Original Research Article

GRIES – ILOSVAY SPECTROPHOTOMETRY FOR DETERMINATION OF NITRITE IN WATER AND VEGETABLES IN VIETNAM

ABSTRACT

Nitrite ions are one of harmful compounds for human health. In addition to the available state, nitrite ions are converted from nitrate ions by microbial reduction in the natural environment. When entering our body, they will react with hemoglobin to form methemoglobin by oxidation of ferrous iron, leading to dangerous methemoglobinemia, also known as blue-baby syndrome. In this study, the qualification of nitrite in water and vegetables samples is based on Griess reaction between sulfanilic acid with 1-Naphthylamine. The absorbance was measured at 524 nm after 15-25 minute reaction in acid environment (pH 2.5) at room temperature. There is a good tolerance towards high amount of interfering ions such as nitrate, chloride and ferric ions. The range of linearity was found to be 0.04 ÷ 1.1 mg/L of nitrite. The limit of detection and limit of quantitation were found to be 3.79 µg/mL and 12.6 µg/mL, respectively. Under these validated conditions, Gries - Ilosvay reagents were successful in quantifying concentrations of nitrite in water and fresh vegetables collected in the South of Vietnam.

Key Words: Nitrate, Nitrite, UV-VIS spectrophotometry, Gries - Ilosvay reagent

Comment [ANH1]: ??
What is the meaning of qualification on this sentence

1. INTRODUCTION

The presence of harmful nitrogen-containing compounds such as nitrate and nitrite with the increasing levels in the soil due to the use of nitrogenous fertilizers and industry wastes have led the high-level accumulation of these compounds in foods and drinks. Many green vegetables that are prepared by pickling method contain high levels of nitrate and nitrite. They are widely used in Asian countries such as Korea, China, Vietnam, Thailand ... The content of nitrate, nitrite varies according to each vegetable species, as well as other conditions such as conditions of cultivation, preparation and preservation.... There have been many studies warning the carcinogenic potentials of these pickled vegetables. However, they are still commonly used because they bring lots of nutritional values to the community. According to many studies, nitrite can react with hemoglobin to form methemoglobin based on the oxidation of ferrous II iron (Fe^{2+}) to the ferric III state (Fe^{3+}). This interaction leads to preventing or reducing the ability of blood to transport oxygen, known as methemoglobinemia which is a dangerous disorder especially in children, so-called blue-baby syndrome (Cemek et al., 2007). Methaemoglobin has no oxygen-binding and carrying ability, thereby producing a leftward shift in oxygen-dissociation curve, one of the symptom of hypoxaemia (Özdestan and Ali Üren (2012). Nitrite, nitrate present in food is one of the precursors to many kinds of cancer, such as esophagus, stomach, colon cancer and other diseases. Nitrite can interact with secondary amines in the body to form nitrosoamines (N-nitroso compounds), some of which are known as teratogenic, mutagenic and carcinogenic diseases especially stomach cancer and esophagus in the stomach (Hsu et al., 2009, Zatar et al., 1999). Oral reduction of nitrate is the most important source of nitrite, accounting for around 70–80% of the human total nitrite exposure. Approximately 5–7% of all ingested nitrate is converted to nitrite by microbial reduction at the tongue. In some cases, due to a high rate of conversion, this number may reach 20% (Özdestan and Ali Üren (2012).

Nitrite ions have been quantitatively determined by various methods, commonly spectrofluorimetry (Huang et al., 2006), chromatography (Ferreira and S. Silva, 2008), potentiometry (Davis et al., 2000),

electrophoresis (Tagliaro et al., 2002), membrane sensors (Hassan et al., 2003). However, almost these methods are expensive, laborious, time-consuming and not environmentally friendly. Spectrophotometric determinations are recommended especially for medium-scale laboratories. Nitrite and nitrate can be determined by spectrophotometric determination using phosphomolybdenum blue complex reaction (Vo et al., 2018). However, this method can be interfered by phosphorus, silicate ions due to their similar rections. Therefore, Griess reaction is the most important widely used as an official nitrite analysis method. In this reaction, nitrite reacts with a primary aromatic amine to form a diazonium salt, which is then coupled with another aromatic compound to form an azo dye with high absorptivity. This principle has been applied using different combinations of reagents. The combinations can be sulfanilamide, sulfamethizole and sulfadimidine with sodium 1-naphthol-4-sulfonate, 4-nitroaniline with 1-naphthol, sulfanilamide with N-naphthylethylenediamine (Sastry et al., 2002), p-nitroaniline and sulfanilamide with ethyl acetoacetate, sulfanilic acid with 1-naphthol (Kiso et al., 2006), sulfanilamide with N-(1-naphthyl)ethylenediamine, 4-amino-5-hydroxynaphthalene-2,7-disulphonic acid monosodium salt, and 4-amino-3-hydroxynaphthalene-1-sulfonic acid (Masoud et al., 2015). This paper uses the reaction between sulfanilic acid as the aromatic amine with 1-Naphthylamine to form the final azo dye in the environment of Hydrochloric acid. The formation of the azo dye is affected by pH, temperature, reaction time, concentration of the reagents and other interfering ions.

2. MATERIALS AND METHOD

2.1. Materials

Nitrite stock solution of 1000 mg/L was prepared by dissolving an exact amount of 0.7575g of dried $NaNO_2$ (P = 99%) in 500 mL. The stock solution is then diluted with distilled water into working standard solutions with the following concentrations: 5 mg/L, 10 mg/L và 25 mg/L NO_2^-. Sulfanilic acid (SAA) solution was prepared by dissolving 1.5 g of sulfanilic acid crystals with 50 mL of hot water, followed by adding 2 mL of concentrated HCl and diluting to 250 mL with distilled water. Stock sulfanilic acid solution was kept in a dark bottle to be stable during the 6-month experiments. N-1-naphthylene-ethylene-diamine (NED) solution was prepared by dissolving 1.5g of α – naphtylamine in 100ml 10% of CH_3COOH, then adding 2mL of concentrated HCl and diluting to 250 mL with distilled water. Stock NED solution was kept in a dark bottle to be stable for 1 month, unless strong brown coloration developed. Buffer solution was prepared from acid monoacetic and adjusted to pH = 2.5. The ions such as $S_2O_3^-$, SCN^-, $C_2O_4^-$, Cl^-, Fe^{3+}, Fe^{2+}, SO_4^{2-}, Cu^{2+}, Zn^{2+} Ca^{2+}, Mg^{2+} interfering the nitrite reaction were prepared from their relevant crystals.

2.2. Apparatus

The absorbance of the purple azo products was measured with UV/Vis spectrometer GENESYS 20 Thermo spectronic, and transparent plastic cells. pH of solutions was measured by pH meter.

2.3. Methods

2.3.1. Development and optimization of Gries – Ilosvay method

The determination of NO_2^- is based on forming complex of NO_2^- with the reagent mixture – sulfanilic acid and alpha naphthylamine (Gries - Ilosvay reagent) at a suitable pH range. To determine the optimal wavelength, separate flasks 25 mL with different concentrations of NO_2^- : 5 mg/L, 10 mg/L và 25 mg/L were prepared with the same amount of reaction mixture including 1 mL of 0.6% sunfanilic acid, 1 mL of 0.6% α - naphtylamine, 1 mL of pH 2.5 buffer solution and finally diluted to 25 mL with the distilled

water. The reaction solution was let stable in about 10 – 15 minutes at room temperature before absorbance measurement.

The components of reaction mixture were kept unchanged and NO_2^- concentration was kept at 5 mg/L for experiments in the order to investigate the effects of pH, time reaction, threshold concentration of sunfanilic acid and 1-naphtylamine. To find out the optimal conditions, pH was changed by changing volume of buffer solution. To determine the best color development time, the reaction solution was let stable within 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 minutes, respectively before absorbance measurement. The effect of reaction reagents and interfering ions (Cl^-, SO_4^{2-}, PO_4^{3-}, Cu^{2+}, Fe^{3+}) were investigated at the optimal pH and time reaction.

After determining the optimal conditions, a calibration curve was built to calculate limit of detection (LOD), limit of quantification (LOQ) and determine nitrite concentration in samples.

2.3.2. Application of the validated method in determining nitrite in water and fresh vegetables

Well water samples are taken in families using water from drilling wells for no more than 3 hours, particularly Nguyen Du St (WT1), Le Duc Tho St (WT2) of Go Vap Distric; Dinh Dien St (WT3), Hoang Van Thu St (WT4) of Tan Binh District; Ly Thuong Kiet St (WT5) of 10 District of Ho Chi Minh city. All of these samples were directly determined by optimal conditions without sample treatment. Pickled mustard green vegetables were purchased from 4 big local markets in Ho Chi Minh city, and in different stores, particularly 3 samples from Pham Van Hai market (PVH1, PVH2 PVH3), 3 samples from Go Vap market (GV1, GV2, GV3), 3 samples from An Nhon market (AN1, AN2, AN3) and 2 samples from Le Thi Hong market (LTH1, LTH2). Total number of samples was 11. Pickled vegetable sample was crushed and immersed in hot water (approximately 60°C) to extract nitrite with the assistant of thermostatic tank, then filtered with activated carbon to remove color organic substances before determining nitrite in the filtered solution with optimized method. A defined amount of standard nitrite was added to standards to reach final concentrations as 0.05, 0.2, 0.4 mg/L, respectively to determine nitrite removal efficiency from sample treatment. The nitrite analysis was repeated three times for each prepared sample (water and pickled mustard vegetable sample).

3. RESULTS AND DISCUSSION

3.1. Development and optimization of Gries – Ilosvay method

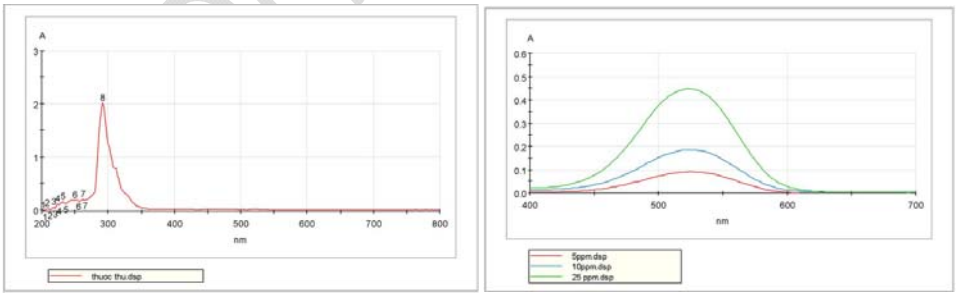


Fig.1 Absorption spectrum of color reagent (a), complex color of nitrite (b)

Condition: 1 mL of NO_2^- 5ppm, 10ppm and 25ppm, respectively. Add 1 mL of 0.6% sulfuric acid, 0.6 mL of 1% α-naphthylamine, 1 mL of buffer pH = 2.5. Response time 10 - 15 minutes, volumetric flask of 25 mL.

Figure 1(a) illustrates the absorption spectrum of the Griess - Ilosvay reagent mixture. The maximum absorbance of the reagent was recorded at at 292 nm. The complex formed by the mixing Griess - Ilosvay reagent with 1 mL of NO_2^- 5 mg/L, 10 mg/L and 25 mg/L, respectively, had the maximum absorption in the range of $\lambda = 400 \div 600$ nm, and the peak was obtained at $\lambda = 524$ nm in Fig. 1(b). Thus, the absorption of reagent did not affect the absorption of nitrite complex, and to obtain the highest sensitivity, 524 nm was chosen as the optimal wavelength for all subsequent experiments.

The mechanism of the complex reaction between nitrite ion and Griess - Ilosvay reagent consists of 2 stages. In stage 1, nitrite reacts with sulfanilic acid to form a diazo salt (Fig.2a) and then the diazonium salts react with α - naphtylamin to form a purple complex in the second stage (Fig.2b).

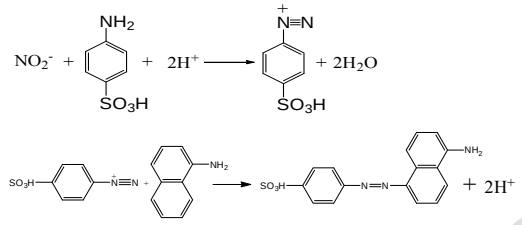


Fig.2 The mechanism of the complex reaction between nitrite and Griess - Ilosvay reagent

The low pH is favorable for the formation of the diazo salts but affects the reaction to form azo compound. Thus, pH affects the formation, durability of the complex, which should be optimized. In Figure 3, when pH was less than 2, or greater than 3, the absorbance was significantly reduced. The absorption was high and stable in the range of pH = 2 - 3, therefore the optimal pH should be in this range, especially at pH = 2.5.

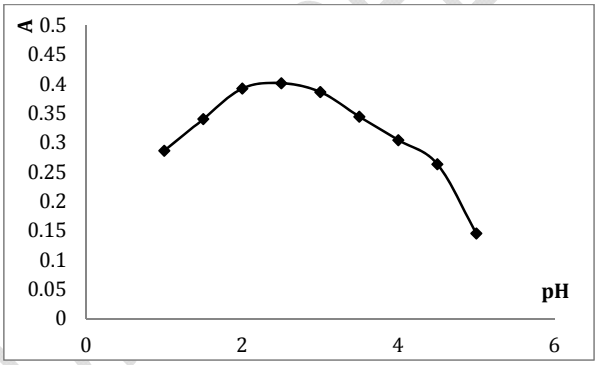


Fig.3 Effect of pH on the absorption of complexes

Condition: 2 mL of NO_2^- 5ppm, 1 mL 0.6% sulfuric acid, 0.6 mL 1% α-naphthylamine, buffer pH 1→5. Response time 10 - 15 minutes, volumetric flask of 25 mL

The formation of nitrite complex with sulfuric acid and α-naphthylamine takes place in two stages which are the formation of diazo salts and the formation of azo dyes, hence the reaction time must be long enough to react completely. Azo compounds were less stable at room temperature. Thus, if the reaction time is too long, the complex will decay. Before 15 minutes, the complex was formed very slowly and the color was not strong enough to be measured. As can be seen from Figure 4, the absorption of color

complex was almost unchanged within 15-25 minutes, the longer the reaction time lasted, the lower the absorption reduced. Therefore, the optimal color development time was within 15 - 25 minutes to obtain the highest sensitivity.

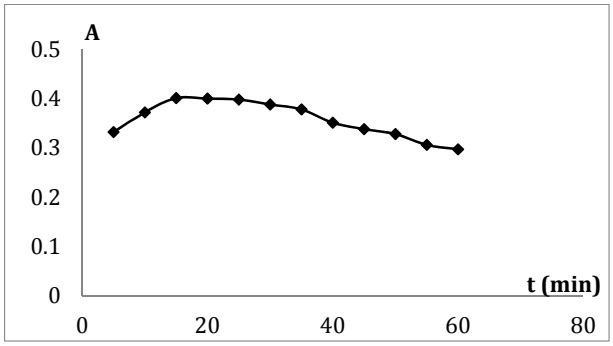
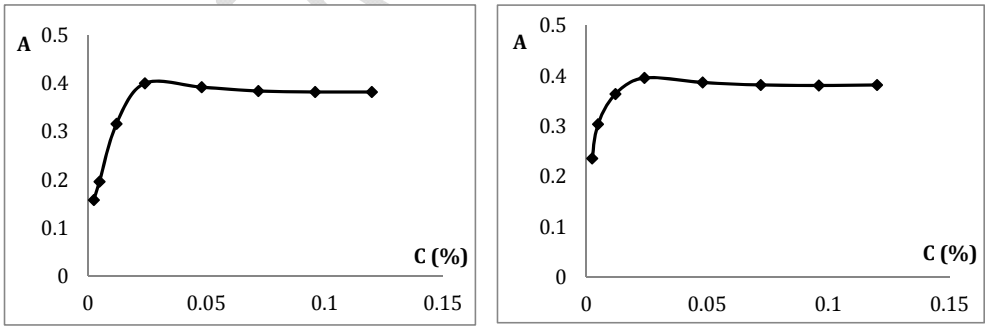


Fig.4 Effect of reaction time on the absorption of the complex

Condition: 2 mL of NO_2^- 5ppm, 1 mL 0.6% sulfuric acid, 1 mL 0.6 % α-naphthylamine, buffer pH = 2.5; response time change 5 - 60 minutes, volumetric flask of 25 mL

The components of solution were kept at 5 mg/L of NO_2^-, 1 mL of α – naptylamin 0.6%, 2 mL buffer solution pH 2.5. Sulfanilic acid 0.6% was added respectively in 8 tubes with different volumes – 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mL equivalent to 0.0024, 0.0048, 0.012, 0.024, 0048, 0.072, 0.096 and 0.12%. Figure 4a shows the effect of sulfanilic acid concentration on the optical absorption. The absorbance reduced significantly when the amount of sulfanilic acid was less than 0.024%, but then the absorbance was stable when the concentration of sunfanilic acid was more than 0.024%. Thus, 0.024% of sulfanilic acid was chosen as the most suitable threshold for the determination of nitrite. The next experiments were carried out with the same procedure, but the volume of α–naptylamine 0.6% was changed instead of sulfanilic acid. The results illustrated in Fig.4b were similar to the results of effects of sulfanilic acid. Thus, the optimal amount of α – naphtylamin was 0.024%.



(a) **(b)**

Fig.4 Effect of reagent concentration on the absorption of the complex (a) sulfanilic acid, (b) α – naphtylamin

Condition: 2 mL of NO_2^- 5ppm, sulfanilic acid (0.0024% – 0.12%), α-naphthylamine (0.0024 – 0.12%), buffer pH = 2.5; response time 15 - 25 minutes, volumetric flask of 25 mL

For the determination of NO_2^- in well water samples and vegetables, the effect of ions NO_3^-, Cl^-, Fe^{3+} was studied. Under the optimal conditions, Sb^{3+}, Au^{3+}, Hg^{2+} and Ag^+ exist as precipitation. Ion Cu^{2+} can reduce the absorption due to its catalytic decomposition of diazonium salt. Ion Fe^{3+} can increase absorbance due to its ability to create complexes with reagents under the testing conditions. As shown in Figure 5, when the concentrations of NO_3^-, Cl^- were 1000 times greater than those of NO_2^- (500 ppm), the percentage difference was about 10%. When concentration of Fe^{3+} was 100 times higher than that of NO_2^- (50 ppm), the influence on the absorption became significant.

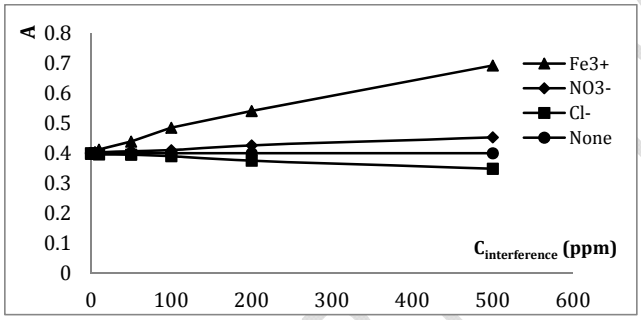


Fig.5 Effect of ion interference on the absorption of the complex (NO_2^- 0.4 ppm, NO_3^-, Cl^-, Fe^{3+} in the range of 0 – 500 ppm)

The linear range was investigated over the nitrite concentration range of 0.04 ÷ 1.4 mg/L. The curve in Fig 6 is compliance with Beer–Lambert law in which the absorbance of solution is proportionally increased with the increase in nitrite concentration. Table 1 shows the regression between nitrite concentration and the number of sample (N). Finally, the linear range was chosen at 0.04 ÷ 1.1 mg/L NO_2^- ($0.995 < R^2 < 1$). Within this range, the standard curve was drawn over the nitrite concentration range of 0.1 ÷ 1.0 mg/L with the regression equation A = 0.9256C + 0.0008 (N=6, Multiple R = 0.999867689, R square = 0.999735396, Adjusted R Square = 0.999669245, Standard Error = 0.005872737).

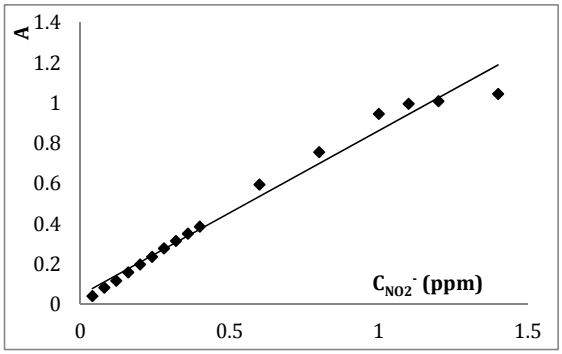


Fig 6. Linear range of method

Condition: NO_2^- 0.4 ppm ÷ 1.4ppm, 1 mL 0.6% sulfuric acid, 1 mL 0.6 % α-naphthylamine, buffer pH = 2.5

NO_2^- (mg/L)	N	R^2
0.04 – 1.4	16	0.9793
0.04 – 1.2	15	0.9936
0.04 – 1.1	14	0.9983
0.04 – 1.0	13	0.9993
0.04 – 0.8	12	0.9990
0.08 – 1.4	15	0.9779
0.08 – 1.2	14	0.9933
0.08 – 1.1	13	0.9982

Table 1. Regression between nitrite concentration and the number of sample (N)

The error of regression coefficients S(a) and S(b), the confidence intervals of the regression coefficients U(a) and U(b) were calculated as below:

S(a) = 0.0046 → U(a) = ± $t_{(0.95;4)}$* S(a) = 0.0127;

S(b) = 0.0075→ U(b) = ± $t_{(0.95;4)}$* S(b) = 0.0209

Table 2. The analysis of standard curve

		Standard Error	Lower 95%	Upper 95%
Intercept (a)	0.000750685	0.004569727	-0.01194	0.013438
Slope (b)	0.925643836	0.007529563	0.904738	0.946549

Limit of detection (LOD) and limit of quantification (LOQ) of this method were determined from absorbance average (Ā) and standard deviation (SD) of triplicate measurements of 21 blank samples containing only developing reagent mixture, without nitrite ions.

$$\text{LOD} = \frac{3SD}{b} = \frac{3 \times 1.17 \times 10^{-3}}{0.9256} = 3.79 \times 10^{-3} \ \frac{\text{mg}}{\text{L}}$$

$$\text{LOQ} = \frac{10SD}{b} = \frac{10 \times 1.17 \times 10^{-3}}{0.9256} = 12.6 \times 10^{-3} \ \frac{\text{mg}}{\text{L}} = 0.0126 \ \frac{\text{mg}}{\text{L}}$$

2.3.2. Application of the validated method in determining nitrite in water and fresh vegetables

Under the optimized conditions, the method was applied to the determination of nitrite in some different well water samples and pickled mustard vegetable samples. The results are shown in Tables 3 and 4.

Table 3. Quantification of nitrite content (mg/L) in five of well water samples

Sample	Concentration of NO_2^- (mg/L)	RSD % [a]	Sample	Concentration of NO_2^- (mg/L)	RSD % [a]

WT1	0.021 ± 0.015	6.98%	WT4	ND	-
WT2	ND	-	WT5	0.037 ± 0.015	4.01%
WT3	0.029 ± 0.015	5.14%			

[a] (n=5)

Table 4. Quantification of nitrite content (mg/kg) in pickled mustard vegetable samples

Sample	Concentration of NO_2^- (mg/kg)[b]	RSD% [a]	Sample	Concentration of NO_2^- (mg/kg)[b]	RSD% [a]
PVH1	0.89 ± 0.03	1.40%	AN1	3.15 ± 0.14	3.68%
PVH2	2.55 ± 0.11	3.57%	AN2	2.78 ± 0.10	3.00%
PVH3	2.84 ± 0.10	2.74%	AN3	3.21 ± 0.11	2.75%
GV1	3.75 ± 0.15	3.21%	LTH1	1.43 ± 0.05	2.63%
GV2	4.29 ± 0.12	2.34%	LTH2	1.90 ± 0.10	4.30%
GV3	2.86 ± 0.11	3.11%			

[a] (n=5); [b] Mean ± CI(P=95%, f=4)

According to the results, nitrite concentrations of well water samples changed from none detected to 0.037 mg/L, of pickled mustard green vegetables changed from 0.89 to 4.29 mg/L. Average nitrite content of baby foods was 2.70 mg/kg.

Table 5. The recovery of nitrite in well water and pickled mustard vegetables samples

Sample	Nitrit added (mg/L)	Nitrit found[a] (mg/L)	Recovery (%)
Pickled mustard vegetable samples PVH1	0.2	0.203 ± 0.003	101.47%
	0.4	0.394 ± 0.003	98.59%
	1	1.009 ±0.002	100.93%
Well water*	0.2	0.203 ± 0.001	101.5%
	0.4	0.400 ± 0.001	99.9%
	1	1.002 ±0.001	100.2%

[a]Mean ± standard deviation, n = 5; [*]Well water have no nitrit (determination of five analyses)

The method accuracy was verified by recovery (%H) of nitrit solution.

4. CONCLUSION

This work investigates factors to determine the optimal conditions for the nitrite analysis based on modified Griess reaction between sulfanilic acid with 1-naphthylamine. In the strong acid environment especially pH 2.5 at room temperature in the present of 0.024%-naphthylamine, the mixture should be stood within 15 - 25 minutes for color development before absorbance measurement. In this condition, the maximum absorption was at 524 nm with high sensitivity (LOD = 3.79×10^{-3} and LOQ = 0.0126 mg/L), and the linear regression was obtained over the range of 0.04 ÷ 1.1 mg/L nitrite. The study of Irandoust et al. (2013) also carried out this reaction in acidic environment with the lower pH (pH=1) and at the wavelength of 515 nm to able to quantify nitrite ions in water as the concentration of up to 0.01 mg/L. Ions particularly NO_3^-, Cl^- and Fe^{3+} are considered to cause interferences in absorbance measurement. Thus, their concentrations are advised to be lower than 500, 500 and 50 ppm, respectively. These conditions of our work were applied in determining the concentrations of nitrite ions in some water samples and some kinds of vegetables. The results achieved high precision and high recovery of more than 100%, similar to percent recoveries (between 99.0% and 102%) of Irandoust's study.

This project is expected to develop a method for co-determination of nitrite and nitrate in samples. Another research approach is photodynamic catalytic method based on the reaction system of Methylene Blue + $KBrO_3$ + NO_2^- which is being developed to detect nitrites in samples.

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