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Effects of Standing Time during Pretreatment on the Nitrite Concentration Detected by Spectrophotometric Method

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Abstract

Food safety problems caused by excessive nitrite addition have been frequently reported and the detection of nitrite in food is particularly important. The standing time during the pretreatment of primary sample has a great influence on the concentration of nitrite tested by spectrophotometric method. In this context, three kinds of food samples are prepared, including canned mustard, canned fish and home-made pickled water. A series of standing times are placed during the sample pretreatments and the corresponding nitrite contents in these samples are detected by spectrophotometric method based on N-ethylenediamine dihydrochloride. This study aims to find out a reasonable standing time during the pretreatment of food sample, providing influence factor for precise detection of nitrite.

Keywords

Standing Time, Spectrophotometric Method, Nitrite Detection

1. Introduction

Nitrite plays a significant role in the nitrogen cycle of natural environment, and has been widely used as fertilizer, colorants or preservatives in modern agricultural and industrial products for a long time [1] [2] [3]. However, it has been proved that the industrial salts of nitrite in water and food pose a great threat to human health. The principal function of these additives is to suppress the prop-

agation of food poisoning microorganisms or to improve the colours and flavours in food products [4]. Commonly, nitrite can interfere with the oxygen transport system in human body, causing the irreversible conversion of haemoglobin to methemoglobin in blood stream and compromising the ability of haemoglobin to exchange oxygen [5] [6]. Nitrite can even react with secondary amines and amides in the stomach to form carcinogenic N-nitrosamines [7] [8]. Particularly, the hazard of nitrite is much more serious for pregnant women and infants [9]. Because nitrate can be easily reduced to nitrite *in-vivo*, it is also recognized as hazardous for human health. Due to its wide existence and toxic effects of nitrite, many countries have placed severe restrictions on the amount of nitrite utilization in processed food products. Therefore, nitrite must be used with caution.

Recently, food safety causes by excessive addition of nitrite for food color were frequently reported, and the detection of nitrite in pickled food is significantly and highly desirable [10] [11]. Many researches have been focused on nitrite detection, including the optimization of detecting method and application of new materials. As studied by Wang *et al*., more than 169 references were covered to review the various techniques for the determination of nitrite and nitrate [10]. The advantages and disadvantages including detection principles, analytical parameters and detection limits and range of each method were tabulated for evaluation and comparison [10]. Jihye *et al.* have realized the rapid detection of nitrite ions based on colorimetric hydrogel biosensor [12]. The colorimetric reaction is specific to nitrite ions, and is completed within a few seconds. So, the sensor is suitable for the detection of nitrite ions in various complex solutions including biological, environmental and clinical samples [12]. Chen *et al.* have developed a simple and convenient method for the highly sensitive and selective determination of nitrite in food [13].

The method of spectrophotometry is based on nitrite reaction (diazotization or nitrosation) with some detecting reagents, and the absorbance of product after reaction is proportional to the nitrite concentration [10]. Spectrophotometry has become an attractive method due to the simplicity, feasibility and low-cost, which is by far the most widely used method for the detection of nitrite [10]. Many spectrophotometric methods are optimized for the purposes of automation and portability. For example, Dong *et al.* has developed an automated and portable spectrophotometric analyzer for nitrite detection based on LabVIVEW, of which the method has low detection limit, high precision, excellent linearity and good repetition [14] [15]. Xiao *et al.* have established a rapid spectrophotometric method for the determination of low concentrations of nitrite in water, which shows the advantages of simple operation, low reagent dosage and high sensitivity [16]. Usually, nitrite can be detected by spectrophotometric method based on N-ethylenediamine dihydrochloride [17]. The effects, including temperature, pH value, standing time for diazotization and other factors, have been investigated [18]. However, there is no research focused on the standing time

during the pretreatment of primary sample, which has also great influence on the concentration of nitrite tested by spectrophotometric method.

In this context, three kinds of food samples are prepared, including canned mustard, canned fish and home-made pickled water. A series of standing times were placed during the sample pretreatments and the corresponding nitrite contents in these samples were detected by spectrophotometric method based on N-ethylenediamine dihydrochloride. Compared to the standing time (15 - 25 min) in the conventional method, the most reasonable standing time is three hours (3.0 h) in this experiment. It is indicated that three hours of standing time is helpful for the detection of the real and accurate value of nitrite in food samples. The aim of this study is to find a reasonable standing time during the pretreatment of food sample, which provides an influence factor for the precise detection of nitrite.

2. Apparatus and Reagents

2.1. Apparatus

PH Meter PHS-3C Model (Shanghai Yueping Scientific Instrument Corporation, China) and visible spectrophotometer 723n Model (Qingdao Juchuang Environmental Protection Corporation, China) were mainly used in the experiments.

2.2. Reagents

Stock solution of standard nitrite: anhydrous sodium nitrite (0.1 g) was firstly dried in oven for 1 h under temperature of 110˚C - 120˚C, and then was dissolved in water. The solution was filled to volume of 500 ml through volumetric flask, and stored in brown bottle after mixing well. The standard solution of sodium nitrite was diluted into the concentration of 5.0 μg/ml, which was formed as standard application solution of sodium nitrite before experiment.

Potassium hexacyanoferrate (II) solution: potassium hexacyanoferrate (II) (106.0 g) was dissolved and mixed well in 1000 ml of water.

P-aminobenzenesulfonic acid solution: p-aminobenzenesulfonic acid (0.4 g) was dissolved and mixed well in 100 ml of hydrochloric acid solution (20%), and then the solution was stored in brown bottle in dark place.

N-ethylenediamine dihydrochloride solution: N-ethylenediamine dihydrochloride (0.2 g) was dissolved and mixed well in 100 ml of water, and then stored in brown bottle in dark place.

Saturated borax solution: borax deca hydrate (5.0 g) was dissolved in 100 ml of hot water, which would be cooled down before experiment.

Zinc acetate solution: zinc acetate (220.0 g) was dissolved and mixed well in 30 ml of acetic acid, which would be diluted to 1000 ml with water.

All the reagents of analytical pure grade were used and all the solutions were diluted with distilled water.

3. Experimental Methods and Results

3.1. Different Absorption Wavelengths

2.0 ml of nitrite solution standard application solution (0.2 μg/ml) and 2.0 ml of P-Aminobenzenesulfonic acid (4 g/L) were respectively added to 50 mL of volumetric flask, which was shook well and set for 5 minutes of standing time. And then, 1.0 ml of N-ethylenediamine dihydrochloride (2 g/L) was added and mixed well, which was set for 15 minutes of standing time in dark. Finally, distilled water was added to the constant volume and shake well. Spectrophotometer was used to measure the absorbance values at the wavelength of 400 nm, 410 nm, 430 nm, 450 nm, 470 nm, 500 nm, 510 nm, 520 nm, 530 nm, 538 nm, 540 nm, 550 nm, 560 nm, 570 nm, 580 nm, 590 nm and 600 nm, respectively. Blank reagent was used as a control to detect the most suitable absorption wavelength of nitrite. Table 1 shows the determination of absorbance value of nitrite solution at different wavelengths. Figure 1 depicts the change of absorbance value of nitrite solution at different wavelengths.

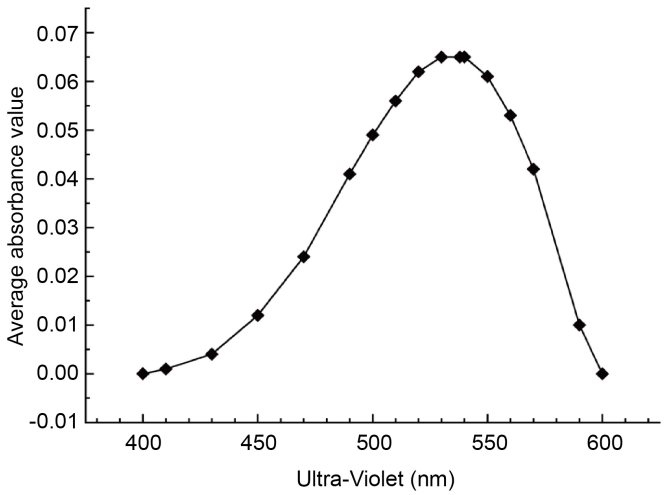

Figure 1. Change of absorbance value of nitrite solution at different wavelength.

Table 1. Determination of absorbance value of nitrite solution at different wavelengths.

Wavelengths (nm)	400	410	430	450	470
Absorbance value	0.001 ± 0.000	0.001 ± 0.002	0.004 ± 0.007	0.012 ± 0.007	0.024 ± 0.007
Wavelengths (nm)	490	500	510	520	530
Absorbance value	0.041 ± 0.006	0.056 ± 0.006	0.062 ± 0.006	0.062 ± 0.006	0.065 ± 0.006
Wavelengths (nm)	538	540	550	560	570
Absorbance value	0.065 ± 0.006	0.065 ± 0.005	0.061 ± 0.006	0.053 ± 0.006	0.042 ± 0.006
Wavelengths (nm)	590	600			
Absorbance value	0.010 ± 0.006	0.000 ± 0.000			

3.2. Detection of Standard Curve

The volume of 0.00 ml, 0.20 ml, 0.40 ml, 0.60 ml, 0.80 ml, 1.00 ml, 1.50 ml and 2.0 ml of standard application nitrite solution with the concentration of 5.0 ug/ml were accurately pipetted into eight volumetric flasks (50 ml). The nitrite concentration in the eight volumetric flasks would be diluted into 0.00 μg/ml, 0.02 μg/ml, 0.04 μg/ml, 0.06 μg/ml, 0.08 μg/ml, 0.10 μg/ml, 0.15 μg/ml and 0.20 μg/ml, respectively. Subsequently, 2.0 ml solution of P-Aminobenzenesulfonic acid was respectively added to each volumetric flask and mixed well, of which the standing time was 5 minutes. Subsequently, 1.0 ml solution of N-ethylenediamine dihydrochloride was added and mixed well, and distilled water was added to the marked volume of 50 ml. The standing time of all the solutions was 15 minutes. And then, absorbance values of all the samples were respectively tested visible spectrophotometer at the wavelength of 538 nm, according to the zero volumetric flask (number 0). Finally, linear regression equation was acquired according to the absorbance values and the nitrite concentrations (μg/ml) of the standard sample solutions. Figure 2 depicts the standard curve for nitrite concentration.

3.3. Different Standing Time of Samples

Three kinds of food samples were pretreated for testing, including canned mustard, canned fish and home-made pickled water. Figure 3 shows the pictures of the three food samples under different standing times (control, 0.25 h, 0.5 h, 1.0 h, 2.0 h, 3.0 h, 4.0 h, 5.0 h, 6.0 h). Firstly, 5.0 g (accurate to 0.01 g) of the slurries of three samples were weighted and put in 50 mL beakers, respectively. 12.5 ml of saturated borax solution was added and mixed well. 300 ml of water (~70˚C) was used to wash the sample solution into volumetric flask (500 ml) and treated in boiling water bath for 15 minutes. The sample solutions would be mixed well

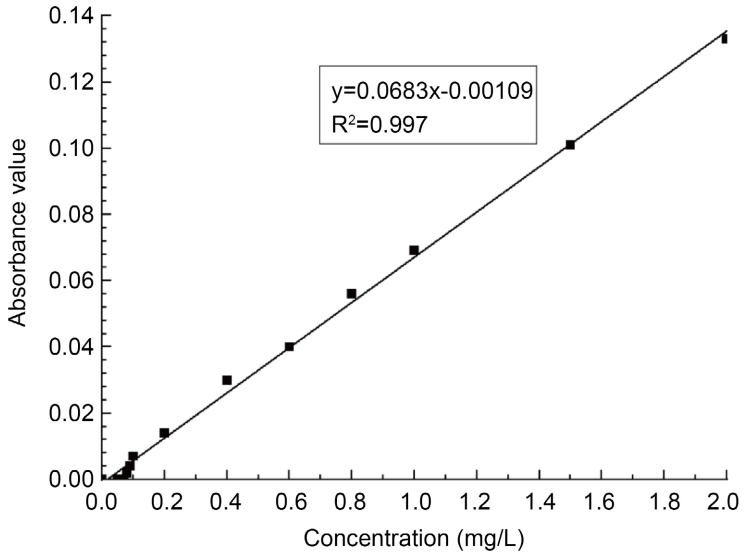

Figure 2. Standard curve for nitrite concentration.

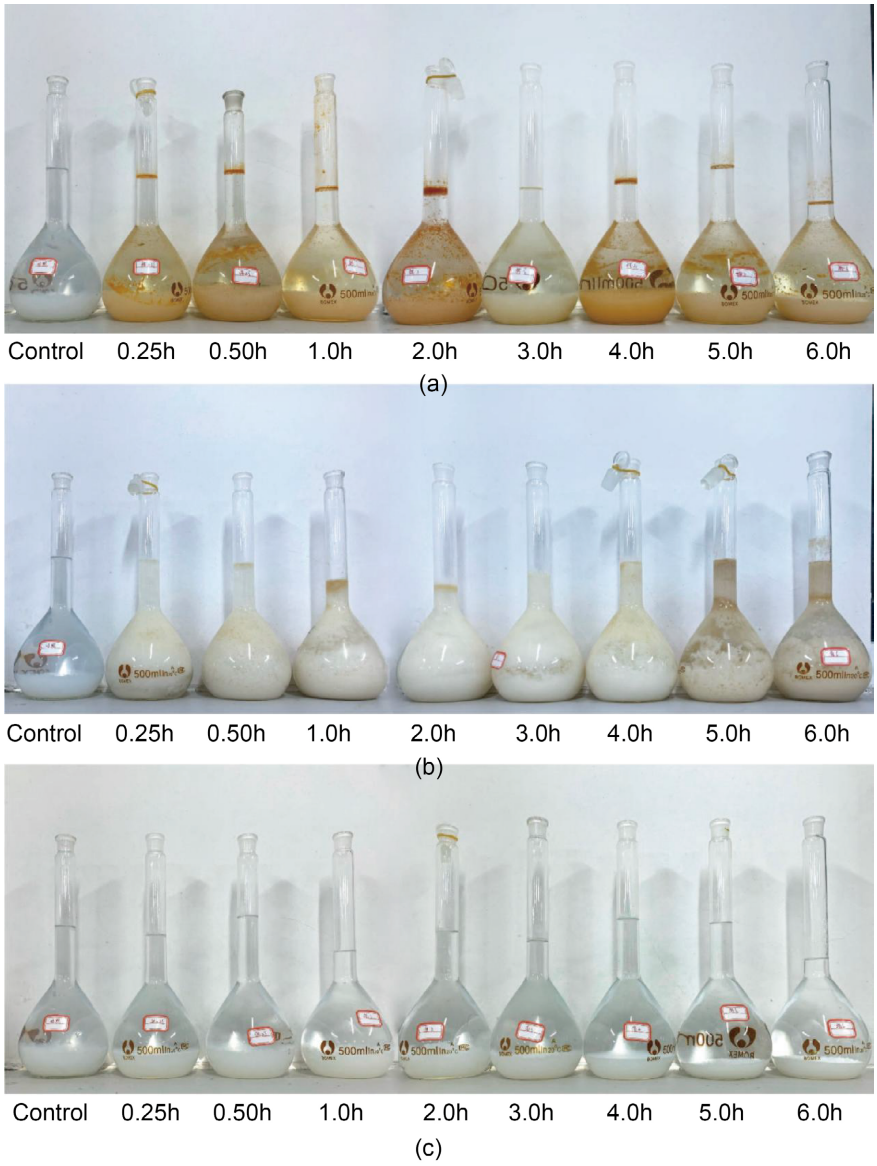

Figure 3. Different standing time (control, 0.25 h, 0.5 h, 1.0 h, 2.0 h, 3.0 h, 4.0 h, 5.0 h, 6.0 h) of the three preserved foods pretreatment solution. (a) Canned mustard pretreatment solution; (b) Canned fish pretreatment solution; (c) Home-made pickled pretreatment solution.

and cooled down to room temperature. Subsequently, 5.0 ml solution of potassium hexacyanoferrate (II) was added and mixed well with the sample solutions, 5.0 ml solution of zinc acetate was added and mixed well with the sample solutions. And then, the sample solutions was filled to 500 ml with distilled water and mixed well with the sample solutions. Nine kinds of standing time of each sample solution were respectively seted, including 0.25 h, 0.5 h, 1.0 h, 2.0 h, 3.0 h, 4.0 h, 5.0 h and 6.0 h. After that, the oil layer floating on top of the sample solution was removed, and the supernatant was filtered with filter paper. Finally, the filtrate, discarding ~30 ml of the primary filtrate, was finally used for nitrite testing. Blank tests of these samples were carried out at the same time. Table 2

Table 2. Absorbance values of three sample solutions under different standing times.

Time (h)	0.25	0.5	1	2
Canned mustard	0.090 ± 0.002	0.012 ± 0.002	0.018± 0.001	0.034 ± 0.002
Canned fish	0.010 ± 0.002	0.015 ± 0.002	0.025± 0.001	0.027 ± 0.002
Home-made pickled	0.080 ± 0.002	0.013 ± 0.002	0.019± 0.002	0.027 ± 0.002
Time (h)	3	4	5	6
Canned mustard	0.040 ± 0.002	0.025 ± 0.001	0.011 ± 0.002	0.004 ± 0.002
Canned fish	0.036 ± 0.001	0.028 ± 0.001	0.021 ± 0.003	0.010± 0.001
Home-made pickled	0.034 ± 0.002	0.025 ± 0.002	0.017 ± 0.001	0.014 ± 0.003

shows the absorbance values of the three sample solutions under different standing times. Figure 4 depicts the changes of absorbance value in the three sample solutions with different standing time.

3.4. Content of the Samples

Appropriate amount of sample solution was enough for determination, which was accorded to the nitrite content in the sample solution. In this experiment, ~20 ml of sample filtrates (three food samples) and blank filtrate were pipetted into two volumetric flasks (50 ml). The additions and treatments of reagents for the three food samples and blank sample were the same as that for the standard samples. Finally, the absorbance values of all the samples could be tested by visible spectrophotometer. After subtracting the blank sample, the nitrite concentrations of the three food samples could be calculated through the linear regression equation.

Nitrite content was calculated by $x = \frac{\rho \times 50 \times 500}{m \times V}$ (In nitrite solution), in which ρ was the nitrite solution concentration (μg/ml) calculated by the regression equation, v was the volume of sample (ml), m was the sample mass (g). Table 3 shows the calculated results of nitrite content in three food samples.

4. Discussions

4.1. Effects of the Absorption Wavelength

The absorption spectra of different substances have different characteristics. As shown in Table 1 and Figure 1, the absorbance of 0.2 μg/ml nitrite solution in the wavelength range of 400 - 600 nm was parabolic, and the absorbance value of nitrite in the range of 530 - 540 nm was the highest, suggesting that the detection of nitrite content was optimal in this range. In this experiment, the absorption wavelength of nitrite detection was 538 nm, which was consistent with the detection wavelength of GB 5009.33-2016.

4.2. Standard Curve Determination

Electrochemistry, chromatography and photometry are the commonly used

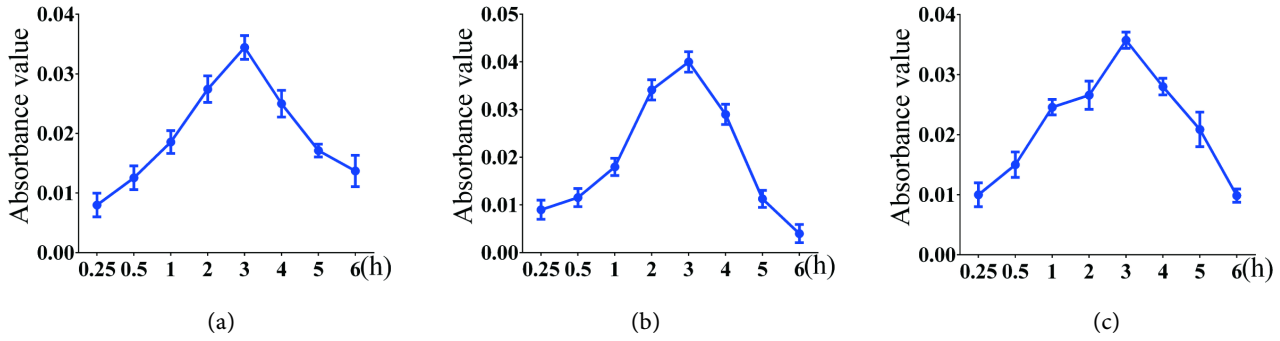

Figure 4. Changes of absorbance value in cured food with different standing time. (a) Different standing times of canned mustard; (b) Different standing times of canned fish; (c) Different standing times of home-made pickled.

Table 3. Determination of nitrite content in three food samples.

Time(h) / Sample (mg/kg)	0.25	0.5	1	2	3	4	5	6
Canned mustard	0.45	0.66	1.10	2.00	2.40	1.50	0.6	0.16
Canned fish	0.50	0.86	1.50	1.60	2.20	1.70	1.2	0.50
Home-made pickled	0.40	0.70	1.10	1.60	2.20	1.50	1.0	0.76

methods for nitrite detection. Electrochemical method can realize fast analysis with high accuracy and high sensitivity. But, the equipment system is expensive and poor repeatability. Chromatography has a wide linear range of detection and strong anti-interference. But, the equipment is really expensive and time-consuming. Spectrophotometric method has good repeatability, economical, high accuracy and simple operation. But, it is easily interfered by the impurities. This study is aimed to find a suitable standing time to overcome the influence of impurities during the detection by N-ethylenediamine dihydrochloride spectrophotometry. Suitable standing time can improve the accuracy of nitrite detection. The absorbance values corresponding to nitrite concentrations (μg/ml) in the eight standard sample solutions were measured. The standard curve in this experiment was shown in **Figure 2**. Linear regression equation ($y = 0.0133x + 0.0015$, $R^2 = 0.9991$) could be acquired according to absorbance values Vs nitrite concentrations (μg/ml). The minimum detection limit of nitrite in this experiment was 0.008 mg/L.

4.3. Effects of the Standing Time

The study of this experiment only focused on the influence of standing time on the tested nitrite concentration. Except for the standing time, all the operations during the pretreatment of food samples were kept the same as the conventional pretreatment method. Totally, eight standing times (0.25 h, 0.5 h, 1.0 h, 2.0 h, 3.0 h, 4.0 h, 5.0 h, 6.0 h) were used to pretreat the sample solutions. According to different standing times, the pictures of pretreatment liquids for the three samples, canned mustard, canned fish and home-made pickled water, were shown in

Figures 3(a)-(c), respectively. And, the absorbance values for the three sample solutions were given in Table 2.

Compared with the control group, the pretreatment liquids of canned mustard and canned fish food showed a distinct three layers, with an oil phase in the upper layer, and the middle layer was the aqueous phase of nitrite. The lower layer was the protein and other macromolecular precipitates, where the three layers of mustard food were more stratified and do not change significantly at different quiescent times. The three layer stratification was less pronounced for canned fish food, and the stratification was more pronounced with increasing time for different standing times. The preconditioned solution of home-made pickled water shows two distinct layers, the upper layer being the aqueous phase of nitrite and the lower layer being the macromolecular precipitate of proteins, among others. However, there was no significant difference between the standing times of different samples.

According to N-neethylenediamine hydrochloride spectrophotometry, the pretreated solutions of food samples standing in alkaline conditions for 15-30 minutes were usually conducted to remove the impurities, including fats and proteins. As depicted in Figures 4(a)-(c), the absorbance values of nitrite in different preserved foods show a parabolic dose-response relationship. Absorbance values of the three samples increase with the standing time during preprocessing, which reach a maximum at 3 h and then gradually decrease.

4.4. Nitrite Content in Food Samples

As shown in Table 3, the measured value of nitrite concentration in the three pickled food samples was presented as parabola according to the standing time. The measured value of nitrite concentration firstly increased with the increasing standing time from 0.25 h to 3.0 h. And then, the measured value of nitrite concentration decreased with the increasing standing time from 3.0 h to 6.0 h. That is, half an hour of standing time during the conventional pretreatment was not enough and three hours of standing time might be the optimum value for the nitrite detection. During the first half of standing time from 0.25 h to 3.0 h, the acidic products such as fatty acids and amino acids would be released from the residual impurities of fat and protein. The acidic solution would facilitate the formation of nitrous acid, making it difficult to extract nitrite from the aqueous solution. As a result, the early detection value of nitrite was lower than the true value. However, nitrite would be oxidized with the increasing standing time from 3.0 h to 6.0 h, causing the detection value of nitrite lower than true value, too. Therefore, three hours of standing time might be most suitable for the nitrite detection by neethylenediamine hydrochloride spectrophotometry. Moreover, the nitrite content of the pickled food used in this experiment was less than the allowable residue of GB2762-2022 (20 mg/kg in nitrite solution), suggesting that the food used in this experiment was safe and reliable, and could be safely consumed.

5. Conclusion

In this research work, the influence of standing time on the nitrite concentration was tested by neethylenediamine hydrochloride spectrophotometry. Except for the standing time, all the operations during the pretreatment of food samples were kept the same as the conventional pretreatment method. Eight standing times (0.25 h, 0.5 h, 1.0 h, 2.0 h, 3.0 h, 4.0 h, 5.0 h, 6.0 h) were totally used to pretreat the sample solutions. It is found that three hours of standing time might be most suitable and accurate for nitrite detection in pickled food. Reasonable standing time could help to acquire stable sample solution, which was useful for removing the impurity residue and reducing the interferences. This study improves the spectrophotometric method at a low cost.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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