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Determination of Nitrite/Nitrate at Trace Level in Organic and Non-organic Processed Vegetables Using 5-(4-Aminophenyl)-1,3,4 Oxadiazole-2-thiol as a Novel Amine

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ABSTRACT

A simple spectrophotometric method for the quantification of nitrite/nitrate has been proposed based on diazo coupling reaction using novel amine, that is, 5-(4-aminophenyl)-1,3,4-oxadiazole-2-thiolandN-(1-Naphthyl) ethylenediamine as a coupling agent under acidic condition. The azo dye formed in this diazo-coupling reaction has been characterized by Fourier Transform Infrared study. The proposed method has exhibited wide linearity in the concentration range 0–200 ppm with a limit of detection 0.05 ppm. The utility of the proposed method has been successfully applied to measure trace level nitrite/nitrate in organic and non-organic farmed vegetables such as spinach, cabbage, beetroot, carrot, and cucumber. The nitrite/nitrate levels in organic farmed vegetables are lower in comparison with non-organic farmed vegetables. The nitrate levels were monitored after its reduction to nitrite using copperized-cadmium reductor column under optimized conditions. The results obtained by the proposed method are in good agreement with the results of the standard protocol.

Key words: Nitrite/nitrate, Spectrophotometry, 5-(4-Aminophenyl)-1,3,4 oxadiazole-2-thiol, Organic and non-organic formed vegetables.

1. INTRODUCTION

Trace level nitrite/nitrate monitoring is one of the challenging tasks especially from food and environmental sample matrices [1]. Human beings are largely exposed to the nitrates primarily through vegetables, meat, fish, milk products, beverages, etc., in which nitrite/nitrate is commonly used as curing agent as well as preservatives [2]. In recent years due to the nutrition fall in diet, people are more conscious about using more and more vegetables in their daily diet. Hence, there is a greater demand for production of vegetables. To improve the vegetables yield, excessive use of fertilizers containing nitrates is employed in the agricultural sector. This leads to massive incorporation of nitrates in the vegetables [3]. Different nitrate/nitrate levels in vegetables were reported earlier. Particularly, green leafy vegetables such as spinach, cabbage, and beetroot contain large amounts of nitrates nearly 80–90% of the total dietary nitrates [4]. Other sources of nitrites/nitrates include drinking water and other foods such as animal-based products (10–20% of the diet) [5-7]. In general, nitrites are involved in the conversion of hemoglobin (Fe^{2+}) into methemoglobin (Fe^{3+}, oxygen deficiency) which can be observed in infants causing blue baby syndrome, the pregnant women and in the adults with carcinogenic problems [8,9]. Most of the vegetables contain milligram levels of nitrate in large quantities when compared to microgram level nitrites [10]. In general, nitrates are good for health, however due to some biological reactions nitrates get converted into nitrites which results in the formation of secondary nitrosamines [11]. Thus, monitoring nitrite/nitrate at trace level from food products and water samples is very important [12]. The acceptable daily intake (ADI) of 3.7 mg/kg of nitrate and 0.0–0.6 mg/kg of nitrites was recommended by the European Food Safety Authority (EFSA) which has been endorsed by the Joint FAO/WHO expert committee on food additives in 2008. According to the EFSA reports, most of the food products are exceeding the ADI threshold limits of nitrite/nitrate [13].

Vegetables are generally grown either by organic or non-organic farming processes. Organic farming means cultivation using natural compost without utilizing any artificial fertilizers, pesticides, dirt mud, genetically tailored organisms, or radiation. Most of the reports have been brought out to compare the levels of nitrite/nitrate within organic and non-organic farmed vegetables. Actually, the nitrite/nitrate concentration levels were less in the organic farmed vegetables than non-organic formed ones. Hence, organic farming is preferred over non-organic farming to reduce the levels of nitrite/nitrates in the vegetables grown for human consumption [14,15]. The excessive use of fertilizers/pesticides causes high levels of these toxins in the vegetables. There are no reports available detailing about the nitrite/nitrate content in many of the organic and non-organic processed vegetables [16]. Hence, it is necessary to compare the nitrite/nitrate levels in different vegetables which were processed by organic and non-organic farming.

Several methods have been reported in the measurement of nitrite/nitrate in vegetables and fruits samples. These, methods

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include high pressure liquid chromatography (HPLC) [17], ionic chromatography [18], capillary electrophoresis [19], gas chromatography [20], membrane sensor [21], and gas chromatography mass spectrometry (GCMS) methods [22]. These methods are accurate and highly sensitive, but time consuming, prolonged sample preparation, contamination, and column selection are too risky. Electrochemical methods have been used in the estimation of nitrite/nitrate at trace level in food samples with good sensitivity, but these methods have poor repeatability and reproducibility. The electrode material is very expensive [23-25]. The spectrophotometric methods have been widely used in the measurement of nitrite/nitrate due to its sensitivity, reproducibility and short analysis time [26-29]. Hence, for routine environmental analysis, spectrophotometry seems to be the most attractive and suitable analytical approach.

Among the spectrophotometric methods, the diazo-coupling reaction is simple and relatively inexpensive protocol. In this approach, a primary aromatic amine reacts with nitrite in acidic medium to form diazonium ion which subsequently couples with a reagent to form an azo dye. Kiso *et al*. have measured low concentration of nitrite (4.0 mgL^{-1}) using sulfanilic acid and 1-naphthol combination [30]. Subrahmanyam *et al*. have reported the formation of azo dye using fenitrothion; 4,4-methylene-bis-(*p*-amino-2_-carboxy benzanilide) and detected minimum concentration of nitrite 0.2 mgL^{-1} [31]. Wang employed sulfanilamide, sulfamethizole, and sulfadimidine coupled with 1-naphthol-4-sulfonate (NS) in the nitrite detection limit were improved [32]. In this modified method of Griess-Ilosvay reaction different combinations of reagents were developed in the quantification of nitrite/nitrate at mM concentration, various pH conditions, and lower temperature 0–5°C. The detection of nitrite/nitrate at trace level (µm) and normal room temperature condition is required. In this report, we have proposed a simple diazo coupling reaction-based protocol in the quantification of trace level nitrite/nitrate using novel amine, that is, APODT and coupling agent NEDA in acid condition. The method has been successfully applied to organic and nonorganic processed vegetables such as spinach, cabbage, beetroot, carrot, and cucumber sample matrices.

2. EXPERIMENTAL

2.1. Chemicals and Reagents

All the chemicals used were of analytical reagent grade and used without any further purification. Double-distilled water was used throughout the experiment. Standard stock solution of sodium nitrite was prepared by dissolving 0.015 g of ultrapure sodium nitrite in 100 mL water. The fresh nitrite solutions were prepared from stock solution. APODT (0.01% w/v), NEDA (0.01% w/v), and sulfanilamide (0.01% w/v) regents were prepared. The nitrate stock solution was prepared by dissolving 0.016 g of ultrapure potassium nitrate dissolved in 100 mL of water. The working standards of nitrate solutions were prepared by diluting the stock solution. The above chemicals KNO_3, $NaNO_2$, amines, and NEDA were procured from Sigma-Aldrich, India. The chemicals such as ammonium chloride, ammonia solution, sodium carbonate, formaldehyde, lead acetate, EDTA, and sodium hydroxide were purchased from SD Fine Chemicals, Mumbai, India. For interference study, stock solutions were prepared using corresponding salts. The working standards were prepared by diluting the stock solution on the day of use.

2.2. Equipment

The absorbance measurements were carried out using a double beam UV-VIS Spectrophotometer (Thermo scientific, Helios Zeta, model: 361003) in wavelength range 400–800 cm^{-1} using 1 cm quartz cuvettes. The synthesized azo dye was characterized using an Fourier Transform Infrared (FTIR) spectrometer (Shimadzu model: 8400S, Japan). pH measurements were carried out using Control Dynamics pH meter (model: APX 175, Mumbai, India).

2.3. Recommended Procedure

Nitrite (0.2 ppm) was added into a 25 mL standard flask containing 1.0 mL of APODT (0.01% w/v) and 2.5 mL of 0.02 N HCl. The solutions were mixed well and allowed to stand for 30 min. in standard flak. Then 1.0 mL of NEDA (0.01% w/v) solution was added and mixed well. It was diluted to 25 mL with double-distilled water. Similarly, a reagent blank was also prepared without nitrite. The sample absorbance values were measured at wavelength 580 nm against reagent blank.

2.4. Copperized-Cadmium Reductor Column

Cadmium granules (25 g, 60–120 mesh size) are splashed using 6N hydrochloric acid, and cleaned with distilled water. Washed granules were swirled in presence of 50 mL of $CuSO_4$ (2% w/v) aqueous solution for 10 min. in a beaker till blue color fades to brown color. This procedure is repeated with a 2% aqueous solution of copper sulfate in a beaker until the appearance of brown colored colloidal precipitate in the beaker. Then, the granules were gently washed with deionized water several times to remove the unreacted copper sulfate solution. The granules were air dried and used in the fabrication of the reductor column. A micro burette of 30 cm × 5 mm column size was used. Glass wool was used to plug the micro burette end and the column was filled with water. The copperized cadmium (Cu-Cd) granules were packed into the column by avoiding air bubbles. The column was washed with 300 mL of NH_4Cl-EDTA buffer solution at a flow rate of 8–10 mL/min. The column parameters, height, and flow rates were optimized for the quantitative reduction of nitrate to nitrite as per the ASTM protocol with known nitrate standards [33]. The Cu-Cd granules can be stored in NH_4Cl-EDTA buffer solution and the column can be used continuously for a period of 2–3 months.

3. RESULTS AND DISCUSSION

3.1. FTIR Spectroscopy Study

The azo dye was prepared on the large scale by the reaction of equimolar volumes of nitrite, APODT, NEDA in the acid media. The synthesized dye was isolated, filtered, and dried. This product was purified by a recrystallization method using alcohol. Proposed dye used for the evidence of the azo group formation has been studied through recording FTIR spectra in ATR mode as shown in Figure 1. The spectra of the proposed azo dye showed that a very broad peak at 2848 cm^{-1} is due to asymmetric stretching vibrations of -NH, -SH, and –CH groups. The stretching bands of C=N at 1585 cm^{-1} and C=C at1537 cm^{-1} were eluted. The IR spectrum of C-C and C-N bands showed sharp bands at 1344 cm^{-1} and 1408 cm^{-1}, respectively. The sharp band at 1247 cm^{-1} indicates the formation of the azo (N=N) group of dye. The N=N band shows the dye formation of the proposed method. IR stretching frequency of -CO band was identified at 1208 cm^{-1}. Remaining bending bands at 1055 cm^{-1}, 989 cm^{-1}, 837 cm^{-1}, and 763cm^{-1}were eluted for -SH, -NH, -CH, and -CO groups, respectively.

3.2. Optimization Study

All the parameters like acid strength, amine volume, diazotization time and NEDA volume were optimized to get a maximum absorbance value at a fixed nitrite concentration (0.4 ppm).

3.2.1. Effect of Acid Strength

The acid strength required in the diazotization process was studied by varying the volume of hydrochloric acid (0.1N) from 0.5 to 4 mL. Maximum absorbance was obtained at 2.5 mL of acid strength. Hence, 3 mL of 0.1N hydrochloric acid was optimized in all further studies (Figure2a).

3.2.2. *Effect of amine concentration*

The effect of amine concentration has been studied in the volume range of 0.5 to 3.5 mL. The highest absorbance value was obtained at 1.0 mL of amine concentration, hence,1mL of amine solution was optimized in all further studies (Figure 2b).

3.2.3 *Effect of diazotization time*

The effect of diazotisation time for the formation of azo dye was studied by varying the time between 10 to 50 min. before adding coupling agent. The absorbance values increased from 10 to 30 min. and remained constant beyond that time. Hence, 25 min. of diazotization time was optimized and used in all other studies (Figure 2c).

3.2.4 *Effect of NEDA concentration*

Finally, the effect of NEDA concertation was evaluated, by varying the volumes from 0.5 to 3.0 mL in order to achieve the maximum sensitivity of the target analyte molecule i.e nitrite. The absorbance values were increased from 0.5 to 2.5 mL, later on it remained constant. Hence, 2.5 mL of NEDA volume was optimized and used in all further studies (Figure2d).

3.3. Species Responsible for Color

Nitrite determination was carried out through spectrophotometric method by diazotization process using a HCl acts as acid catalyst, APODT novel amine, and NEDA have been a coupling agent. The synthesized purple color azo dye absorbance was measured at 580 nm (Scheme 1).

3.4. Absorption Spectra

To find the λ_{max} of the present synthesized dye and records the spectra in the wavelength region 400–800 nm. The recorded spectrum was shown at 580 nm. Hence, all the absorbance values measured were at 580 nm throughout the study.

3.5. Calibration Plot

The sensitivity of the proposed diazotization method has been examined by recording the absorbance peaks using optimized conditions by measuring the absorbance with increasing nitrite ion concentrations ranging from 0.1 to 2.5 ppm. Obtained absorbance values were plotted (Figures 3 and 4) against nitrite concentrations. Constructed calibration plot showed a linearity range from 0.1 to

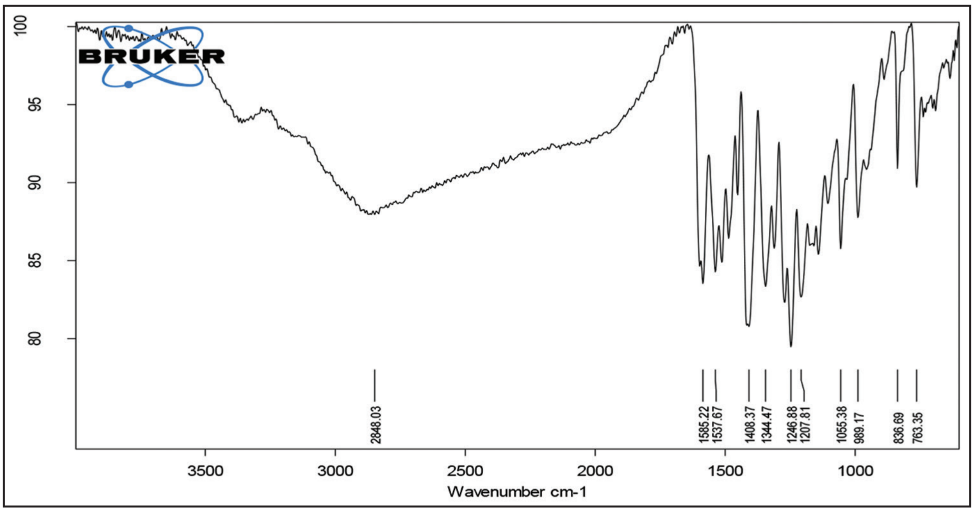


Figure 1: Fourier transform infrared spectrum of the azo dye.

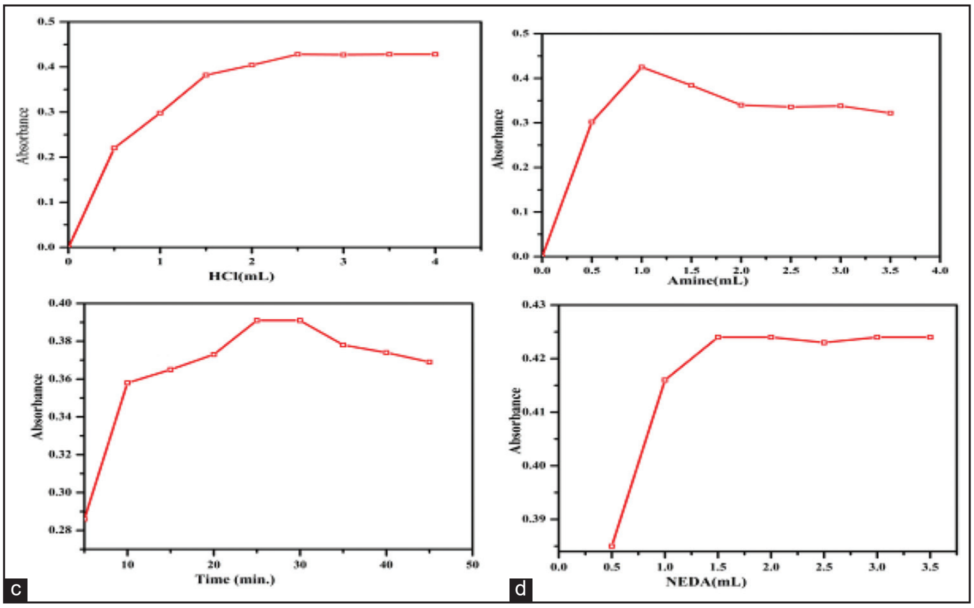
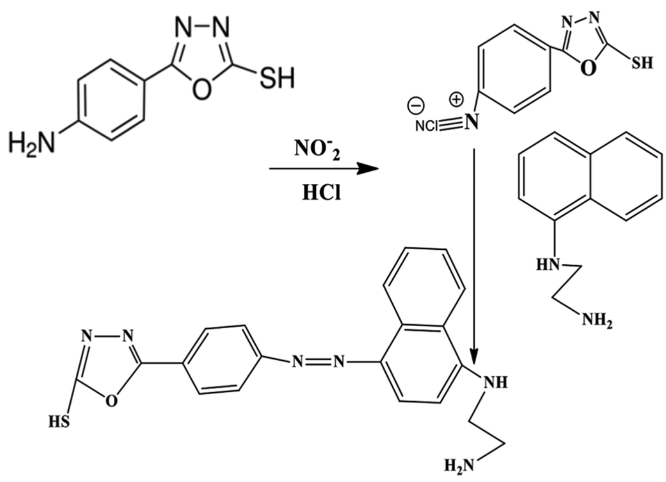


Figure 2: Effect of strength of acid (a), amine (b), diazotization time (c), and NEDA (d).



5-(4-((4-((2-aminoethyl)amino)naphthalen-1-yl)diazenyl)phenyl)-1,3,4-oxadiazole-2-thiol

Scheme 1: 5-(4-((4-((2-Aminoethyl)amino)naphthalen-l-yl) diazenyl)phenyl)-1,3,4-oxadiazole-2-thiol.

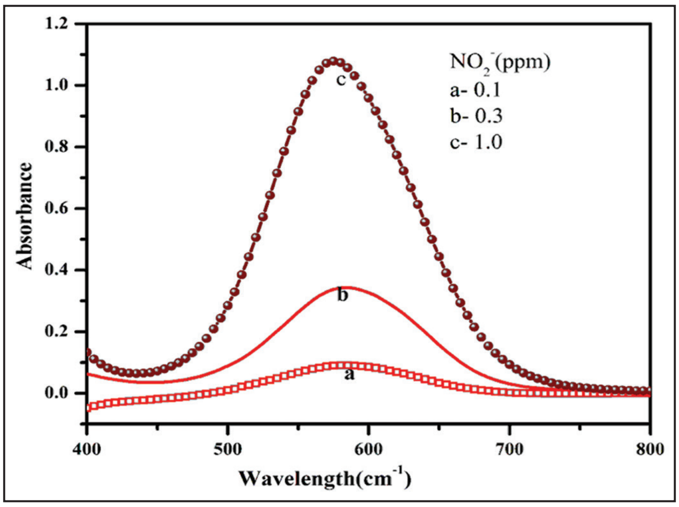


Figure 3: Absorption spectra of azo dye.

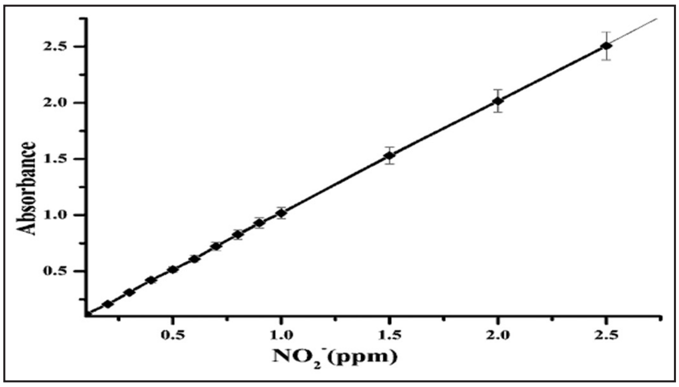


Figure 4: Calibration plot.

2.5 ppm with a linear regression coefficient of 0.999 (Table 1). High molar absorptivity (ε) 1.1 × 10^6 ($Lmol^{-1}cm^{-1}$) and low level limit of detection 0.051 (μgL^{-1}) were found in this plot and also many of the merits are mentioned in Table 2. The proposed diazotization method has been compared with the standard ASTM method. Hence, it can be used for the nitrite/nitrate measurements at trace concentrations. This protocol was applied for the organic and non-organic processed vegetable samples.

Table 1: Interference study.

Interferent	Tolerance limit (ppm)
NH_4^+, Fe^{2+}, $Ca^{2+,}$ Mg^{2+}, Zn^{2+}, Hg^{2+}, Mn^{2+}, Cd^{2+}, Cr^{3+}, Pb^{2+}, K^+, Na^+, Cl^-, CO_3^{2-}, HCO_3^-, $PO4^{3-}$, ClO_4^-, acetate, oxalate	10000
Ni^{2+}, Cu^{2+}	5000
HCHO	20
SO_3^-	5
SO_3^{-b}	10
SO_3^{-a}	20
SO_4^{2-}	150
SO_4^{2-a}	300
Fe^{3+}	100
Fe^{3+}c	300
Cu^{2+}d	7000

[a] 1 mL of 0.05% formaldehyde (v/v) was added before the addition of the coupling agent. [b] 1 mL of 0.01% lead acetate (w/v) solution was added before the addition of the coupling agent. [c] 1 mLof 1 N NaOH (w/v) solution was added before the color development. [d] 2 mL of 0.05 M EDTA (w/v) solution was added before coupling

Table 2: Analytical merits of the proposed method.

Parameters	Proposed method	Standard method
Wavelength (λ_{max})	580 nm	540 nm
Color	Purple	PINK
Working range (ppm)	0.1–100	0.3–10
Molar absorptivity (ε) ($Lmol^{-1}$ cm^{-1})	1.1×10^6	1.5×10^5
Sandell's sensitivity (μgcm^{-2})	0.0004	0.0017
Correlation coefficient (R^2)	0.9995	0.9996
LOD ($\mu g\ L^{-1}$)	0.051	0.123

3.6. Interference Study

To check the suitability of the present method for real sample analysis of nitrite/nitrate in presence of other common cations and anions coexist along with the target nitrite ion in the real sample matrices. Hence, the effect of the foreign ions was studied in presence of 1 ppm nitrite concentration. This study has been carried out by adding cations or anions in different concentration ranges in their respective salts and listed in Table 1. The maximum concentration of the nitrite absorbance error was ±5% value of 1ppm nitrite concentration was considering the tolerance limit. By, addition 1 mL of a, b, c, and d masking agents respective of SO_3^-, SO_4^{2-}, Fe^{3+}, and Cu^{2+}ions tolerance limits were increased, it is shown in Table 1.

3.7. Application Study

The proposed diazo coupling modification has been validated by applying the real sample matrices, such as organic and nonorganic processed vegetable samples, these samples were collected in the Bengaluru market. In this work, we were determined the nitrite/nitrate levels in the real samples and also known concentration of nitrite/

nitrate was spiked, recovery % also carried out. The obtained recovery % has been compared with the standard method.

3.7.1. Sample preparation

For the analysis purposes required quantitie of the samples were taken out and remaining samples are immediately stored at –20°C until completion of the analysis. 70 g of sample matrices were first cleaned with tap water and rinsed with distilled water, then dabbed with tissue paper and dried. The samples were chapped and homogenized with an electrical blender for 3 min, 10 g of weighed sample was used for analysis. The samples were transferred into 100 mL volumetric flasks as per quantitatively and added 70 mL water, then stirred and heated up to 60–70°C for 30 min. These samples are cooled at room temperature and make up with distilled water. The solutions are filtered with Whatman no 41 filter paper, then 20 mL filleted solution was decolorized using 10 mg of activated charcoal and was used for removing of samples turbidity and samples pH have been adjusted pre pH = 7. Finally, samples were used to determination of nitrite/nitrate levels.

Table 3: Determination of nitrite.

Sample name	Nitrite (μg kg^{-1} [*n*=3])		Total nitrite (μg kg^{-1} [*n*=3])			
	Originally present	**Added**	**Proposed protocol**	**Recovery% (RSD)**	**Standard protocol**	**Recovery% (RSD)**
Spinach						
Sample a	18	5	23.2	101.1±2	21.6	100.2±2
Sample b	21	5	27.5	103.4±4	24.4	99.4±4
Cabbage						
Sample a	1.2	5	6.3	103.2±4	5.3	101.3±4
Sample b	1.4	5	6.5	100.5±4	6.1	99.8±4
Beetroot						
Sample a	22	5	27.2	100.3±3	26.4	102.4±1
Sample b	26	5	29.4	101.2±2	28.9	100.5±3
Carrot						
Sample a	ND	5	5.1	100.5±4	4.5	100.3±5
Sample b	ND	5	5.3	101.6±3	5.1	101.2±6
Cucumber						
Sample a	ND	5	4.9	98.6±3	5.2	101.2±2
Sample b	ND	5		100.2±2	5.1	102.1±4

[a]Vegetables by organic farming, [b]Vegetables by non-organic farming, ND: Not detected

Table 4: Determination of nitrate.

Sample name	Nitrate (mg kg^{-1})		Total nitrate (mg kg^{-1} [*n*=3])			
	Originally present	**Added**	**Proposed Protocol**	**Recovery% (RSD)**	**Standard protocol**	**Recovery% (RSD)**
Spinach						
Sample a	362.5	10	372.7	102.4±2	364.6	100.5±2
Sample b	384.3	10	394.3	101.6±2	390.3	101.6±5
Cabbage						
Sample a	118.4	10	127.9	100.5±2	125.4	100.2±4
Sample b	123.6	10	135.2	102.2±4	129.5	103.6±3
Beetroot						
Sample a	408.6	10	419.1	102.2±4	415.83	100.9±3
Sample b	421.5	10	433.1	104.3±2	430.65	101.4±1
Carrot						
Sample a	70.6	10	80.7	101.2±1	75.36	101.2±4
Sample b	80.2	10	90.3	100.3±2	85.52	100.3±3
Cucumber						
Sample a	48.5	10	59.1	101.4±3	58.9	99.4±3
Sample b	56.3	10	66.3	100.4±4	64.4	97.6±1

3.7.2. Nitrite determination

1 mL of the vegetable solution was taken in to the 25 mL volumetric flask and 1 mL of amine solution, 2.5 mL of HCl solution were added and mixed well, and stand for 30 min at room temperature and finally added 2 mL of NEDA solution, then immediately purple color azo dye was formed and make up with water. The formed azo dye absorbance values were measured against reagent blank. The results are shown in Table 3. In this evaluation, organic processed vegetables were given low level nitrites comparatively non-organic processed vegetables.

3.7.3. Nitrate determination

10 mL of vegetable sample solution was taken out into the 50 mL beaker and added the 5 mL of NH_3-NH_4Cl buffer solution (pH = 8.5) was added and allowed to stand for 5 min then transfer into the redector column, sample solution was collected into the 25 mL volumetric flasks makeup with water. This solution was used for the preparation of azo dye by following the above procedure. The concentrations of the reduced nitrite were calculated with reference to the standard calibration plot constructed using standard nitrite through diazo-coupling reaction. The results of nitrate were calculated using below formula. Obtained values were represented in Table 4. By, results comparisons of organic processed vegetables concentration low level nitrate values then, non-organic processed vegetables. The nitrate content is determined using this formula.

$$\text{Nitrate (mg mL}^{-1}) = ([\text{Total } NO_2^{-} (\mu\text{g L}^{-1}) - NO_2^{-} \text{ originally present } (\mu\text{g mL}^{-1})]/\text{sample volume} \times 1000]) (62/46).$$

4. CONCLUSIONS

A simple protocol based on diazo-coupling reaction has been proposed using a novel amine. The method provided a very low detection limit, wide linear range with high molar absorptivity. The protocol has been successfully utilized to measure trace level nitrite/nitrate from organic and non-organic processed vegetable sample matrices. The results by proposed method are in good agreement with the results of the standard protocol.

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