

Study on Mechanism and Application of High Concentrations of Nitrite-Induced Specific Color Fading

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Abstract: Nitrite and edible salt are easy to confuse for their similar appearance and taste, thus possibly causing nitrite poisoning. Therefore, it is particularly crucial to figure out how to screen high concentrations of nitrite, how to discriminate NaNO_2 and NaCl , and how to accurately quantify high concentrations of nitrite. In this study, the phenomenon of high concentrations of nitrite fading to orange-yellow color in colorimetric solution of naphthalene ethylenediamine hydrochloride was studied and accurately quantified by dilution colorimetry. It is to prevent false negative detection while NaCl presented no such phenomenon. Through spectrophotometry, high-performance liquid chromatography, and liquid mass spectrometry, we discovered that the principle of high concentrations of nitrite fading to orange-yellow after colorimetry was that high concentrations of nitrite nitrosated azo compounds and naphthalene ethylenediamine hydrochloride in hydrochloric acid medium. This principle can make the solution appear orange-yellow while had almost no matrix interference and this demonstrates no difference in food and water. We thus argue that this principle can be used for the screening of high concentrations of nitrite, and discriminating NaNO_2 and NaCl to prevent poisoning events caused by misuses. In this sense, we believe the principle in question has a high applying value and is worth promoting. It can be made into a commercial hydrochloric acid acidified naphthylethylenediamine hydrochloride solution for further commercialized applications.

Keywords: Nitrite, Discoloration, Study, Application

1. Introduction

Nitrite is a relatively common chemical substance in nature. In the food industry, it is often used as food additives, such as colorants, preservatives, etc [1-2]. Because it also has the effect of inhibiting *Clostridium botulinum*, coloring meat, and enhancing flavor, it is often used as an additive in meat processing [3-5]. However, Excessive nitrite can react with amine foods and convert to toxic N-nitrosamines, which may cause deformities and cancer [6-9], Methemoglobinemia of Infants (Blue Baby Syndrome) [10], spontaneous abortion [11], and central system problems [12]. In addition, nitrite can combine with hemoglobin in human blood to form methemoglobin, reducing the oxygen transport capacity of the blood, leading to tissue hypoxia [13]. It is easy to confuse nitrite with edible salt for their similar appearance and taste,

resulting in nitrite poisoning [14-23].

Nitrite can be used as a color-retaining agent and preservative in cured bacon, sauce-braised meat and other foods. In China's national standard GB 2760-2014, the maximum nitrite use acceptable in food is 0.15g/kg [24]. In China today, the determination of nitrite in food mainly adopts GB 5009.33-2016 "National Food Safety Standard - Determination of Nitrite and Nitrate in Food" [25]. In spectrophotometry, since the standard curve is prepared with low-content samples for calculation, excessively high concentration will affect the curve calculation or the calculating results, and even lead to false negatives. It is reported that when poisoning samples with high-concentration nitrite contact reagents in few seconds, the purple-red color fades to orange-yellow completely [18, 26], and this is easy to form false negatives, then eventually resulting in mis judgment. However, concerning the question

of how the fading reaction occurs, there are comparatively few in-depth studies exploring that. In this paper, we addressed the gap through the utilization of low, medium, and high concentrations of nitrite standard solutions and sample solutions in colorimetry, and used high performance liquid chromatography and high-performance liquid chromatography-mass spectrometry to qualitatively study the solution before and after fading, eventually figured out the mechanism of fading reaction. The principle has certain scientific research value.

2. Experimental Part

2.1. Instruments and Reagents

UVmini-1285 UV-Vis Spectrophotometer (Shimadzu, Japan); Agilent 1200 High Performance Liquid Chromatograph (Agilent, USA); Waters ACQUITY TM Ultra-High Performance Liquid Chromatography-Xevo®-TQ-S Mass Spectrometer (Waters, USA), Mass Lynx V4.1 workstation; Oasis HLB (200 mg, 6 mL) solid phase extraction column (Waters, USA).

Sodium borate, potassium ferrocyanide, zinc acetate (dihydrate), p-aminobenzenesulfonic acid, naphthylethylenediamine hydrochloride, sodium nitrite, etc. are analytical reagents.

The experimental samples were the samples collected after a food incident by the Hunan Provincial Center for Disease Control and Prevention in 2020 and two kinds of Chinese marinating sauces purchased by ourselves. There were three kinds of Chinese marinating sauces, a marinated beef tripe, a salted egg yolk, and a marinated steamed bean curd roll.

2.2. Sample Preprocessing

Marinated beef tripe, marinated steamed bean curd roll, and salted egg yolk: used the quartering method to take an appropriate amount or all of it, used a food grinder to make a homogenate, and set [25].

Chinese marinating sauces: Mixed all samples well by stirred or repeatedly shook and inverted the container [25].

2.3. Spectrophotometry

2.3.1. Extract

For extraction of solid samples and liquid samples, we used China's GB 5009.33-2016 National Food Safety Standard Determination of Nitrite and Nitrate in Food as the reference to determine [25].

2.3.2. Determination of Nitrite

(i). Standard Curve Configuration and Color Rendering

(a) Sodium nitrite standard solution (60 mg/mL)

We accurately weighed 3.000g of sodium nitrite, then had it dried to constant weight at 110°C to 120°C, and put it in a 50 mL volumetric flask. We then added water to dissolve and diluted to the mark, shook well for later used.

(b) The configuration and color development of low,

medium and high concentration series of nitrite

We took an appropriate amount of 2.3.2.(i).(a) sodium nitrite standard solution (60 mg/mL) and diluted it step by step, referred to GB 5009.33-2016 National Food Safety Standard for Determination of Nitrite and Nitrate in Food [25] for color development step, then measured the absorbance, and did the reagent blank at the same time.

The concentrations of low concentration series solutions were 0.00 µg/mL, 0.02 µg/mL, 0.04 µg/mL, 0.08 µg/mL, 0.12 µg/mL, 0.16 µg/mL, 0.20 µg/mL; the concentrations of the medium concentration series solutions were 4.00 µg/mL, 8.01 µg/mL, 16.02 µg/mL, 20.02 µg/mL, 30.05 µg/mL, 40.05 µg/mL, 49.98 µg/mL, 60.08 µg/mL, 80.10 µg/mL; the concentrations of the high concentration series solutions were 0.200 mg/mL, 0.400 mg/mL, 1.00 mg/mL, 2.00 mg/mL, 4.00 mg/mL, 6.00 mg/mL.

(ii). Determination of Sample Solutions

We pipetted 40.0 mL of the filtrate in the extraction step 2.3.1 into a 50 mL colorimetric tube with a stopper, and the rest of the operation steps were the same as in 2.3.2.(i).(b).

(iii). Color Development of Blank Solution

We took three 10 mL colorimetric tubes with stoppers, and numbered them respectively as A, B and C. And added 2 mL of 4 g/L sulfanilic acid solution to tube A, then we added 1 mL of 2 g/L naphthalene ethylenediamine hydrochloride solution, mixed them well; then we added 2 mL of 20% hydrochloric acid to tube B, plus 1 mL of 2 g/L naphthylethylenediamine hydrochloride solution, and mixed them well; we added then 2 mL of 4 g/L p-aminobenzenesulfonic acid solution to tube C, plus 1 mL of pure water, and mixed them well. At the final step we added 1 mL of standard sodium nitrite solution (60 mg/mL) to tubes A, B and C respectively, mixed them well, and observed the phenomenon.

2.4. High Performance Liquid Chromatography-Diode Array Detection

2.4.1. Sample Processing

We took the reagent blank after color reaction in 2.3 spectrophotometry, the 0.20 µg/mL and 1.00 µg/mL sodium nitrite standard solutions after color reaction, and passed through 0.22 µm filter membrane to be tested. We took another part of the 0.20 µg/mL and 1.00 µg/mL sodium nitrite standard solutions that had undergone color reaction, then added 1 mL of sodium nitrite standard solution (60 mg/mL), and next mixed until the color fade reaction occurred which purple red all faded and turned into orange-yellow. After passing through 0.22 µm filter membrane, it was tested.

2.4.2. Liquid Phase Conditions

HPLC column: XBridge® C₁₈ (250 mm×4.6 mm, 3.5 µm); column temperature: room temperature; mobile phase: water-methanol, 75:25 (V/V), filtered through 0.22 µm microporous membrane; flow rate: 1.0 mL/min; visible wavelength: 538 nm; injection volume: 100 µL.

2.5. High Performance Liquid Chromatography-Mass Spectrometry

2.5.1. Sample Processing

We took an HLB solid-phase extraction column (200 mg, 6 mL), then activated it with 6 mL of methanol, next equilibrated with 6 mL of water, then added 3 mL of the sample solution in 2.4.1 to control speed in 2~3 drops/second. Then we rinsed with 6 mL of water, after drying it, next eluted with 6 mL of methanol, and at last passed through a 0.22 μm filter to be tested.

2.5.2. LC and MS Conditions

Liquid phase conditions: HPLC column: WATERS ACQUITY UPLC® BEH C₁₈ (100 mm×2.1 mm, 1.7 μm); column temperature: room temperature; mobile phase: 0.1% formic acid water-methanol, 75:25 (V/V), filtered through 0.22 μm microporous membrane before used; flow rate: 0.30 mL/min; injection volume: 5 μL .

MS conditions: Electrospray ion source (ESI), positive ion mode, capillary voltage: 3.0 kV; ion source temperature: 150°C; desolvation temperature: 500°C, desolvation gas flow rate: 800 L/H; collision chamber pressure: 3.1×10^{-3} mbar. Scanning mode: precursor ion scanning mode (PIS) and multiple reaction monitoring mode (MRM) and the conditions of multiple reaction monitoring mode (MRM) before and after the fading reaction are shown in table 1.

Table 1. Table of multi-reaction monitoring mode (MRM) condition before and after fading reaction.

Parents	Daughters	Collision energy (V)	Taper hole voltage (V)
371.4	354.4	18	55
	328.4	21	55
	311.4	22	55
217.2	187.2	12	25
	186.2	12	25
	172.2	18	25

2.6. Validation Experiment

We took a small amount of 1.00 $\mu\text{g/mL}$ sodium nitrite standard solution that had undergone color reaction in 2.5.1 sample processing and 2 g/L naphthalene ethylenediamine hydrochloride solution and put them in a 10 mL centrifuge tube, and next added an appropriate amount of 20% hydrochloric acid solution, and then added 1 mL of sodium nitrite standard solution (60 mg/mL), next added an appropriate amount of pure water, at last shook well.

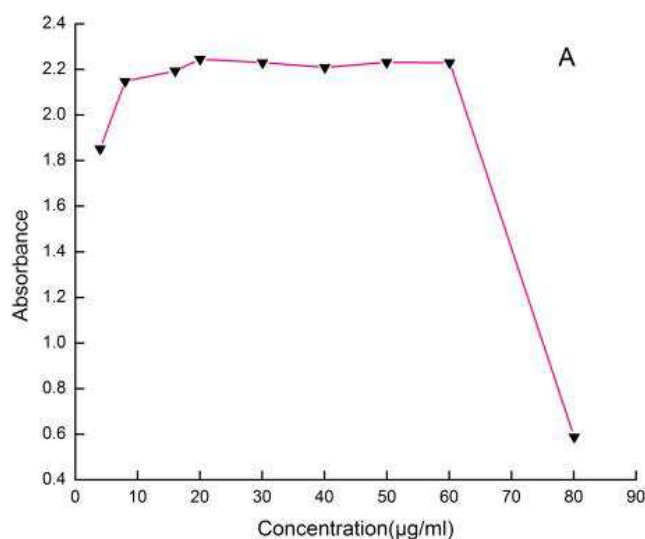
3. Results and Discussion

3.1. Analysis of Spectrophotometric Results

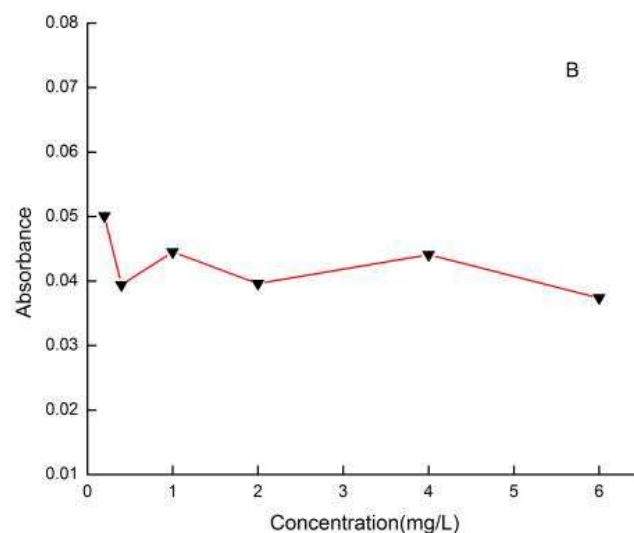
3.1.1. Low, Medium and High Concentration Series of Nitrite Determination Results

For the low concentration series, we took the concentration C ($\mu\text{g/mL}$) as the abscissa x and the absorbance A as the ordinate y, then drew the standard curve $y=3.1226x+0.0294$, $r=0.9998$. In the medium concentration series, we found that the absorbance increased slightly at the beginning, then

maintained at about 2.2, and next decreased rapidly when the concentration was 80.10 $\mu\text{g/mL}$. In the high concentration series, we found that the absorbance was maintained at 0.04-0.05, as shown in figure 1.



A. medium concentration series;



B. high concentration series

Figure 1. Relationship between nitrite concentration and absorbance.

3.1.2. Investigation and Analysis of Fading Time

After adding the color developer, when the nitrite concentration exceeded 20.02 $\mu\text{g/mL}$, we left it for 15 minutes, and the color development would be unstable. When the concentration reached 80.10 $\mu\text{g/mL}$, obvious discoloration began to appear; when it reached 0.20 mg/mL~6.00 mg/mL, the liquid level quickly turned purple-red, and then gradually faded to orange-yellow. The fading time is shown in table 2, it is believed that high concentrations of nitrite may react with the color-developing substance in a specific manner, thereby destroying the structure of the color-developing substance and fading. The higher the nitrite concentration, the faster the reaction occurs, the shorter the fading time will be.

Table 2. Fading time.

Concentration (mg/mL)	0.20	0.40	1.00	2.00	4.00	6.00
Fading time	15min	2min	1min	20s	5s	fade immediately

3.1.3. Matrix Interference Experiment

We took a set of practices in accordance with the recovery rate to spiked extraction and the other group used extracts such as Chinese marinating sauces, marinated beef tripe, marinated steamed bean curd roll, and salted egg yolk as diluents respectively which color development experiments were performed on the nitrite concentrations of 16 $\mu\text{g/mL}$, 40.05 $\mu\text{g/mL}$, 0.20 mg/mL and 4.00 mg/mL in the two groups respectively. The result was the same as that of the nitrite standard solution of the same concentration; it showed that the fading reaction has little relationship with the matrix, and there was no matrix interference phenomenon.

Table 3. The contents of nitrite in three Chinese marinating sauces, marinated beef tripe, salted egg yolk and marinated steamed bean curd roll.

Name	Content (mg/kg)
Chinese marinating sauce 1	6684
Chinese marinating sauce 2	<1
Chinese marinating sauce 3	<1
marinated beef tripe	1.5
Salted egg yolk	<1
marinated steamed bean curd roll	<1

3.1.4. Sample Measurement Results

One serving of Chinese marinating sauces contained a high concentration of nitrite (as sodium nitrite) at 6684 mg/kg. The detected amount in marinated beef tripe was 1.5 mg/kg,

which met the standard of residual amount ≤ 30 mg/kg in terms of sodium nitrite (potassium) [24]. The rest were not detected, as shown in table 3. Among them, during the determination of Chinese marinating sauce 1, after adding the chromogenic agent, the solution quickly turned purple-red, and then quickly turned orange-yellow, and the result could not be determined. The sample was diluted 1000 times and re-measured.

3.1.5. Color Development of Blank Solution

Tube A and tube B turned orange-yellow, tube C was achromatic, as shown in table 4. The upper end of the C tube produced a brown-red gas. After shaking, the color faded. There were bubbles in all tubes, and most bubbles appeared in tube A and B. It could be seen that the nitrite in tube C generated nitrous acid when it encountered hydrochloric acid. This weak acid was unstable and easy to decompose, and emitted colorless NO gas, which was oxidized into brown-red NO₂ gas in the presence of oxygen in the air [18]. After shaking, there was no orange-yellow color and the color faded, the absence of orange-yellow color indicated that NO₂ cannot oxidize the p-aminobenzenesulfonic acid solution while the color fading indicated that it dissolves in water and forms nitric acid. The presence of orange-yellow color in both tube A and B demonstrated that the nitrite content may be too high, nitrosating or oxidizing the azo compound and naphthylethylenediamine hydrochloride.

Table 4. Added reagent and solution color of A and B and C colorimetric tube with stoppers.

Reagent and solution color	Pipe number	A	B	C
4-aminobenzenesulfonic acid		+	-	+
Naphthylethylenediamine hydrochloride		+	+	-
Color		Orange-yellow	Orange-yellow	Achromatic

Note: "+" means adding this reagent, "-" means not adding this reagent; there are hydrochloric acid matrix in tubes A, B and C.

3.2. Results and Analysis of High Performance Liquid Chromatography-Diode Array Detector

Figure 2 shows the blank chromatogram, 0.20 $\mu\text{g/mL}$ and 1.00 $\mu\text{g/mL}$ sodium nitrite standard solutions after color development and fading. It can be seen from the figure that the 0.20 $\mu\text{g/mL}$ and 1.00 $\mu\text{g/mL}$ sodium nitrite standard solutions both yielded a chromatographic peak at 3.51 min after color development, while the chromatographic peak disappeared in the solution after the blank solution and the 0.20 $\mu\text{g/mL}$ and 1.00 $\mu\text{g/mL}$ sodium nitrite standard solutions faded. Combined with figure 3, the spectra of 0.20 $\mu\text{g/mL}$ and 1.00 $\mu\text{g/mL}$ sodium nitrite standard solutions were basically the same after color development, but the intensity was slightly different. It is not difficult for us to conclude that this chromatographic peak was the chromatographic peak of the color-developing substance after color development.

After adding a high concentration of nitrite, the chromatographic peak disappeared, the color-developing substance was destroyed, and some chemical reaction may occur, which changed the structure of the color-developing substance.

3.3. Results and Analysis of High Performance Liquid Chromatography-Mass Spectrometry

Figure 4 shows the color development and fading of the blank solution and the sodium nitrite standard solutions in concentration of 0.20 $\mu\text{g/mL}$ and 1.00 $\mu\text{g/mL}$. It can be seen from the figure that after the color rendering of both sodium nitrite standard solutions, the parent ion scanning m/z was 371.4, and a chromatographic peak appeared at 1.50 min, while the chromatographic peak disappeared in the solution after the blank solution and the 0.20 $\mu\text{g/mL}$ and 1.00 $\mu\text{g/mL}$ sodium nitrite standard solutions faded. Combined with the

color reaction principle [27] (figure 5) and the molecular weight of the parent ion, the chromatographic peak was the chromatographic peak of the color-developing substance after color development. After exploring the mass spectrometry conditions, some product ions were scanned in MRM mode under the optimal conditions. The results are shown in figure 6. Combined with the m/z of the product ion, a mass spectral fragmentation process can be demonstrated in figure 7. After adding a high concentration of nitrite, the chromatographic peak disappeared, the color-developing substance was destroyed, and some chemical reactions occurred, thus changing the structure of the color-developing substance.

At the same time, we scanned the parent ions after the color development of the 0.20 $\mu\text{g/mL}$ sodium nitrite standard

solution after fading, and we found out that the m/z was 217.2 and a chromatographic peak appeared at 1.20 min (see figure 8). After exploring the mass spectrometry conditions, some product ions were scanned in MRM mode under the optimal conditions. The results are shown in figure 9. Combined with the m/z of the product ion its mass spectral fragmentation process can be demonstrated as Figure 10 shows. It can be inferred that the fading reaction principle consist of high concentrations of nitrite nitrosated azo compounds and naphthylethylenediamine hydrochloride in a hydrochloric acid medium. The specific process is shown in figure 11, it was consistent with the phenomenon that aromatic secondary amines underwent nitrosation with sodium nitrite in hydrochloric acid to form yellow oily substances [28].

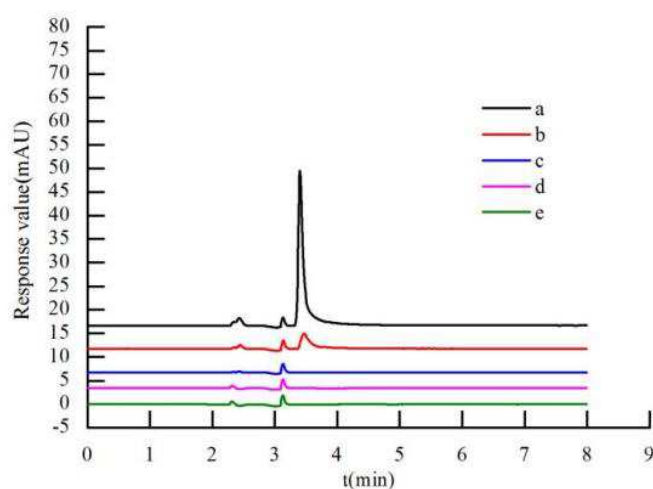


Figure 2. Chromatogram in blank, chromatogram after colour reation and fading reaction in 0.20 $\mu\text{g/mL}$ and 1.00 $\mu\text{g/mL}$ of sodium nitrite standard solutions.

- a. Chromatogram after colour reation in 1.00 $\mu\text{g/mL}$ of sodium nitrite standard solution
- b. Chromatogram after colour reation in 0.20 $\mu\text{g/mL}$ of sodium nitrite standard solution
- c. Chromatogram in blank
- d. Chromatogram after fading reaction in 0.20 $\mu\text{g/mL}$ of sodium nitrite standard solution
- e. Chromatogram after fading reaction in 1.00 $\mu\text{g/mL}$ of sodium nitrite standard solution

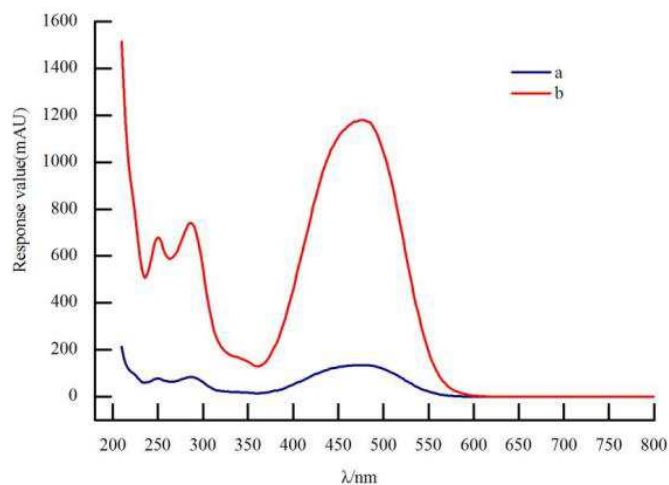


Figure 3. Spectrograms after colour reation in different concentrations of sodium nitrite.

- a. Spectrogram after colour reation in 0.20 $\mu\text{g/mL}$ of sodium nitrite standard solution
- b. Spectrogram after colour reation in 1.00 $\mu\text{g/mL}$ of sodium nitrite standard solution

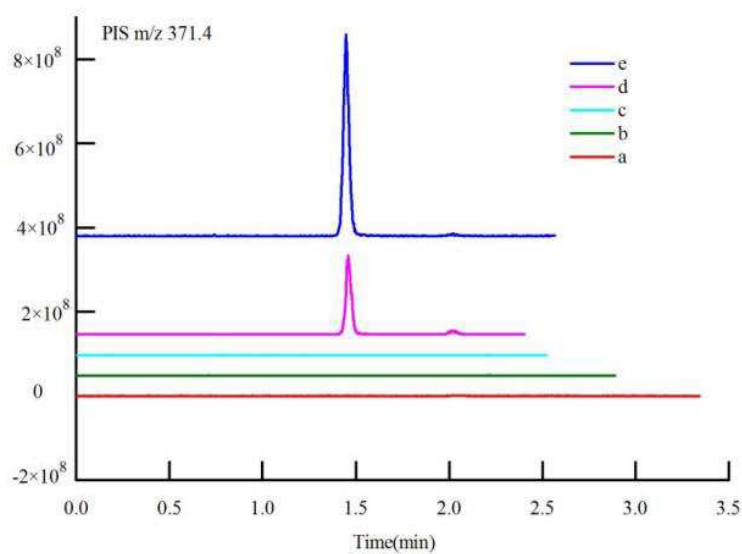


Figure 4. Parent ion scan in blank, parent ion scan after colour reation and fading reaction in 0.20 $\mu\text{g/mL}$ and 1.00 $\mu\text{g/mL}$ of sodium nitrite standard solutions.

- a. Parent ion scan in blank
- b. Parent ion scan after fading reaction in 0.20 $\mu\text{g/mL}$ of sodium nitrite standard solution
- c. Parent ion scan after fading reaction in 1.00 $\mu\text{g/mL}$ of sodium nitrite standard solution
- d. Parent ion scan after colour reation in 0.20 $\mu\text{g/mL}$ of sodium nitrite standard solution
- e. Parent ion scan after colour reation in 1.00 $\mu\text{g/mL}$ of sodium nitrite standard solution

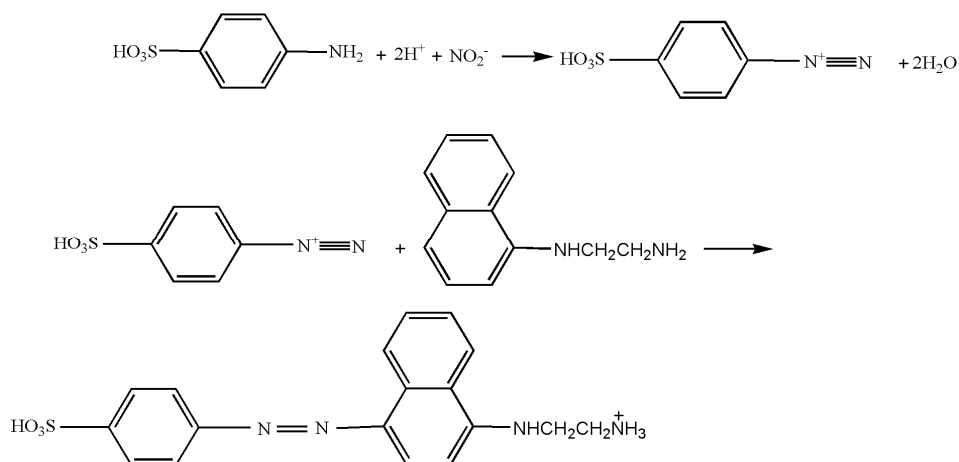
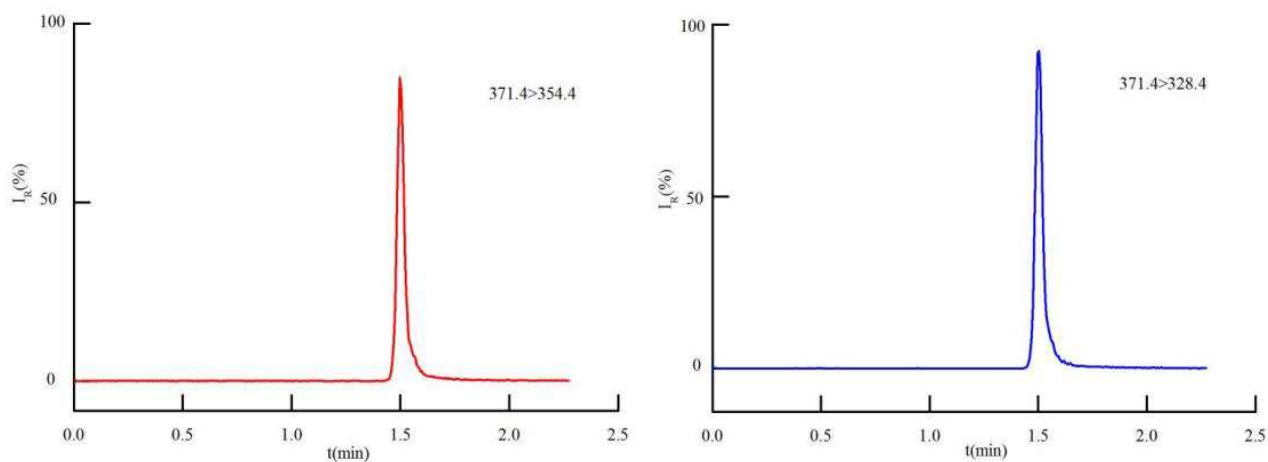


Figure 5. Figure of color rendering principle of nitrite.



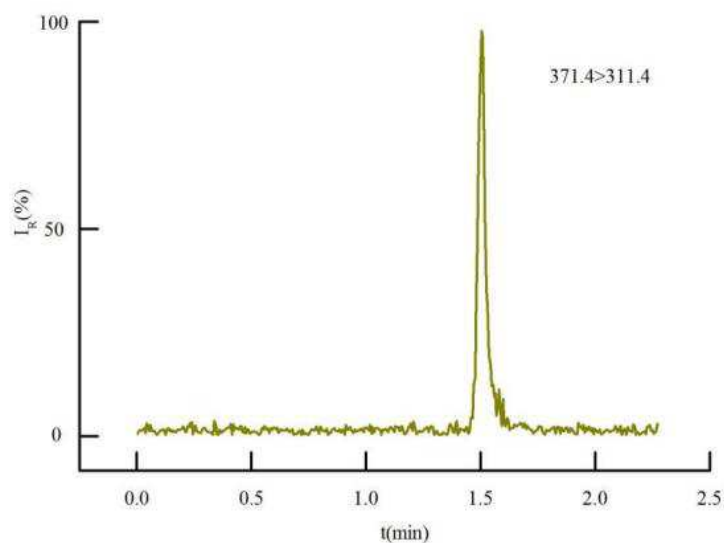


Figure 6. Chromatograms of partial daughter ions in chromogenic substance after colour reation.

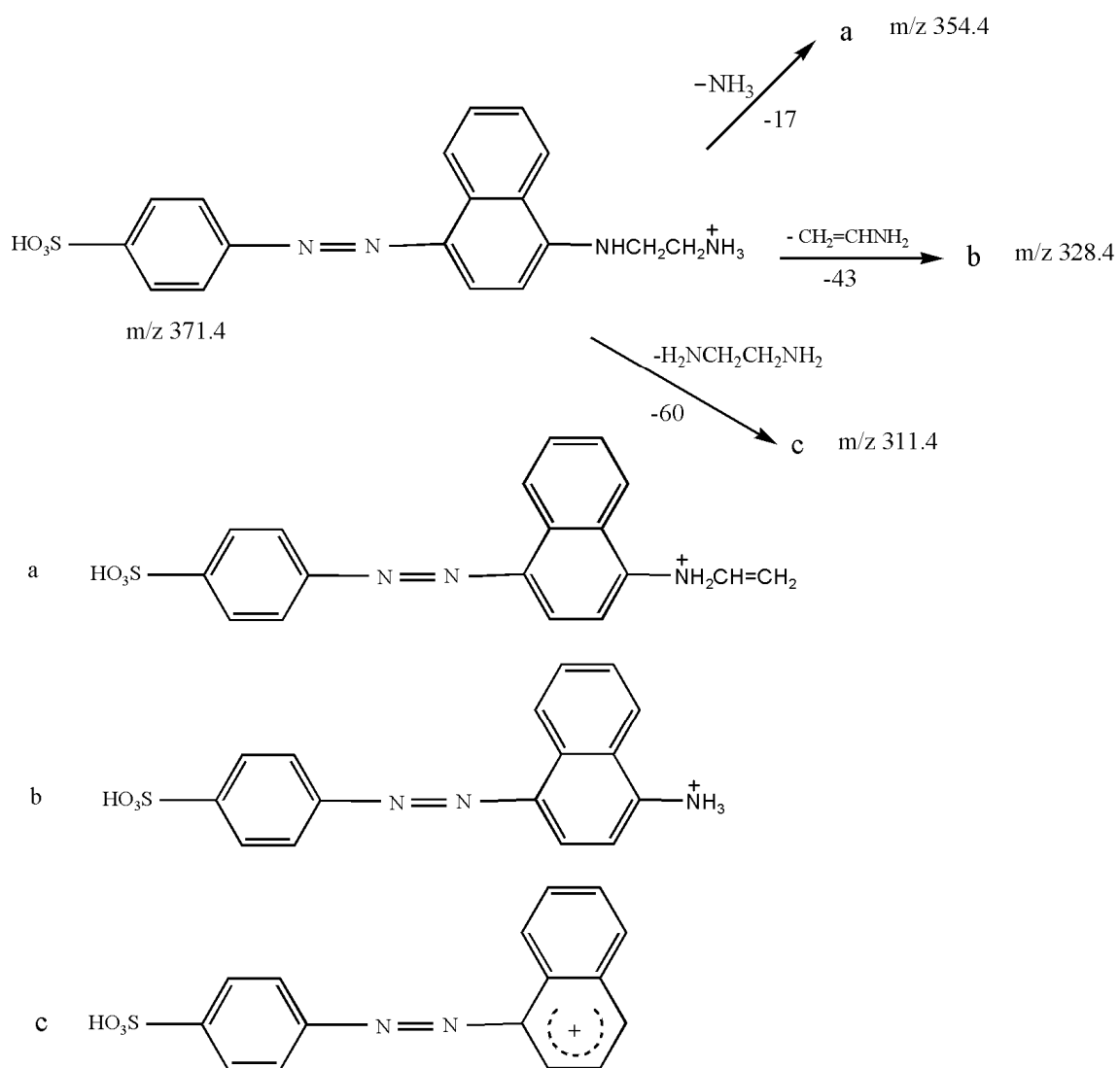


Figure 7. Partial mass spectrogram of chromogenic substance after colour reation.

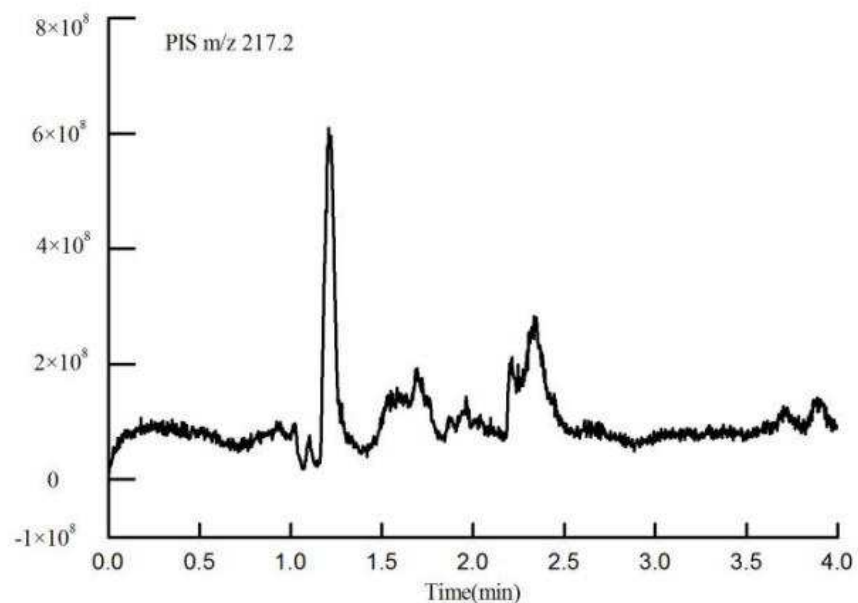


Figure 8. Parent ion scan after fading reaction in 0.20 µg/mL of sodium nitrite standard solution.

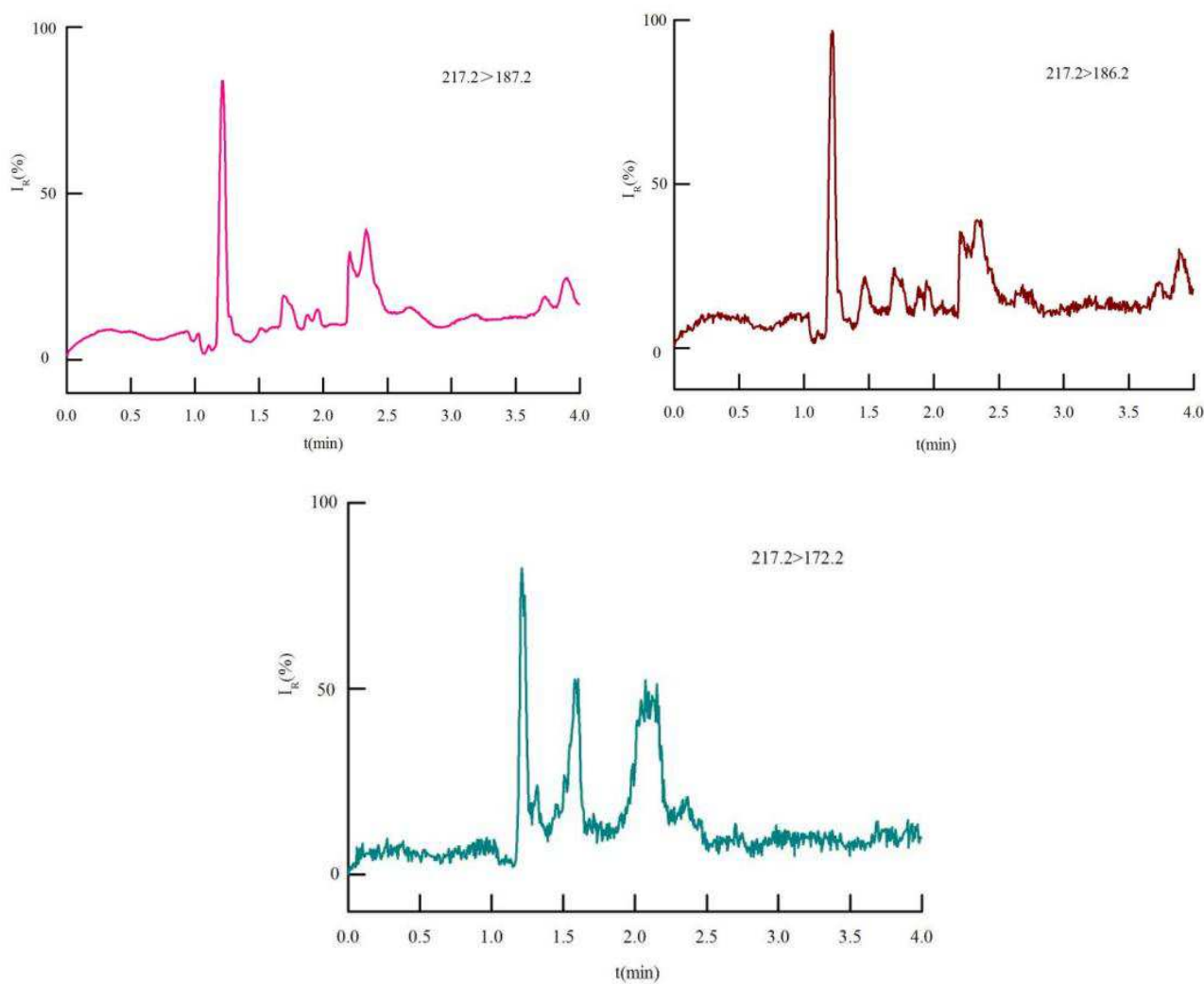


Figure 9. Chromatograms of partial daughter ions in chromogenic substance after colour reaction and fading reaction.

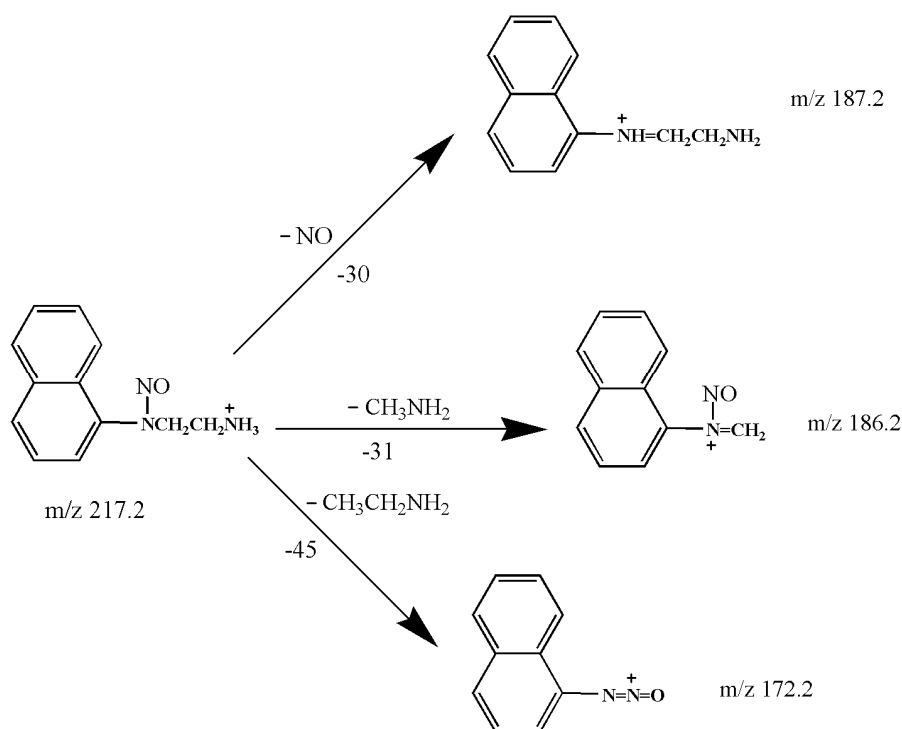


Figure 10. Partial mass spectrogram of chromogenic substance after fading reaction.

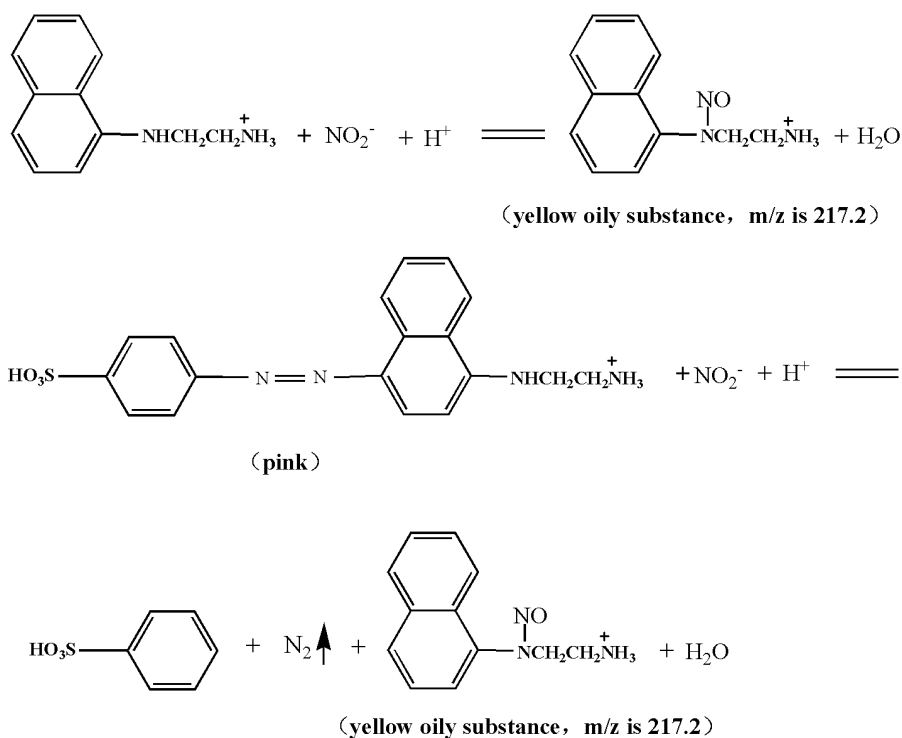


Figure 11. Principles of fading reaction.

3.4. Verify the Experimental Results

The 1.00 µg/mL sodium nitrite standard solution that had undergone color reaction was treated with the sample in 2.5.1 and the 2 g/L naphthylethylenediamine hydrochloride solution showed orange-yellow after reacting with a high concentration of nitrite in the medium of hydrochloric acid

(see figure 12). It suggested that when high concentrations of nitrite nitrosated azo compounds and naphthalene ethylenediamine hydrochloride were both in hydrochloric acid medium, the solution appeared orange-yellow. Red gas was produced at the upper end of the tube containing 2 g/L naphthaleneethylenediamine hydrochloride solution, which was similar to the color development study of the blank solution in 3.1.5.

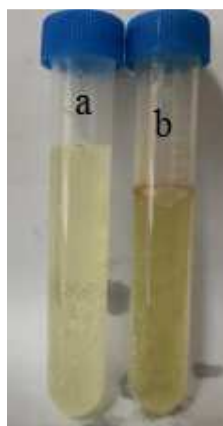


Figure 12. Figure of verification experiment.

- a. 1.00 µg/mL of sodium nitrite standard solution after fading reaction;
b. 2 g/L naphthylethylenediamine hydrochloride solution after fading reaction.

4. Conclusion

Through spectrophotometry, high performance liquid chromatography, liquid mass spectrometry, and verification experiment, it can be inferred that high concentrations of nitrite nitrosated azo compounds and naphthalene ethylenediamine hydrochloride in hydrochloric acid medium can make the solution appear orange-yellow. When in the food matrix and water, the same phenomenon appeared and barely had matrix interference. The reaction principle is shown in figure 11. Therefore, we can argue that the occurrence of such phenomenon contains a high concentration of nitrite, which can be diluted for further quantification to prevent the false negative and misdetection. The first reaction in figure 11 can be used for the primary screening of high concentrations of nitrite, and it can also be used to screen NaNO_2 and NaCl to prevent poisoning caused by the misuse of NaNO_2 as NaCl . It can be made into a commercial hydrochloric acid acidified naphthylethylenediamine hydrochloride solution for further commercialized applications.

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