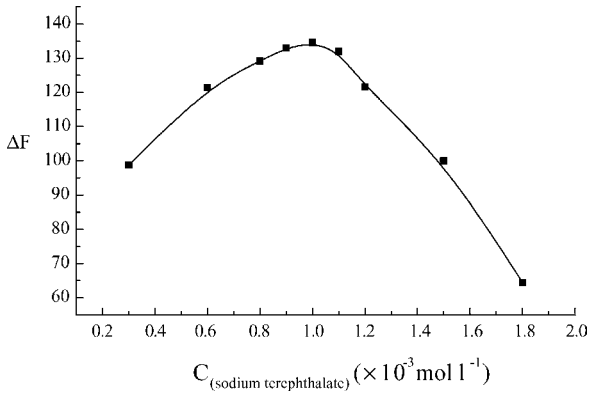


Fig. 6. Effect of sodium terephthalate concentration.

The concentration of sodium terephthalate (trapping agent of HO•) is the key parameter for the quantitative HO• trapping for best precision of the method. The ΔF increased with the trapper concentration increasing upto 8.0×10^{-4} mol l^{-1}, and the increasing tendency became slow and reached the highest level at 1.0×10^{-3} mol l^{-1}, above which it decreased (Fig. 6). A suitable concentration of trapping agent was advantageous, which guaranteed HO• could be trapped completely, while superfluous trapping agent could lead to collision quench of fluorescence. Therefore, the concentration of sodium terephthalate solution injected was 1.0×10^{-3} mol l^{-1}.

3.9. The relation between H_2O_2 and the HO• yield

The amount of HO• was influenced directly by H_2O_2 concentration. The relationship between H_2O_2 and the HO• yield was tested. The results showed that hydroxyl radicals had a linear relation with H_2O_2 concentration in the range from 0.03 to 0.33% (Fig. 7). In this paper 0.3% of H_2O_2 was chosen.

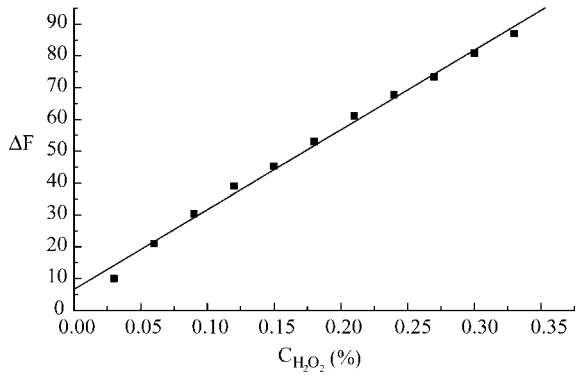


Fig. 7. The relation between H_2O_2 and the HO• yield.

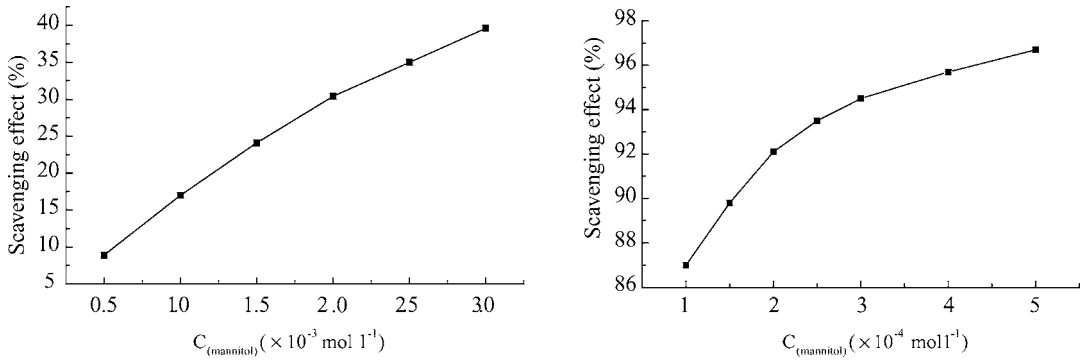


Fig. 8. The relation between scavenging effect and scavengers concentration.

3.10. Effect of interferences

The influence of foreign ions was studied with 0.3% H_2O_2, which yielded the same amount of HO• as 1.0×10^{-6} mol l^{-1} sodium 2-hydroxyterephthalate calculated according to the linear equation. An error of ±5% in the relative fluorescence intensity was considered tolerable. No interference was encountered from (tolerance ratio in mol): K^+, Zn^{2+}, Cl^-, Na^+, F^-, $SO_4{}^{2-}$, Br^-, $NO_3{}^-$, $B_4O_7{}^{2-}$ (over 1000); I^-, $NH_4{}^+$, table sugar, glucose (500); $PO_4{}^{3-}$, Mn^{2+}, Cd^{2+}, sorbitol (300); Ba^{2+}, Al^{3+}, Mg^{2+}, lactin, DL-tyrosine, DL-tryptophan (100); Ca^{2+}, Ba^{2+} (20); Pb^{2+} (10); Cu^{2+} (5); Fe^{2+} (2); Sn^{2+} (1). The interference experiment proved that most components in food extracts had no or little effect on the determination of HO• (shown in Table 2). This indicated that the proposed method had better selectivity from other methods. Therefore, the proposed method may be useful for determination of HO• in vivo as well as for scavenging activities.

3.11. The reproducibility of the method

Eleven determinations were made, according to the experimental method. The standard deviation for the peak height was 0.78 and the relative standard deviation of the method was 0.57%, which showed that the reproducibility of the proposed method was very good.

3.12. Determination of scavenging effect of HO•

Thiourea and mannitol are the scavengers of HO•. They can reflect their scavenging capacity of HO• well, because they have no fluorescence at the measuring wavelength. Their scavenging effects on HO• can be used to verify the efficiency of the proposed method. The results shown in Fig. 8 indicate that there is a correlation between scavenging effect and scavenger concentration based on FI–spectrofluorimetric measurements, which proves that the proposed method is effective.

Table 3
Comparison of scavenging effect on HO• of some foods extracts

Food	Scavenging percent (*P*,%)
Walnut	68.7
Blank sesame	60.1
Garlic	39.3
Ginger	34.6
Peanut	28.7
Chili	21.6
Soybean	15.3
Peepery	6.8

3.13. The antioxidant capacity of samples

3.13.1. The antioxidant capacity of natural foods

The proposed method was applied to the determination of scavenging capacity of eight natural foods. The results are shown in Table 3. Among the selected natural foods, walnut and blank sesame have strong scavenging capacity of HO•, which is in agreement with the work of X-Y Xu [23] based on spectrofluorimetric detection.

3.13.2. The antioxidant effect of maize pollen polysaccharide

The scavenging effect of different-grade maize pollen polysaccharide was determined and compared to the determination with spectrophotometry [18,19]. The results are shown in Table 4. Due to the small value of kinetic rate constant of Fenton reaction, short-life of HO• and relatively low sensitivity of spectrophotometry, longer time and high concentration of H_2O_2 are needed to generate enough HO• for detection, so condition of 30 min in water bath and 3.0% H_2O_2 are chosen. Because of the long reaction time and high concentration of H_2O_2, the HO• amounts generated in spectrophotometry were much more than in the proposed method (only 3 min needed). Therefore, the reagent blank value (without scavengers) in spectrophotometry was higher than that in the proposed method; further, the scavenging percent (*P*) values were much smaller than these in the proposed method. For these reasons, the data in Table 4 were different. Being relative values, the data (*P*) themselves could account for nothing. The sequence provided by the data was significant indeed. The orderliness obtained from the two methods was accordant. The scavenging capacity of different-grade polysaccharide was in the same sequence: crude polysaccharide > 60 > 40 > 80%, and pure polysaccharide did not exhibit

Table 4
Comparison of scavenging effect on $HO^{\bullet}$ of different-grade maize pollen polysaccharide

Different-grade maize pollen polysaccharide	20%	40%	60%	80%	Crude polysaccharide	Pure polysaccharide (PBMIV)
The proposed method (*P*, %)	21.89	82.52	84.42	75.60	89.42	0
Spectrophotometry (*P*, %)	16.05	24.08	26.09	22.41	41.81	0

antioxidant capacity by two methods. The reported antioxidant capacity of polysaccharide was presumed to be related with impurity.

It is rather difficult to determine the real concentration in determination for the short-life and high activity of $HO^{\bullet}$. In this paper, the introduction of FIA led to the $HO^{\bullet}$ generation and its capture took place in an airtight system. This reduced the contacts of $HO^{\bullet}$ with oxygen and other substances in the environment, and then the chain reaction caused by $HO^{\bullet}$ could be prevented. In addition, FIA could achieve the real-time effect that $HO^{\bullet}$ was trapped immediately after its generation. All these advantages brought the results of $HO^{\bullet}$ determination more close to the real values. Compared with spectrophotometry, the developed method only needed 3 min to obtain enough amounts of $HO^{\bullet}$, so the sensitivity was higher and the precision was better. In addition, it was simple, rapid and automatic.

4. Conclusion

Hydroxyl radicals are highly reactive and are the strongest oxidants to be known. Many diseases are related to $HO^{\bullet}$. In this paper, a method was developed using sodium terephthalate as the trapper of $HO^{\bullet}$, H_2O_2 and Co^{2+} as the $HO^{\bullet}$ source; flow injection spectrofluorimetry to determine $HO^{\bullet}$ was developed. The proposed method was applied to verify scavenging effect of thiourea and mannitol in which there was obvious quantitative correlation between scavenging effect and scavengers concentration. In addition, the relationship between antioxidant capacity and the purity of maize pollen polysaccharide was validated and the experimental data indicated that pure polysaccharide had no antioxidant capacity. The results showed that the determination could exhibit the real existence of $HO^{\bullet}$, compared with spectrophotometry. Therefore, the proposed method could be used to search protecting fresh reagent and antioxidants. The method is simple, reliable and stable technique for the determination of $HO^{\bullet}$ in which instruments needed are simple, the operation is automatic, reaction product is single and its analytical speed is rapid. It is beneficial to determine samples in block. The proposed method has important application value for sieving antioxidant medicines on a large scale.

Acknowledgements

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