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A Solid-phase Fluorescent Quenching Method for the Determination of Trace Amounts of Nitrite in Foods with Neutral Red

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ABSTRACT

A simple and sensitive solid-phase fluorescent quenching method for the determination of trace amounts of nitrite in food samples has been developed. The method is based on the reaction between nitrite and neutral red, which is used as an emission reagent, to form a non-fluorescent compound in 0.1 mol/L hydrochloric acid. The fluorescent intensity was measured in 5-mm quartz cells with excitation and emission wavelengths of 548 and 609 nm, respectively. The degree of fluorescent quenching showed good linearity at concentrations of nitrite between the ranges of 40-200 μg/L. The detection limit is 8.1 μg/L and the RSD is 1.7%. The general coexisting ions do not interfere with the reaction of neutral red with nitrite. The proposed method was applied to the determination of trace amounts of nitrite in food samples with satisfactory results. In addition, the mechanism involved in the reaction was discussed.

Key words: nitrite, neutral red, solid-phase spectrophotofluorimetry, fluorescence quenching, β-cyclodextrin epichlorohydrin polymer (β-CDEP)

INTRODUCTION

Nitrite exists widely in food, natural water, and soil. In the food industry, sodium nitrite has been permitted as a curing agent for use in meat, poultry, and fish products. When nitrite is added to meat, it reacts with the muscle protein myoglobin and with the blood hemoglobin to form the cured meat color and eventually causing the pink pigment of nitrosyl haemochrome. Nitrite is a toxic compound and also reacts with the primary and the secondary amines in acidic media to form nitrosamines, which are carcinogens. Therefore, quantitative analysis of nitrite is very important for environment and food quantity control. Some papers have reported the determination of nitrite. These methods mainly focused on spectrophotometry[(1-5)], flow-injection spectrophotometry[(6-14)], gas chromatography[(15)], liquid chromatography[(16-20)], and capillary electrophoresis[(21-24)]. In addition, Mesaros and co-workers reported an electrochemical biosensor method[(25)] and Jie et al. reported using spectrophotofluorimetry with tyrosine, tryptophan, or indole[(26, 27)]. In a recent review, the authors cited 179 references on the determination of nitrite[(28)], but not all the methods are suitable for routine trace determinations. Spectrophotometry often suffers from poor sensitivity and interference from some anions. Electrochemometry is severely subjected to interference from nitrate. HPLC and CE suffer from more or less time-consuming procedures.

In the present work, neutral red was used as an emission reagent, nitrite as a fluorescence quenching reagent and β-cyclodextrin epichlorohydrin polymer (β-CDEP) as a support. A novel solid-phase fluorescence quenching method for the determination of trace amounts of nitrite in foods has been developed. This method has shown several important advantages such as higher sensitivity than those of spectrophotofluorimetry and spectrophotometry, low interference level, the use of conventional instrumentation and the simultaneously preconcentration and fluorescent quenching of nitrite. Solid-phase fluorescent quenching method using β-CDEP seems to be an excellent and useful technique for the determination of trace amounts of constituents. This paper describes a procedure for β-CDEP solid-phase fluorescent quenching method and the determination of trace amounts of nitrite in ham and sausage samples using neutral red and a 5-mm quartz cell.

MATERIALS AND METHODS

I. *Reagents and Apparatus*

(I) *Apparatus*

A model Hitachi 850 spectrofluorometer (Hitachi Ltd., Japan) matched with a 5 mm quartz cell was used for all fluorescence measurements. The band passes were at 20 nm for both excitation and emission monochromators. The

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light source was a 150 W Xenon lamp. A Nicolet 20SXC FT-IR spectrometer (Nicolet Instrument Corporation, Madison, WI, USA) was used to record the IR spectra of β-CDEP. All pH-values of solutions were measured using a digital pH-meter, model Orion 290A (Orion Research Inc., Boston, MA, USA). A thermostatic rotatory shaker model Peking SHZ-2 (Beijing Analytical Instrument Company, Beijing, China) was used for the inclusion procedure.

(II) *Reagents*

All chemicals are analytical reagent grade or better. Milli-Q water (Millipore Company, Bedford, Mass, USA) was used throughout the experiment.

A standard stock solution of 200 μg/mL sodium nitrite was prepared by dissolving 0.1 g of ultra-pure grade sodium nitrite (Aldrich, Aldrich Chemical Company Inc., Milwaukee, WI, USA) in 500 mL of Milli-Q water. Working solution of 10 μg/mL sodium nitrite was prepared from the stock solution by appropriate dilutions.

The 0.01% neutral red solution was prepared by dissolving 0.05 g of neutral red (Aldrich, Aldrich Chemical Company Inc., Milwaukee, WI, USA) in 500 mL of Milli-Q water.

Ham and sausage samples were purchased from local markets of Tianjin. Before use, the samples were pretreated according to the standard procedure[29]. The extraction solutions of the samples were stored in a refrigerator at about 4˚C.

II. *Procedure*

(I) *Synthesis of β-cyclodextrin epichlorohydrin polymer (β-CDEP)*

β-CDEP was synthesized according to the following procedure. 40 g of β-CD (β-cyclodextrin), 10 g of soluble starch, and 100 mL of 20% sodium hydroxide were added into a beaker. The mixture was vigorously stirred at 50~60˚C for an appropriate period until the reactants were dissolved. Sixty mL of epichlorohydrin (ECH) was added drop-wise into this solution, and β-CDEP was formed in 30 min. After washing seriatim with Milli-Q water and acetone 5~6 times, the polymer was dried at 100˚C, ground and sieved into 40~60, 60~80, 80~100, and over 100 mesh fractions. The polymers were stored at room temperature (20˚C) in a desiccator before use.

(II) *Procedure of determination*

0.6 mL of 0.01% neutral red solution, 0.8 mL of 3.0 mol/L hydrochloric acid, and 0.5 g of β-CDEP were added to a 50-mL stoppered conical flask. An appropriate quantity of working solution of 10 μg/mL sodium nitrite or sample solution was added. Finally, the mixture was made to 25 mL with water. After shaking mechanically at a rotatory rate of 40 rpm at room temperature (20˚C) for 10 min, the insoluble neutral red-included β-CDEP would settle and be transferred to a 5-mm quartz cell using a pipette. The fluorescence intensity of the neutral red-included β-CDEP was measured at 609 nm, with excitation at 548 nm. All fluorescence intensity measurements were corrected with a blank.

RESULTS AND DISCUSSION

I. *Characterization of β-CDEP*

Figure 1 shows the FT-IR spectra of β-CD and β-CDEP. The differences between the FT-IR spectra suggest cross-linkage of β-CD in β-CDEP. The peaks of C-H stretching (2890-2880 cm^{-1}) and bending (1480~1280 cm^{-1}) from normal alkanes, CH_2Cl rocking and wagging band (1190~1070 cm^{-1}) and the C-Cl broad band (700~420 cm^{-1}) confirm the formation of a polymer with the addition of ECH. Meanwhile, the similarities between the spectra of β-CD and β-CDEP also indicate that the basic structural units are preserved in the polymer.

II. *Excitation and Emission Spectra of Neutral Red*

The excitation and emission spectra of neutral red in the presence of nitrite and of the reagent alone obtained under the condition of 0.1 mol/L hydrochloric acid and 60~80 mesh β-CDEP are shown in Figure 2. It can be seen that the excitation and emission peaks of neutral red appeared at 548 and 609 nm, respectively, and that the fluorescence intensity at the maximum emission peak (609 nm) decreased as the concentrations of nitrite increased. Therefore, E_x 548 nm and E_m 609 nm were chosen as the operating wavelengths for our experiment.

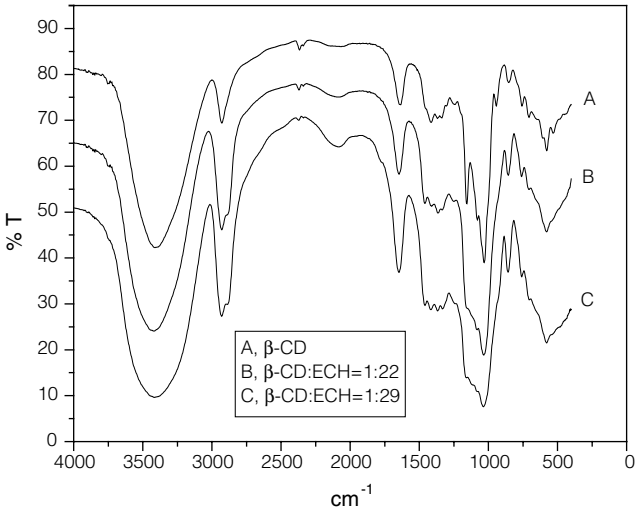

Figure 1. The FT-IR spectra of β-cyclodextrin epichlorohydrin polymer (β-CDEP) and β-cyclodextrin (β-CD).
A: β-CD; B: β-CDEP (β-CD: ECH = 1:22); C: β-CDEP (β-CD: ECH = 1:29)

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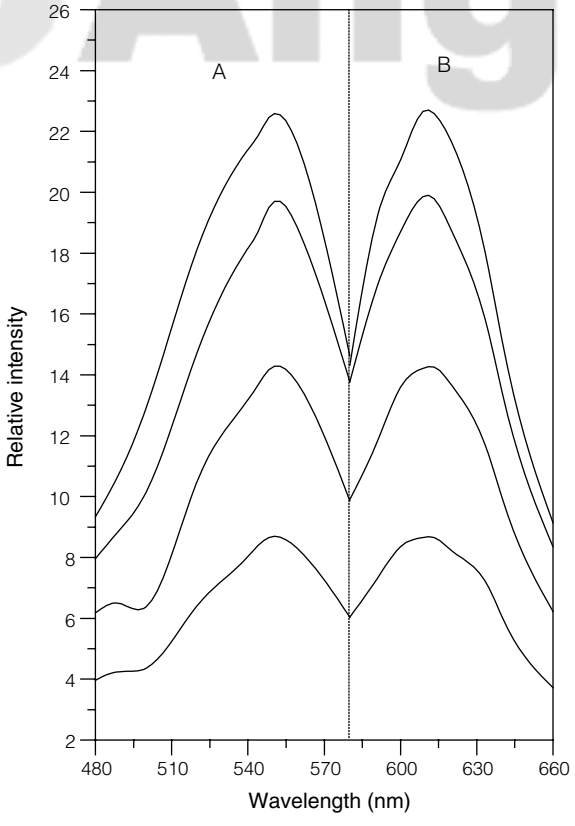

Figure 2. Fluorescence spectra of neutral red.
Excitation with 548 nm (A) and emission with 609 nm (B); nitrite concentration: (from top to bottom) 0, 40, 120, 200 μg/L, respectively; hydrochloric acid concentration: 0.1 mol/L; 0.01% neutral red: 0.6 mL; β-CDEP (60-80 mesh): 0.5 g; total volume: 25 mL; shaking time: 10 min.

III. *Conditions of Measurement*

(I) *Effect of the concentrations of hydrochloric acid*

The effect of the concentrations of hydrochloric acid on the fluorescent quenching of the neutral red-nitrite system was determined according to the above procedure. The results are shown in Figure 3. The maximum fluorescent quenching is obtained when the added quantities of 3.0 mol/L hydrochloric acid are more than 0.8 mL. This added quantity of hydrochloric acid was therefore chosen for further experiments.

(II) *Effect of the concentration of neutral red*

The effect of the concentration of neutral red on its fluorescence intensities is shown in Figure 4. It can be seen that the maximum fluorescence intensity is obtained when the added quantity is 0.6 mL. Therefore, 0.6 mL of 0.01% neutral red was selected as the optimum quantity of neutral red for further studies.

(III) *Effect of the β-CDEP size*

The effect of the β-CDEP size is shown in Figure 5. It can be seen that neutral red that was included by 40~60 or 60~80 mesh β-CDEP has the same fluorescence intensities and as particle sizes increased, the fluorescence intensities decreased. This is due to the poor transparency of the small particle sized β-CDEP. In a previous study[30], the authors found that β-CDEP with small particle size had larger inclusion capacity than β-CDEP with large particle size. It is desirable that neutral red in the solution is included by β-CDEP as completely and quickly as possible. In addition, the separation of the polymer from the equilibrated solution and its packing into a sample cell should be simple. For this reason the 60~80 mesh β-CDEP was used for our analysis.

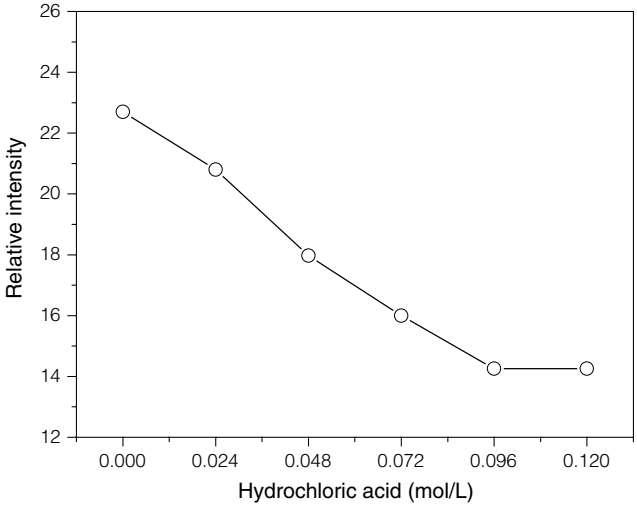

Figure 3. Effect of hydrochloric acid concentration on fluorescence quenching of neutral red.
E_x = 548 nm; E_m = 609 nm; 0.01% neutral red: 0.6 mL; β-CDEP (60~80 mesh): 0.5 g; 10 μg/mL sodium nitrite: 0.3 mL; total volume: 25 mL; shaking time: 10 min.

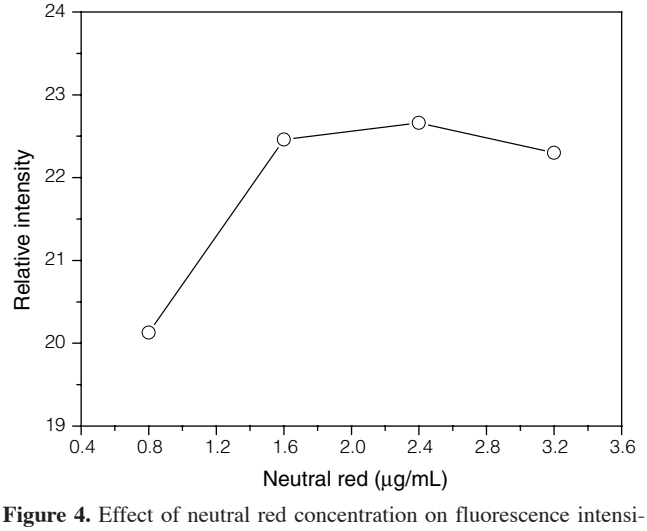

Figure 4. Effect of neutral red concentration on fluorescence intensities.
E_x = 548 nm; E_m = 609 nm; 3.0 mol/L hydrochloric acid: 0.8 mL; β-CDEP (60~80 mesh): 0.5 g; total volume: 25 mL; shaking time: 10 min.

(IV) *Fluorescent life of neutral red*

The fluorescent lives in polymer and in solution were determined and the results are shown in Figure 6. We found that the fluorescent life is shorter in solution (15 min) than that in polymer. In the presence of β-CDEP, neutral red has very stable fluorescent intensity, which can maintain about 600 min. This is because neutral red molecules were included into the cavities of β-CD to from a solid supramolecular complex. Therefore, the fluorescent stability and intensity of neutral red increased.

(V) *Effect of shaking time*

In general solid-liquid separations, in order to extract the analytes on the solid adsorbent, the liquid sample was often stirred with the adsorbent for a fixed time. Shaking was adopted instead of stirring in the present work. In such conditions, no destruction of the polymer particles occurred, but often observed when stirred very rapidly. The shaking time required for attaining the adsorption equilibrium depended on the system concerned. All neutral red in a 25 mL sample solution was extracted onto β-CDEP within 10 min by shaking, as shown in Figure 7.

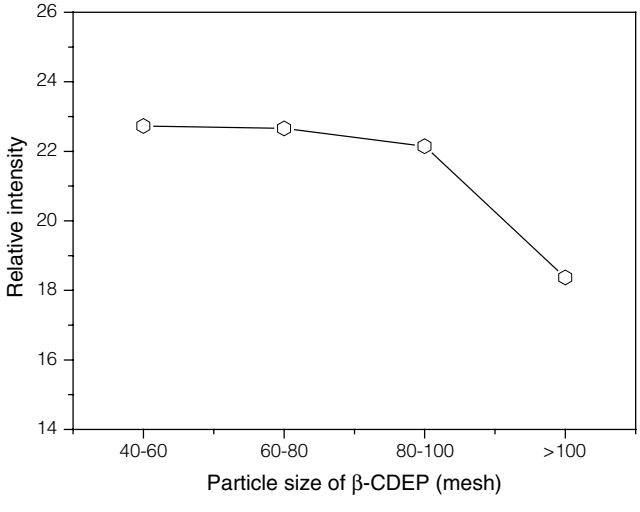

Figure 5. Effect of β-CDEP particle size on fluorescence of neutral red.
E_x = 548 nm; E_m = 609 nm; 0.01% neutral red: 0.6 mL; 3.0 mol/L hydrochloric acid: 0.8 mL; β-CDEP: 0.5 g; total volume: 25 mL; shaking time: 10 min.

(VI) *Calibration, precisions, and detection limit of nitrite*

The calibration curve of nitrite was constructed in the concentration range of 40~200 μg/L under the optimum conditions. The calibration curve of nitrite with good linearity is expressed by the equation:

$$\Delta F = 22.66 - 0.07 \bullet C_{NaNO_2}, \quad R = 0.9991$$

Wherein, ΔF denotes the relative fluorescent intensity of neutral red, and C_{NaNO_2} represents the concentration of nitrite (μg/L) in sample solutions, respectively.

The precision of the proposed method for five replicate determinations at 40 μg/L of sodium nitrite was 1.7% of relative standard deviation (RSD) according to the method of IUPAC[31].

The detection limit of the method for sodium nitrite was 8.1 μg/L, which was calculated as the concentration corresponding to a three-fold standard deviation (3s method)[31] obtained by five subsequently repeated measurements of a blank sample.

(VII) *Effect of foreign ions*

The effect of foreign ions on the fluorescent intensity of the neutral red-nitrite system was studied with 4.5 μg of

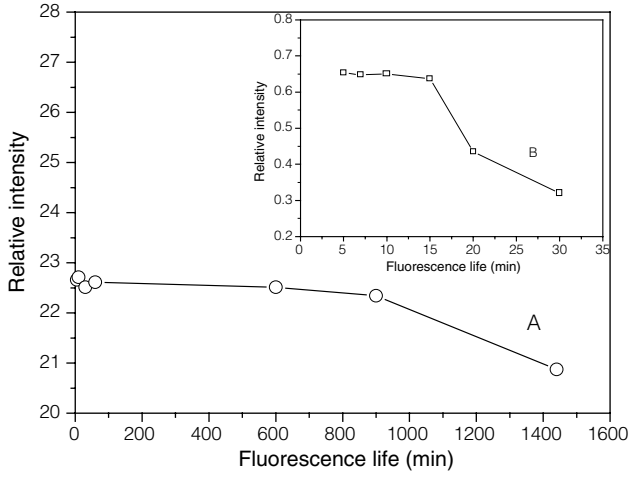

Figure 6. Fluorescence stabilities of neutral red in polymer phase (A) and in solution (B).
E_x = 548 nm; E_m = 609 nm; 0.01% neutral red: 0.6 mL; 3.0 mol/L hydrochloric acid: 0.8 mL; β-CDEP (A) (60~80 mesh): 0.5 g; total volume: 25 mL; shaking time: 10 min.

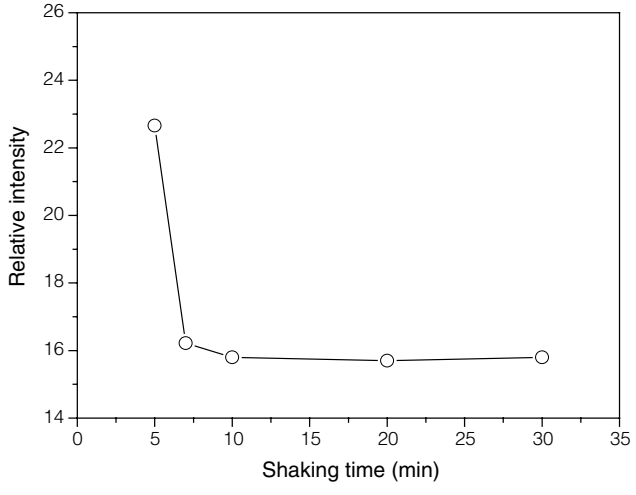

Figure 7. Effect of shaking time on fluorescence quenching of neutral red in the presence of β-CDEP.
E_x = 548 nm; E_m = 609 nm; 0.01% neutral red: 0.6 mL; 3.0 mol/L hydrochloric acid: 0.8 mL; 10 μg/mL of sodium nitrite: 0.25 mL; β-CDEP (60~80 mesh): 0.5 g; total volume: 25 mL.

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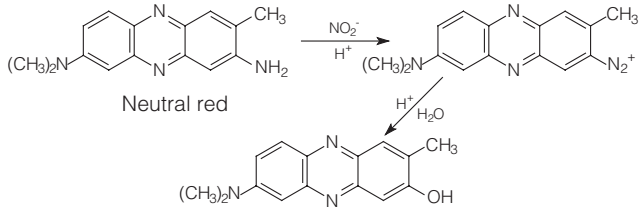
Table 1. Tolerance limits of foreign ions for the determination of nitrite*

Foreign ion	Added (μg/mL)	Error (%)	Foreign ion	Added (μg/mL)	Error (%)
F^-	40	–2.3	NH_4^+	160	–5.5
Cl^-	240	0.3	Mg^{2+}	40	4.7
Br^-	32	3.8	Ca^{2+}	120	3.1
I^-	0.2	1.9	Ba^{2+}	40	3.5
NO_3^-	4	2.4	Zn^{2+}	40	–2.3
$CH_3CO_2^-$	40	2.6	Co^{2+}	40	–3.9
SO_4^{2-}	60	–3.8	Cu^{2+}	80	–4.1
$S_2O_3^{2-}$	0.04	–5.5	Al^{3+}	12	–5.8
PO_4^{3-}	32	–4.4	Fe^{3+}	4	1.7

*: 10 μg/mL nitrite: 0.45 mL; 0.01% neutral red: 0.6 mL; 3.0 mol/L hydrochloric acid: 0.8 mL; β-CDEP (60~80 mesh): 0.5 g; total volume: 25 mL; shaking time: 10 min.



Scheme 1. The reaction mechanism of neutral red with nitrite

nitrite. The tolerance limits were determined as the maximum added quantities of foreign ions, which resulted in less than 5% RSD in the fluorescent intensity of neutral red. The results are shown in Table 1. Most common ions, including Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Cl^-, and SO_4^{2-}, were tolerated in relatively high concentration or did not interfere. In addition, it is worth mentioning that nitrate did not interfere with the determination even if the concentration of nitrate was twenty-two times of nitrite.

IV. *Mechanism of the Reaction*

Based on the above experiments, the possible reactions between neutral red and nitrite are put forward and shown in Scheme 1. Under acidic condition, nitrite was first transferred into nitrous acid, which is a strong oxidant. Then, neutral red was oxidized by nitrous acid to form its azide salt. Finally, the azide salt was hydrolyzed to obtain the corresponding phenolic species. Therefore, the fluorescence of neutral red disappeared.

V. *Determination of Samples*

The proposed method was applied to the determination of nitrite in ham and sausage samples. The results are given in Table 2. The nitrite contents in two different meat samples are 5.88 and 6.33 μg/g, respectively. In order to further check the validation of the proposed method, nitrite contents in the samples were determined simultaneously by Chinese standard method[29] and the results are also shown in Table 2. As we can see, the results obtained by the proposed method are in good agreement with those obtained by Chinese standard method.

Comparing the proposed method with the Chinese standard method, we found that the proposed method is

Table 2. The determination of nitrite in ham and sausage samples

Sample	Nitrite (μg/g)		Error (%)
	This method	Chinese standard method[29]	
Ham	5.88 ± 0.07*	5.81 ± 0.08	1.2
Sausage	6.33 ± 0.09	6.42 ± 0.10	–1.4

*: Average ± standard deviation for three determinations

obviously superior to the Chinese standard method. The sensitivity and accuracy of the present method are much higher than those of the Chinese standard method. Most of the common ions do not interfere with the measurement of nitrite with the use of the proposed method. Furthermore, the proposed method has lower detection limit (8.1 μg/L) than the Chinese standard method (50 μg/L)[29]. Concerning meats and the related products, the recovery and RSD of the Chinese standard method were 93.5% and 3.6%[29], while these for our method were 99.0% and 1.7%, respectively.

CONCLUSIONS

A novel and sensitive solid-phase spectrofluorimetry for the determination of trace amounts of nitrite with neutral red was developed. In this method, neutral red molecules were included by β-CDEP to form a solid supramolecular complex of neutral red-included β-CDEP. The fluorescent stability and intensity of neutral red were then prolonged comparing with those in solution. It is easy to pack the neutral red-included β-CDEP into a 5-mm quartz cell with the use of a pipette. The method is very convenient for the determination of 40~200 μg/L levels of nitrite. In addition, the polymer used can be regenerated by 80% ethanol. Solid-phase fluorescent quenching method seems to have more widely applicable value than traditional liquid-phase fluorescent quenching method.

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