

Quantification of Nitrite/Nitrate in Food Stuff Samples Using 2-Aminobenzoic Acid as a New Amine in Diazocoupling Reaction

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Abstract 2-Aminobenzoic acid has been used as an amine in diazocoupling reaction to form an azo dye in the quantification of nitrite/nitrate at trace level. The formed azo dye has an absorption maximum at 550 nm in aqueous phase, and the resulted dye can be extracted into organic solvent to lower the detection limit. The method obeys Beer's law in the concentration range 0–10 μg of nitrite in 25 ml of aqueous solution with a molar absorptivity of $3.6 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ and 0–2 μg of nitrite in 5 ml of organic phase. The detection limit of the dye has been found to be 0.056 $\mu\text{g ml}^{-1}$. Nitrate is determined by reducing it to nitrite after passing through a copperized cadmium reductor column. The effect of interfering ions on the determination of nitrite/nitrate has been described. The developed method has been applied to determine the nitrite/nitrate trace level in vegetable, fruit juice, and milk powder samples.

Keywords Diazocoupling Reaction · 2-Aminobenzoic Acid (ABA) · *N*-(1-naphthyl) Ethylenediamine Dihydrochloride (NEDA) · Reductor Column · Nitrite and Nitrate

Introduction

The determination of nitrite/nitrate ion is an important aspect in the analysis of food samples as well as water

samples. Nitrite/nitrate ions are intimately involved in the overall nitrogen cycle in soil and higher plants (Hsu et al. 2009). Both of these ions occur wide spread contaminants in aqueous environment and serve as significant indicators of quality of natural waters. The increasing level of nitrate in ground water results mainly from agricultural application of fertilizers as well as from many industrial processes (Van Staden and Makhafola 1996). Nitrate is one of the principal nutrients which stimulates the growth of macrophytes and phytoplankton present in water causing eutrophication. Nitrite formed during the biodegradation of nitrate and ammonical nitrogen or nitrogenous organic matter is an important indicator of fecal pollution of natural water. The simultaneous determination and speciation of nitrite/nitrate in water and food stuffs has attracted the scientific community significantly in recent years because of its harmful impact on human health (Leonardo 2009). The permissible level of nitrite in drinking water is 1 $\mu\text{g ml}^{-1}$ (US Environmental Protection Agency 2004). Excessive contamination of nitrite in drinking water could be hazardous to health, especially for infants and pregnant women. Nitrite oxidizes iron in hemoglobin of red blood cells to form methemoglobin, which reduces the oxygen carrying ability which is known as methemoglobinemia (blue baby syndrome). In addition, the reaction between nitrite and secondary or tertiary amines can result in the formation of *N*-nitroso compounds which are known to be carcinogenic, teratogenic, and mutagenic (Puckett 1995; Hartman 2006; Erkekoglu et al. 2009; Santamaria 2005). Due to the significant impact of nitrite/nitrate toxicity on human health, it is essential to monitor their concentration at low levels and to examine the mechanism involved in their production, transport, and decomposition in water matrices. The concentration level of nitrite/nitrate in milk is normally at trace level, but dietary intake of these

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contaminants via diary foods is a minor significance in adult humans. However, in newborn infants, it is highly susceptible to the detrimental effects of these contaminants (Gapper et al. 2004). Leafy vegetables are an excellent source of vitamins, minerals, and biologically active compounds (McMullen et al. 2005; DJ Favell 1998; Jiang et al. 2009). The incidence of coronary heart disease, atherosclerosis, and stroke can be reduced by increasing vegetable consumption as that of the major cancers such as cancer of the stomach, lung, mouth, esophagus, colon, and rectum (Dauchet et al. 2009). Leafy vegetables are one of the major sources of nitrite in our bodies. Generally, nitrates are abundant in foods because plants take up nitrogen from the soil in the ionic form. The nitrates in foods can be reduced to nitrite by the bacterial action (Kidmose et al. 2001). Nitrite is widely used as preservative in meat products due to their ability to inhibit the growth of spores of *Clostridium botulinum*. It is added (particularly to ground meat products) in the meat curing process to speed curing and the formation of the required colors and flavors. The reduction of nitrate to nitrite is possible in the stomach of infants, where low acidity allows the growth of nitrite-reducing microorganisms. Nitrite levels when correlated with other forms of nitrogen in water provide indexes of organic pollution in water (Marshall and Trenery 1996; Burakhama et al. 2004). It also affects the dissolved oxygen content of water. Due to these toxic effects, it is very important to develop new methods with high sensitivity and selectivity for its continuous monitoring in food products. Many analytical methods available for the determination of nitrite are not suitable for routine ultra-trace determinations. The widely used spectrophotometric method for the determination of nitrogen dioxide in air and nitrite/nitrate in water and soil samples are mainly based on the Griess–Ilosvey reaction. This was modified several times after fixing it as nitrite ion in a suitable trapping solution. Many other methods other than diazotization reaction were also reported for the determination of nitrite (Yue et al. 2004; Nam et al. 2008). They are prone to problems of sensitivity and lack of selectivity mainly due to severe interference from other ions. The spectrophotometric determination of nitrite/nitrate is generally carried out by using the diazocoupling reaction due to its simplicity, high reproducibility, and easy adoptability in an analytical procedure (Prasad and Chetty 2008; Wootton et al. 2006). Many of these methods have low sensitivity and require long sampling periods. The proposed method is based on the diazotization of nitrite with 2-aminobenzoic acid and its subsequent coupling with *N*-(1-naphthyl) ethylenediamine dihydrochloride in aqueous medium to form an azo dye. The proposed method has been applied to determine trace levels of nitrite/nitrate in a variety of food sample matrices.

Materials and Methods

Reagents All reagents used were analar grade without further purification, and distilled water was used throughout the experiment.

Standard Nitrite Stock Solution (1,000 $\mu\text{g/ml}$) Prepared by dissolving 0.15 g of predried sodium nitrite (at about $105 \pm 5^\circ\text{C}$ for an hour) in distilled water and diluting to 100 ml. Suitable aliquots of this solution were diluted using sodium arsenite absorber solution to give 2 $\mu\text{g/ml}$ of nitrite.

2-Aminobenzoic Acid (0.05%) Prepared by dissolving 0.05 g of 2-aminobenzoic acid (ABA) in 3 ml of 2 N HCl and diluting to 100 ml with distilled water.

***N*-(1-naphthyl) Ethylenediamine Dihydrochloride (0.05%)** Prepared by dissolving 0.05 g of *N*-(1-naphthyl) ethylenediamine dihydrochloride (NEDA) in distilled water and diluting to 100 ml.

Sodium Arsenite Absorber Solution Prepared by dissolving 4 g of NaOH and 1 g of sodium arsenite in 1 l of water.

$\text{NH}_3\text{--NH}_4\text{Cl}$ Buffer Solution (pH= 10) Prepared by dissolving 0.531 g of NH_4Cl in 80 ml of water and adjusting the pH to 8.5 with 1:1 ammonia (vol/vol) and diluting it to 100 ml with water.

$\text{NH}_4\text{Cl}\text{--EDTA}$ Buffer Solution (pH= 8.5) Prepared by dissolving 3.25 g of NH_4Cl and 0.43 g of disodium ethylenediamine tetra acetate (EDTA) in 225 ml of water. The pH was adjusted to 8.5 using NH_4OH solution and diluted to 250 ml.

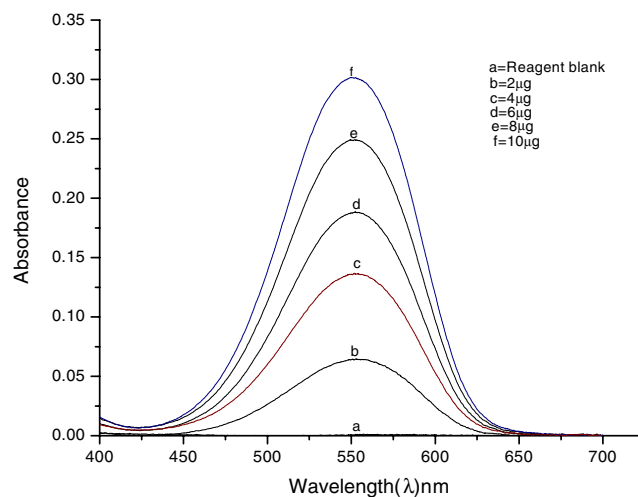


Fig. 1 Absorption spectra

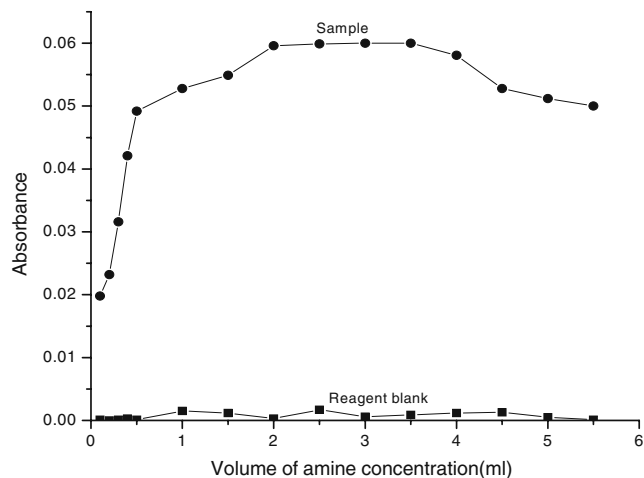


Fig. 2 Effect of amine concentration

Acetate Buffer (pH 3.5) Dissolve 6.8 g of sodium acetate in 3 ml of acetic acid and adjust the pH to 3.5 with acetic acid and diluting to 100 ml with distilled water.

Sodium Carbonate (0.5%) Prepared by dissolving 0.5 g of Na_2CO_3 in distilled water and diluting to 100 ml.

Formaldehyde (0.5%) Prepared by diluting 1.3 ml of formaldehyde (38%) to 100 ml with water.

Solvent for Extraction 1-butanol

Copperized Cadmium Reductor Column

Wash 25 g of 20–100 mesh Cd granules with 6 N HCl and rinse with water. Swirl Cd granules with 100 ml of 2% CuSO_4 solution for 5 min or until blue color partially fades. Decant and repeat with fresh CuSO_4 until a brown colloidal precipitate begins to develop. Gently flush with water to remove all precipitated Cu. Insert a glass wool plug into the

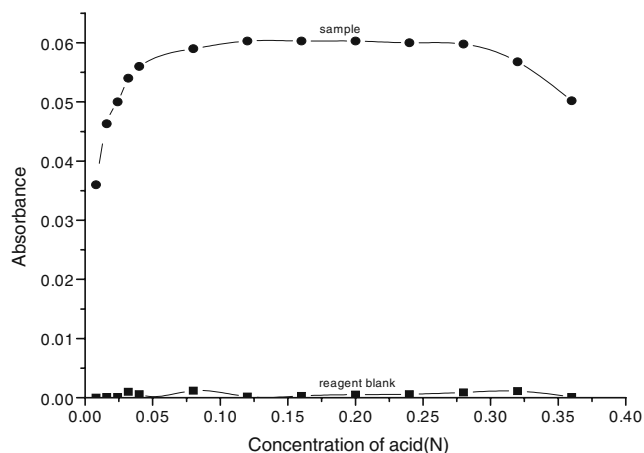


Fig. 3 Effect of acid concentration

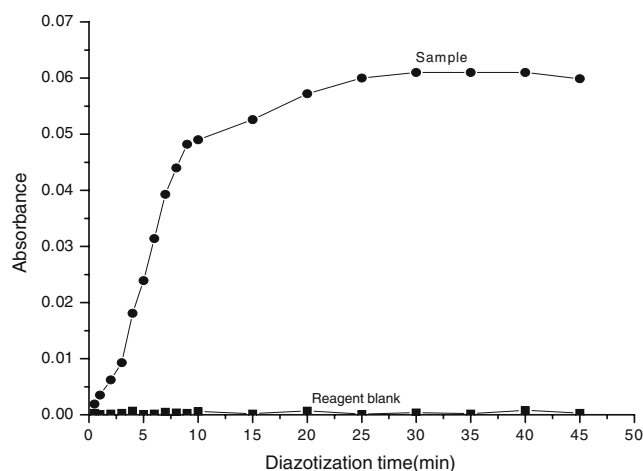


Fig. 4 Effect of diazotization time

bottom of a glass column (30 cm long \times 5 mm id) and fill with water. Add sufficient Cu–Cd granules to produce a column of 18.5 cm long. Maintain water level above Cu–Cd granules to prevent entrapment of air. Wash column with 200 ml of dilute NH_4Cl –EDTA buffer solution. The column was activated by passing NH_4Cl –EDTA buffer solution at a flow rate 7–10 ml min^{-1} . The flow rate was adjusted in such a way that the nitrate solution quantitatively reduces to nitrite after passing through the reductor column. The column was stored using NH_4Cl –EDTA solution. The column should not be allowed to dry. Under these conditions, the column can be used for several months. All the column conditions were optimized according to the standard method (APHA 1995).

Apparatus

Absorbance measurements were made using Shimadzu UV-VIS-NIR Scanning Spectrophotometer (model-UV-3101PC)

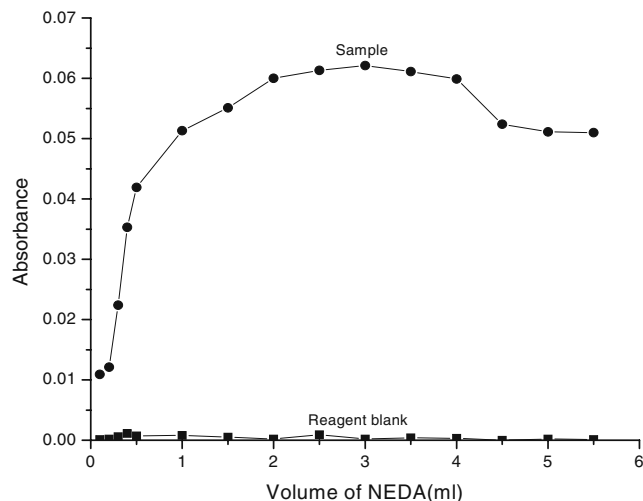


Fig. 5 Effect of coupling agent concentration

Table 1 Effect of variation of solvents during extraction

Solvent no.	Solvent (ml) ^a	Absorbance	
		Blank vs. solvent	Sample vs. blank
1	Isoamyl acetate (5)	0.027	0.238
2	Isoamyl alcohol (5)	0.028	0.244
3	1-Butanol (7.5)	0.002	0.309
4	Isoamyl alcohol + isoamyl acetate (5) ^b	0.021	0.251
5	MIBK (5)	0.021	0.205
6	MBK (5)	0.020	0.213

^a Based on the solubility of solvents in aqueous phase, different volumes have been used. In all these cases, the extract was collected into 5-ml standard flasks and made up to mark with methanolic HCl to restore the original color

^b 1:1 by vol/vol

with 1 cm quartz cuvettes; Controlled Dynamics digital pH meter (model APX 175) was used for all pH measurements.

Standard Procedure

Aqueous Procedure

Into a series of 25-ml standard flasks containing 3.5 ml of 2-aminobenzoic acid and 1.5 ml of 2 N hydrochloric acid, 10-ml aliquots of sodium arsenite solutions containing 0–10 µg nitrite were added. The contents were mixed and allowed to stand for 20 min. Then, 3 ml of *N*-(1-naphthyl) ethylenediamine dihydrochloride was added as coupling agent and diluted to 25 ml with distilled water, and the absorbance values were measured at 550 nm using 1 cm quartz cuvettes (Fig. 1).

Extraction Procedure

In order to lower the detection limit, into a series of 25-ml standard flasks containing 3.5 ml of 2-aminobenzoic acid and 1.5 ml of 2 N hydrochloric acid and 10 ml aliquots of sodium arsenite solution containing 0–2 µg nitrite were added; the contents were mixed well and allowed to stand for 20 min. Then, 3 ml of *N*-(1-naphthyl) ethylenediamine dihydrochloride was added as coupling agent and diluted to 25 ml with distilled water. The solutions were then transferred into 60-ml separating funnels and treated with 7.5 ml of 1-butanol and 5 ml of acetate buffer solution. After extraction, the organic phase was collected into 5-ml standard flask, and the absorbance of the extract was measured at 555 nm against reagent blank.

Analysis of Real Samples

Determination of Nitrite/Nitrate in Tomato Juice (*Lycopersicon esculentum cerasiforme*)

Preparation Two hundred fifty grams of weighed tomato fruits were crushed using juice maker, filtered, and made up

to 100 ml with distilled water. A 10-ml aliquot of prepared juice sample was used for the analysis.

Nitrite Determination

Ten milliliters of the made-up solution was transferred into a 25-ml standard flask containing 3.5 ml of ABA and 1.5 ml of 2 N hydrochloric acid. The contents were mixed well and allowed to stand for 20 min. Then, 3 ml of NEDA solution was added as coupling agent and diluted to 25 ml with distilled water, and the absorbance was measured at 550 nm.

In case the color intensity is low, the solution was transferred to a 60-ml separating funnel and treated with 5 ml of acetate buffer solution. The contents were equilibrated with 7.5 ml of 1-butanol and extracted. The extracted organic layer was collected in 5-ml standard flask, and the absorbance was measured at 555 nm against reagent blank.

Nitrate Determination

Ten milliliters of made-up solution was treated with 5 ml of NH₃–NH₄Cl buffer solution and passed through

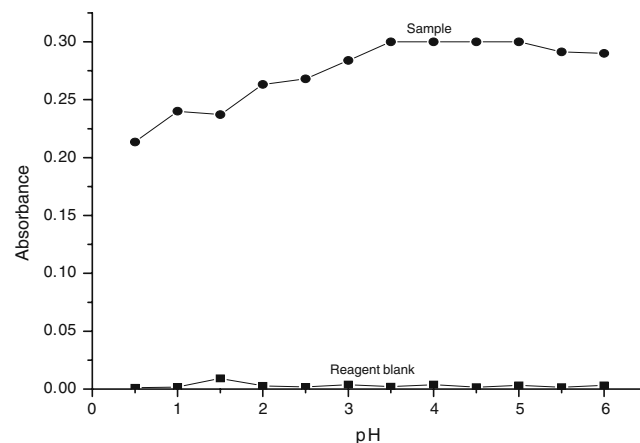
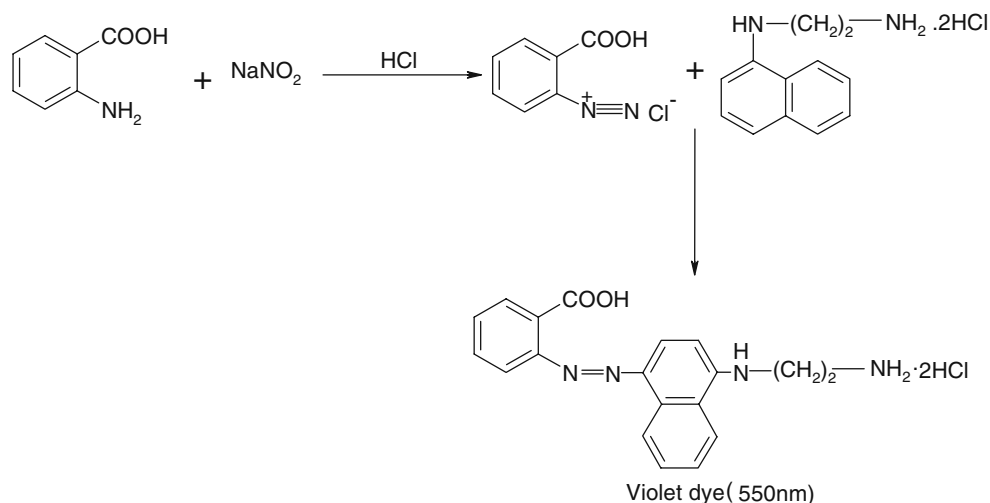


Fig. 6 Effect of extraction pH

Scheme 1 Species responsible for color

copperized cadmium reductor column at a flow rate of 1 ml/min. The column was washed with 5×3 ml portions of water, and the eluents were collected in to a 50-ml standard flask and diluted to the mark with distilled water. Ten milliliters of the made-up solution was taken and analyzed for total nitrite content using the procedure described above (Table 7).

Determination of Nitrate in Sweet Orange Fruit Juice (*Citrus sinensis*)

Preparation One hundred grams of weighed sweet orange fruits were peeled and squeezed to juice using juice maker, filtered, and made up to 100-ml standard flask with distilled water. Ten milliliters of the made-up solution was used for analysis.

Nitrite/Nitrate Determination

Ten milliliters of the made-up solution was used for color development, and the absorbance was measured using the procedure described above.

Ten milliliters of the made-up solution was used to reduce nitrate to nitrite by passing through the Cu–Cd column. The reduced nitrite was analyzed using the procedure described above (Table 8).

Determination of Nitrate in Sweet Lime Juice Samples (*Citrus lemettioides*)

Preparation One hundred grams of weighed sweet lime fruits were peeled and squeezed to juice using juice maker, filtered, and made up to 100-ml standard flask with distilled water. From the made-up solution, 10 ml was used for analysis.

Nitrite/Nitrate Determination

Ten milliliters of the made-up solution was used for color development, and the absorbance was measured using the procedure described as above.

Ten milliliters of the made-up solution was used to reduce nitrate to nitrite by passing through the Cu–Cd column. The reduced nitrite was analyzed using the procedure described above (Table 9).

Determination of Nitrite in Milk Powder Samples

Preparation A known weight of milk powder was dissolved in water and treated with 1 ml of acetic acid to deproteinate the sample. The precipitated protein was removed by centrifugation, and the residue was washed with 5-ml portions of water for several times and

Table 2 Analytical parameters of proposed method and standard method

	Proposed	Standard (APHA 1995)
λ max, nm	540	540
Color stability, h	>24	24
Linearity, $\mu\text{g ml}^{-1}$	0.08–0.4	0.08–0.4
Molar absorptivity, $\text{L mol}^{-1}\text{cm}^{-1}$	3.6×10^3	3.0×10^3
Sandell sensitivity, $\mu\text{g cm}^{-2}$	0.0012	0.0013

Table 3 Comparison of limit of detection and limit of quantification

Sample	Proposed method		Standard method (APHA 1995)	
	Limit of detection (LOD), $\mu\text{g ml}^{-1}$	Limit of quantification (LOQ), $\mu\text{g ml}^{-1}$	Limit of detection (LOD), $\mu\text{g ml}^{-1}$	Limit of quantification (LOQ), $\mu\text{g ml}^{-1}$
Tomato	0.059	1.07	0.056	1.10
Orange	0.062	1.80	0.066	2.00
Moosambi	0.078	2.30	0.080	2.40

LOD limit of detection, *LOQ* limit of quantification

centrifuged again. All the centrifugates were mixed well and made up to 100 ml in a standard flask. From the made-up solution, 10 ml was used for analysis.

Nitrite/Nitrate Determination

Ten milliliters of the made-up solution was used for color development, and the absorbance was measured using the procedure described above.

Ten milliliters of the made-up solution was used to reduce nitrate to nitrite by passing through the Cu–Cd column. The reduced nitrite was analyzed using the procedure described above (Table 10).

Results and Discussion

Initial studies were carried out by using different primary aromatic amines for diazotization process and phenols or naphthols as coupling agents for the determination of nitrite by using 10-ml aliquots of sodium arsenite containing 6 μg of nitrite. This solution was introduced into a 25-ml standard flask containing 1 ml of amine and 2 ml of 5 N HCl. The contents were allowed to stand for 10 min, and 1 ml of coupling agent dissolved in 2 N sodium hydroxide was added and diluted to the mark with distilled water. The

absorbance measurements were carried out over the wavelength range 400–800 nm. Among these, 2-aminobenzoic acid and *N*-(1-naphthyl) ethylenediamine dihydrochloride combination gave low blank and high sample absorbance values; hence, the reaction conditions were optimized using these reagents.

Optimization of Experimental Conditions

Effect of Amine Concentration

The optimum amount of amine required for maximum sample absorbance was studied using the proposed procedure by adding 0.1–5 ml of 0.05% amine and 1.5 ml of 2 N hydrochloric acid to a series of 25-ml standard flasks. The solutions were treated with 10-ml aliquots of sodium arsenite solution containing 2 μg of nitrite. Then, the solutions were allowed to stand for 20 min. This was then treated with 3 ml of NEDA as coupling agent and diluted to mark with distilled water. A reagent blank was prepared simultaneously for each concentration of amine, and absorbance was measured at 550 nm (Fig. 2). It has been evident from the graph that 3.5 ml of 0.05% amine was sufficient enough to get maximum sample absorbance with minimum blank value. Higher concentrations of amine did not enhance the sample absorbance; but, it gave higher

Table 4 Accuracy and precision studies

Method	Added nitrite ($\mu\text{g ml}^{-1}$)	Intra-day accuracy and precision			Inter-day accuracy and precision		
		Nitrite found ^a ($\mu\text{g ml}^{-1}$)	RE (%)	RSD (%)	Nitrite found ^a ($\mu\text{g ml}^{-1}$)	RE (%)	RSD (%)
Proposed	3.0	3.10	3.3	2.13	3.08	3.1	1.66
	6.0	6.06	2.0	1.98	6.09	1.8	2.02
	9.0	8.96	1.1	1.25	9.04	1.3	1.16
Standard	3.0	3.02	3.2	2.21	3.06	3.0	2.06
	6.0	6.03	2.4	2.80	6.02	2.2	2.30
	9.0	8.98	1.8	2.00	9.00	1.6	1.95

RE relative error, *RSD* relative standard deviation

^a Mean value of five determinations

Table 5 Recovery studies

Sample ^a	Method	Nitrite found ($\mu\text{g ml}^{-1}$)	Nitrite added ($\mu\text{g ml}^{-1}$)	Total nitrite found ($\mu\text{g ml}^{-1}$)	Percent recovery + SD
Sample-1	Proposed	–	2.5	2.54	103±1.06
		–	3.0	3.02	102±1.85
		–	3.5	3.50	100±2.56
	Standard	–	2.5	2.51	101±2.64
		–	3.0	3.00	100±1.98
		–	3.5	3.50	99.9±2.04
Sample-2	Proposed	–	2.5	2.49	99.3±1.43
		–	3.0	3.02	101±2.00
		–	3.5	3.52	102±1.63
	Standard	–	2.5	2.48	99.7±2.41
		–	3.0	2.98	99.8±1.98
		–	3.5	3.49	99.9±1.99

^a Nitrite/nitrate was not detected in these samples

blank absorbance value; hence, 3.5 ml of 0.05% of amine has been fixed as an optimum concentration in all further studies.

Effect of Acid Strength

The optimum strength of the acid required for diazotization reaction was studied. In these experiments, 10-ml aliquots of alkaline sodium arsenite solution containing 2 μg of nitrite were added to series of 25-ml standard flasks containing 3.5 ml of amine and in various volumes of (0.1–5 ml) of 2 N hydrochloric acid. These solutions were allowed to stand for 20 min and treated with 3 ml of NEDA as coupling agent and diluted to mark with distilled water. A reagent blank was prepared simultaneously for each concentration of the acid, and the absorbance was measured at 550 nm (Fig. 3). It was evident from the graph that 1.5 ml of acid was sufficient enough to get minimum blank and maximum sample absorbance values.

Effect of Diazotization Time

The standing time required for diazotization of amine with nitrite was next investigated. In these experiments, 10 ml of alkaline sodium arsenite solution containing 2 μg of nitrite were added to a series of 25-ml standard flasks containing 3.5 ml of amine and 1.5 ml of 2 N hydrochloric acid. These solutions were allowed to stand for different time intervals and then treated with 3 ml of NEDA as coupling agent and diluted to mark with distilled water. A reagent blank was prepared simultaneously for each concentration of the time, and the absorbance was measured at 550 nm (Fig. 4). It is evident that at least 20 min standing time is needed for the complete diazotization before the addition of coupling agent.

Effect of Coupling Agent Concentration

The effect of variation of coupling agent concentration in the range of 0.1–5 ml of *N*-(1-naphthyl) ethylenediamine dihydrochloride was next investigated. In these experiments, 10-ml aliquots of alkaline sodium arsenite containing 2 μg of nitrite were added to a series of 25-ml standard flasks containing 3.5 ml of amine and 1.5 ml of 2 N hydrochloric acid, and various volumes of coupling agent were added and diluted to mark with distilled water. A reagent blank was prepared simultaneously for each concentration of the coupling agent. Absorbance measurements were made at

Table 6 Interference studies

Interferent	Tolerance limit (μg)
Formaldehyde	2×10^3
Sulfite	3×10^3
Sulfite ^a	5×10^3
Sulfide	5
Sulfide ^b	50
$\text{CO}_3^{2-}, \text{C}_2\text{O}_4^{2-}, \text{Citrate}, \text{NO}_3^-, \text{tartarate}$	1×10^4
$\text{Ni}^{2+}, \text{Co}^{2+}, \text{Zn}^{2+}, \text{Ba}^{2+}, \text{Mg}^{2+}$	1×10^4
Cu^{2+}	2×10^3
Fe^{2+}	5×10^3
Fe^{3+}	2×10^2
${}^c\text{Fe}^{3+}$	1×10^3

^a Treated with 2 ml of 0.05% formaldehyde solution before the addition of coupling agent

^b Treated with 1 ml of 0.01% lead acetate solution centrifuged and washed the residue, the centrifugate and washings were mixed and used for color development

^c Treated with 1 ml of 1 N NaOH solution centrifuged and washed the residue, the centrifugate and washings were mixed and used for color development

Table 7 Determination of nitrite/nitrate in tomato juice samples

Sample	Original nitrite found ($\mu\text{g l}^{-1}$)		^a Total nitrite found ($\mu\text{g l}^{-1}$)	
	Standard method	Proposed method	Standard method	Proposed method
Sample-1 ^b	147.20	155.20	516.00	546.40
Sample-2 ^c	162.24	163.20	541.12	567.20

^a Total nitrite (μg)=nitrite originally present (μg) + nitrite formed by the reduction of nitrate after passing through the copperized cadmium reductor column
 Nitrate = [total nitrite (μg) – nitrite originally present (μg)] $\times \frac{62}{46}$

^{b,c} Samples have been collected from different departmental stores

550 nm in 1 cm quartz cuvettes against water and respective reagent blank (Fig. 5). It was evident from the graph that 3 ml of 0.05% coupling agent was sufficient enough to get maximum sample absorbance with minimum blank value.

Effect of Variation of Solvents During Extraction

Several solvents like 1-butanol, isoamylalcohol, isoamylacetate, methyl isobutyl ketone, and methyl butyl ketone were used for extracting the azo dye. Among all the above solvents, 1-butanol gave the lower blank absorbance and higher sample absorbance values; hence, 1-butanol was chosen as solvent for extraction. In these experiments, 10-ml aliquots of sodium arsenite solution containing 0–10 μg nitrite were added to series of 25-ml standard flasks containing 3.5 ml of amine and 1.5 ml of 2 N hydrochloric acid. The contents were mixed well and allowed to stand for 20 min. Then, treated with 3 ml of NEDA as coupling agent and diluted to 25 ml with distilled water, and absorbance was measured (Table 1).

Effect of Extraction pH

In order to establish the most suitable pH range for the quantitative extraction of the azo dye, the variation of pH was next investigated. In these experiments, 10-ml aliquots of alkaline sodium arsenite solution containing 2 μg of nitrite were added to a series of 25-ml standard flasks containing 3.5 ml of amine and 1.5 ml of 2 N hydrochloric acid. After standing time of 20 min, the solutions were treated with 3 ml of NEDA as coupling agent. A reagent blank was prepared simultaneously for each concentration of the solution. These solutions were treated with various buffer solutions of pH range 0.1–12 and then transferred to 60-ml separating funnels to equilibrate with 7.5 ml of 1-butanol as organic solvent. After extraction, the extracts were collected in to 5-ml standard flasks, and the absorbance was measured at 555 nm (Fig. 6). It has been evident from the graph that the extraction was quantitative in the pH range 3–5; hence, the required pH range was maintained by the addition of 3.5 ml of acetate buffer

before its extraction into organic solvent to get maximum sample absorbance.

Species Responsible for Color

2-Aminobenzoic acid on treatment with nitrite undergoes diazotization in acidic medium to form corresponding diazonium ion which couples with *N*-(1-naphthyl) ethylenediamine dihydrochloride instantaneously in aqueous media to form a pink colored azo dye which has λ_{max} at 550 nm. However, the resulted dye was extracted in acidic condition with 1-butanol as organic solvent. The extracted organic phase was collected in 5-ml standard flasks, and the absorbance of the extract was measured at 555 nm against reagent blank (Scheme 1).

Method Validation

Analytical Merits of the Method

A linear correlation was found between the absorbance values and concentration of nitrite in the concentration range 2–10 μg in 25 ml of solution. The optical parameter such as Beer's law, molar absorptivity, and Sandell sensitivity values of the proposed and standard methods were shown in Table 2. The limits of detection and quantification were calculated according to International Conference on Harmonization (ICH) guidelines [ICH

Table 8 Determination of nitrate in sweet orange juice samples

Sample	Nitrate in orange samples (mg l^{-1})	
	Standard method	Proposed method
Sample-1 ^a	468	495
Sample-2 ^b	424	461

^{a,b} Nitrite was not detected

Nitrate = [Total nitrite (μg) – nitrite originally present (μg)] $\times \frac{62}{46}$

^{a,b} Samples have been collected from different departmental stores

Table 9 Determination of nitrate in sweet lime juice samples

Sample	Nitrate (mg l ⁻¹)	
	Standard method	Proposed method
Sample-1 ^a	575	617
Sample-2 ^b	580	613

^{a,b} Nitrite was not detected

Nitrate = [Total nitrite (μg) – nitrite originally present (μg)] × $\frac{62}{46}$

^{a,b} Samples have been collected from different departmental stores

Guidelines, dated 06 November 1996, incorporated in November 2005, London, Shabir 2003 and Ermer 2001]. All these reveal that the proposed method is sensitive when compared with standard method which can be used as an alternative method (Table 3).

Precision

The precision of the methods were evaluated in terms of intermediate precision (intra-day and inter-day) [ICH Guidelines, dated 06 November 1996, incorporated in November 2005, London, Shabir 2003 and Ermer 2001]. Three different concentrations of nitrite within the Beers law range in each method were analyzed for five replicate samples during the same day (intra-day precision) and five consecutive days (inter-day precision). The relative standard deviation (RSD) values of intra-day and inter-day studies for nitrite shows that the precision of the method was good and compared well with standard method (Table 4).

Accuracy

The accuracy of the analytical method expresses the closeness between the reference value and measured value [ICH Guidelines, dated 06 November 1996, incorporated in November 2005, London, Shabir 2003 and Ermer 2001]. Accuracy was evaluated as percentage relative error between the measured mean concentration and added concentrations for nitrite. The results have been described in the (Table 4) from which it was evident that the accuracy obtained was satisfactory. In terms of accuracy, the

proposed method with an intra-day relative error of $\leq 1.1\%$ has been found to be more accurate than the standard method which has a higher relative error of 1.8%. Similarly, the proposed method with RSD $\leq 1.25\%$ has been more precise than the standard method (RSD, 2%). A similar trend has been observed with respect to inter-day accuracy and precision.

Recovery studies To assess the accuracy of the method, recovery experiments were performed by standard addition technique. The recovery was assessed by determining the agreement between the measured concentration of nitrite of samples and spiked samples. These studies were carried out by spiking the samples with known amounts of nitrite at three different concentration levels (2.5, 3.0, and 3.5 μg) in the milk powder, and the total nitrite was measured by the proposed and standard methods. The percentage recovery values of nitrite were found in the range 99.3 to 103 with SD in the range 1.06 to 2.64. All these studies showed that the proposed method has been found to possess good accuracy and compared well with standard method (Table 5).

Effect of Interfering Species

The tolerance limits of interfering species were established as those concentrations, which did not cause more than $\pm 1.5\%$ error in the recovery of added nitrite at 2-μg level.

The effect of various gasses on the determination of nitrogen dioxide in air was studied after fixing it as nitrite ion in sodium arsenite or triethanolamine absorber solution. Formaldehyde at levels of 2,000 μg did not interfere in the proposed method, while sulfite at concentration above 3,000 μg interfered, causing decrease in absorbance value. However, higher concentrations (up to 5,000 μg) of sulfite can be tolerated by the system if it is held as bisulfite adduct, which was achieved by the addition of 2 ml of 0.5% formaldehyde solution to the sample prior to nitrite determination. Sulfide at levels of 5 μg interfered and caused decrease in absorbance value. However, higher concentrations (up to 50 μg) of sulfide can be tolerated with the addition of 1 ml of 0.01% lead acetate solution prior to nitrite determination. The interference of several anions and cations were studied to check the suitability of the method

Table 10 Determination of nitrite in milk powder samples

Samples	Nitrite found (μg)	Nitrite added (μg)	Total nitrite found (μg)		% of added nitrite recovered	
			Proposed method	Standard method	Proposed method	Standard method
Sample-1 ^a	Nil	2	1.98	1.93	99	96.5
Sample-2 ^b	Nil	2	2.00	1.96	100	98.5

^{a,b} Samples have been collected from different departmental stores

for the determination of nitrite and nitrate in water or soil samples. Anions like oxalate, carbonate, citrate, nitrate, and tartarate did not interfere up to 10,000 μg levels. Similarly, cations like Ni^{2+} , Co^{2+} , Ba^{2+} , Mg^{2+} , and Zn^{2+} up to 10,000 μg level did not interfere. But copper (II) and Iron (II) did not interfere up to 2,000 μg and 5,000 μg levels, respectively. Iron (III) gave a negative interference even at 200 μg level; however, it was overcome by precipitating it as hydroxide and removing by centrifugation method. By this method, up to 1,000 μg level the iron (III) can be overcome (Table 6).

Conclusions

The proposed method based on the diazocoupling reaction between 2-aminobenzoic acid and *N*-(1-naphthyl) ethylenediamine dihydrochloride for the quantification of nitrite/nitrate is simple and sensitive. The reaction conditions have been optimized, and the method obeys Beer's law over the concentration range 0–10 μg in 25 ml of aqueous phase and 0–2 μg in 5 ml of organic phase. The proposed method has been applied to determine nitrite/nitrate in vegetables, fruit juices, and milk powder samples. The results obtained by the proposed method are in good agreement with standard method (APHA 1995). It has been applied to measure nitrite and nitrate levels in a variety of vegetable samples and fruit juice samples. The application of this method to measure nitrite/nitrate levels in foodstuffs will be a useful analytical procedure, and it can serve as an alternative method to other existing procedures (Tables 7, 8, 9 and 10).

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