

Simultaneous flow-through determination of nitrites, nitrates and their mixtures in environmental and biological samples using spectrophotometry

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Received 31 October 2004; received in revised form 10 November 2004; accepted 11 November 2004

Available online 4 January 2005

Abstract

Newly synthesised 4,4'-methylene-bis-*m*-nitroaniline has been used to detect nitrites, nitrates, their mixtures in environmental and biological samples. The method is based on the reaction of nitrite with 4,4'-methylene-bis-*m*-nitroaniline in HCl medium and the concentration of diazonium salt formed, monitored through simultaneous flow by spectrophotometrically. The absorbance of the resultant red colored diazonium salt was measured at 495 nm and stable for more than 12 h. The determination of nitrates can be carried out by using the same reaction prior to reduction in a flow-through reductor column containing zinc dust coated polyurethane foam. The reaction and flow conditions of the full experimental design were optimised. The molar absorptivity and Sandell's sensitivity were found to be $7.5 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$ and $0.00062 \mu\text{g ml}^{-1}$. Detection limits for nitrite and nitrate were 0.10 and 0.2 $\mu\text{g ml}^{-1}$, respectively. The detailed study of various interference made the confirmation of high selectivity of the method. The method was successfully applied to the determination of nitrites and nitrates in water, soil and biological samples. The results obtained were agreed with the reported methods at the 95% confidence level.

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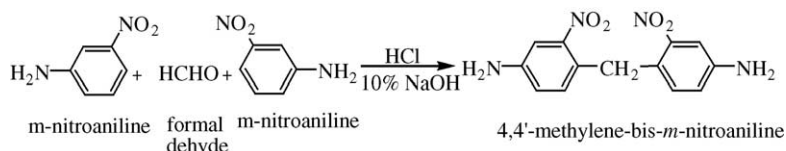
Keywords: 4,4'-Methylene-bis-*m*-nitroaniline; Nitrite ion; Nitrate ion; Spectrophotometry; Water; Soil; Human serum and urine samples

1. Introduction

Nitrite is undesirable in water due to its toxicity [1], hence a great deal of interest has been generated concerning potential health hazards of nitrite. Nitrites are frequently used as preservatives in food products and their precursors are widely distributed in nature because of nitrogen fertilisers [2]. The important and mysterious physiological roles [3–5] for nitric oxide have led to the extensive growth in the studies of NO in the fields of life science and its related subjects [6,7]. Determination of nitrites, nitrates and their mixtures, especially in environmental samples, is required at many control laboratories and thus the subject of a great many

professional communications. Most spectrophotometric methods for the determination of nitrite in natural and waste water were based on Griess–Iosvey reaction [8], Saltzman [9] and Jacobs–Hochheiser [10], which was modified several times. Classical batch, flow injection analysis (FIA) or sequential injection analysis (SIA) and spectrophotometric determination of nitrites (nitrates after their reduction) are mostly based on their ability to undergo the diazotization reaction with aromatic amino groups frequently followed by coupling with the formation of azodyes [11–22], the effect on fluorescence of suitable dyes [23–25] and other methods [26,27] were recently used. All these methods have certain limitations such as less stability, selectivity, sensitivity, interference of non-target species and laborious process (diazonium salt coupled with *N*-(1-naphthyl) ethylene diamine hydrochloride (NEDA)). Ion-selective nitrate electrodes, which have recently become popular but suffer from severe

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Scheme 1. Synthesis of 4,4'-methylene-bis-*m*-nitroaniline.

interference effect caused by other anions present in the sample [28].

This paper deals with the flow-through analysis, coupled with a spectrophotometric detector it is simple, rapid, economically advantageous (low reagent consumption), provides reproducible results in statistical importance (elimination of subjective analytical errors and foreign species) and a useful tool for quantification of samples in a variety of real natural samples at low detection level for series analysis of nitrite, nitrate and their mixtures in environmental and biological samples.

2. Experimental

2.1. Reagents

All the chemicals used were of the highest purity available and used without further purification. Sodium nitrite, ammonium chloride were purchased from S.D. fine chemicals, Mumbai, India. Hydrochloric acid, sodium hydroxide, formaldehyde and *m*-nitroaniline were purchased from Merck Chemicals, Mumbai, India. Doubly-distilled water was used throughout the experiment.

A standard stock solution of nitrite (1.0 mg/l) was prepared by dissolving 0.15 g sodium nitrite in 100 ml of doubly-distilled water. The reagent 0.5 M 4,4'-methylene-bis-*m*-nitroaniline was prepared by dissolving 14.40 g of 4,4'-methylene-bis-*m*-nitroaniline in 100 ml of 5 M HCl and diluted to 100 ml with double-distilled water. Finally, 1 M HCl solution was prepared by adding 8.33 ml concentrated HCl in 100 ml double-distilled water. A 0.5 M NH_4Cl solution was prepared by dissolving 2 g of NH_4Cl in 100 ml of double-distilled water.

2.2. Instrumentation

Flow-through measurements were carried out using a Cole Parmer programmable peristaltic pump with an Ismatec eight-channel head (Cole Parmer, USA). In the factor experiments, an SC4 pump from the developmental workshops of the Academy of Science of CR was employed as a second peristaltic pump for pumping the solutions of carrier, HCl and color forming reagent. Sample flow-through was carried out using a six-way 5020 valve (Rheodyne, USA).

Solutions were pumped through TYGON tubes (Cole Parmer, USA) with various internal diameters. The liquids

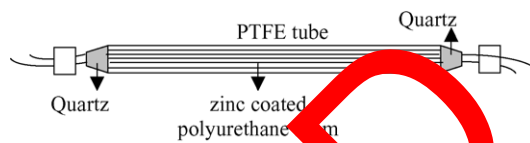


Fig. 1. Reduction column.

were then fed through Teflon capillaries with an internal diameter of 0.5 mm (Supelco, USA). The reaction coils consisted of Teflon tubes (internal diameter 0.5 mm) coiled in a spiral of 80 mm and reductor column of 200 mm. A HITACHI U 2001 spectrophotometer with a flow-through quartz cuvette with an internal volume of 80 μl and absorption layer thickness of 1 cm cell was employed as the flow-through detector.

2.3. Synthesis of 4,4'-methylene-bis-*m*-nitroaniline

12.7 g of *m*-nitroaniline was dissolved in 125 ml acetone and 25 ml of 36.5% hydrochloric acid at 50 °C. The reaction mixture was then treated with 35 ml of 3% aqueous formaldehyde solution at 60 °C with stirring for 1 h and neutralised with 5 M sodium hydroxide. The solid obtained was filtered, washed with hot water, dried and recrystallized from acetic acid [29] (m.p., 102 °C; yield, 91%, as shown in Scheme 1).

2.4. Preparation of reductor column

The column for reduction of nitrate (Fig. 1) consists of a Teflon tube with an internal diameter of 2 mm, whose inlet and outlet are sealed with silicon wool. The reductor column was filled with zinc dust coated polyurethane foam. The reductor can be reactivated four to five times, but its lifetime is decreased after each activation. The reductor column works with good efficiency for a period of 12 h.

2.5. General procedure

Fig. 2 depicts a scheme of the flow-through apparatus (FTA) for determining the nitrites and Fig. 3 depicts the apparatus for determining nitrates (reduction to nitrites) and for simultaneous determination of nitrites and nitrates. Two solenoid valve (A, B) are included in the system for determining both nitrite and nitrate anions. Adjustment of these valves permits feeding of the sample through the reduction column, where the nitrates present in the sample are reduced

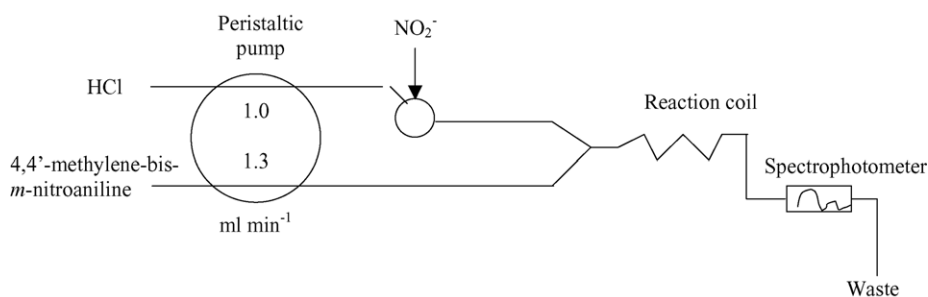


Fig. 2. Flow-through apparatus for determining nitrites.

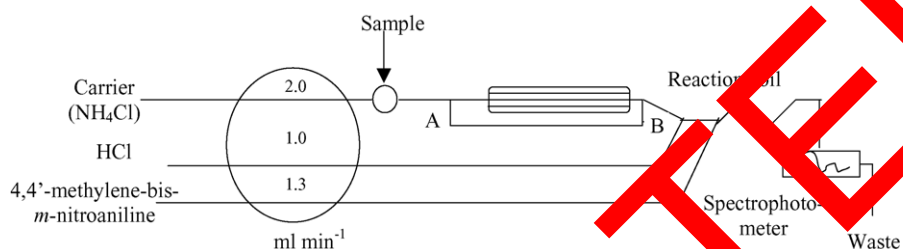


Fig. 3. Flow-through apparatus for determining nitrites and nitrates.

to nitrites and the signal obtained then corresponds to the sum of nitrites present in the sample and nitrites formed by reduction of the nitrates present in it. If both valves are switched to the other position, the sample is fed around the reduction column and the signal obtained corresponds only to the concentration of nitrites originally present in the sample. The concentration of nitrates in the sample is then determined as a difference between these two signals without Saito effect.

2.6. Procedure for the determination of nitrate and nitrite ions in water sample

An aliquot of the sample containing not more than $12 \mu\text{g ml}^{-1}$ of nitrate was treated with 5 M HCl solution in 5:1 ratio (sample:acid). The solution was mixed and then centrifuged to remove any precipitate formed. The centrifugate was transferred to 50 ml evaporated flask as reported in literature [30] and injected into the flow-through system for determination of nitrate and nitrite ions as described in general procedure.

2.7. Procedure for the determination of nitrate and nitrite ions in soil samples

Soil samples were collected from several selected sites in and around Tirupati and treated in the standard manner [30]. First the soil samples were dried in an oven at $110 \pm 15^\circ\text{C}$ for a period of one day and then they were crushed in a mortar to yield fine dust and was used for analysis. Hundred millilitres of double-distilled water was added to 10 g of prepared sample, the mixture was shaken in a shaking machine and filtered

after a prescribed time of 5 min. NO_2^- and NO_3^- ions were determined in the filtrate as discussed in aforesaid procedure.

3. Procedure for the determination of nitrate and nitrite ions in biological samples

One millilitres of 0.5 M NaOH solution and 1 ml of 0.5 M ZnSO_4 solution were added to 10 ml serum and the volume was made up to 100 ml. After protein was removed as described in literature [25]. The nitrate and nitrite ions were determined as mentioned in aforesaid procedure.

Ten millilitres urine was diluted by 5 ml methanol. The protein present in the urine was removed [25] and sample is subjected to the determination of nitrite and nitrate as discussed above.

3. Results and discussion

The reaction of nitrite with 4,4'-methylene-bis-m-nitroaniline in an HCl medium produces red colored product with λ_{max} 495 nm as shown in Fig. 4.

3.1. Flow-through determination of nitrates following reduction to nitrites

Several methods of reducing nitrates to nitrite are described in the literature [8,19–27] (e.g., by hydrazine using Cu^{2+} ions as a catalyzer, zinc in $\text{Mn}(\text{OH})_4$ medium or in H^+ medium). The reaction conditions for these methods require long time for reduction and the necessity of mixing large volume of reagent. However, it do not allow these types

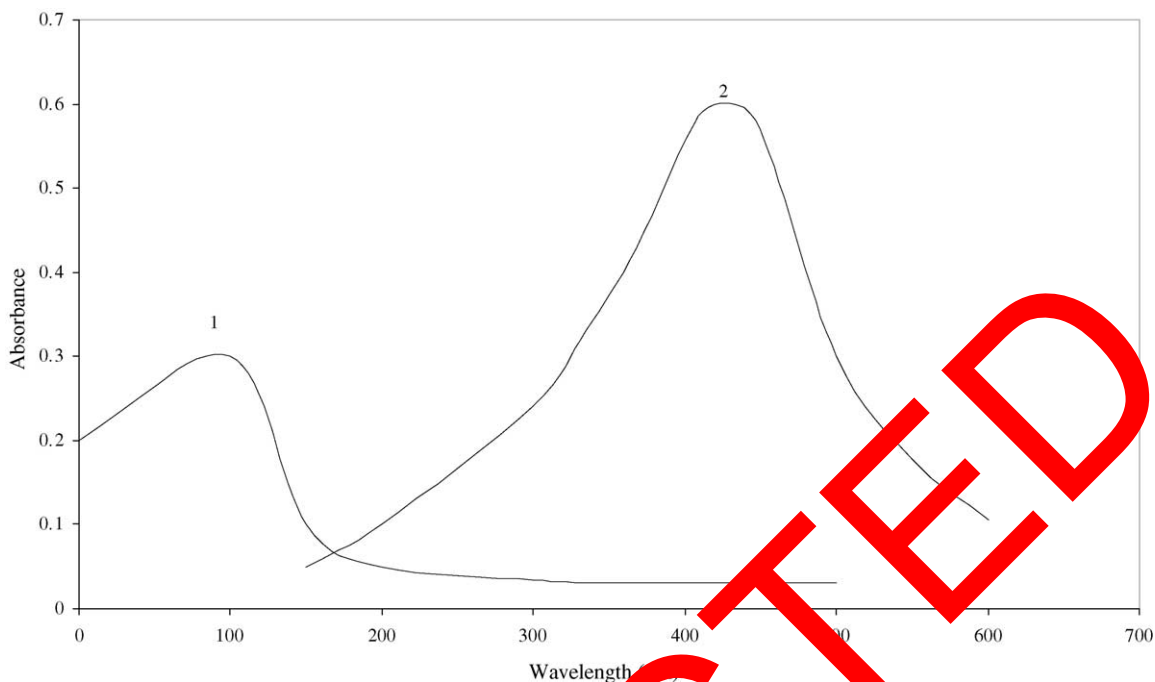


Fig. 4. Absorption spectrum of nitrite ion with: (1) reactant blank; (2) 4,4'-methylene-bis-*m*-nitroaniline.

of reductions for flow-through determination. In the present work, the reducing agent employed in reductor was zinc coated polyurethane foam. The nitrites formed were determined by reaction with 4,4'-methylene-bis-*m*-nitro aniline in presence of HCl medium.

A 0.5 M NH_4Cl solution was used as a carrier stream into which the sample is injected (Fig. 3) and passes through the reductor column. The presence of hydrochloric acid in reductor would lead to destruction of polyurethane foam if it is in excess. Hence, the channel of hydrochloric acid, which is necessary for the diazotization reaction, was connected to the system after the exit of the sample from the reductor.

3.2. Simultaneous flow-through determinations of nitrites and nitrates

We employed the apparatus depicted in Fig. 3 for the determination of nitrite and nitrate present in given samples by the above reagent employed. As the reaction conditions in the reactor are different than in the apparatus for the determination of nitrites as shown in Fig. 2, i.e., a carrier channel for NH_4Cl instead of HCl, was necessary to measure the calibration curve of nitrites under the same conditions as the calibration curve of nitrates, i.e., in the presence of ammonium chlorides into which the sample is injected and with the solenoid valves switched to a position so that the injected NO_2^- sample is carried by the carrier stream around the reduction column.

The basic characteristics of flow through determination for NO_2^- were compared with direct determination for NO_2^- were presented in Table 1. It indicates from comparison that

when the NH_4Cl carrier is used, there is a slight decrease in the sensitivity of the method, i.e., apparently caused by the greater dispersion of the sample in the reductor. On the other hand, the detection and determination limits were decreased in flow-through arrangement, probably due to lower resultant pulsation of the flow. Thus, there is a significant increase in the linear dynamic range of the calibration.

3.3. Optimisation of flow-through system

3.3.1. Effect of flow-rate of a sample

The effect of flow-rate on the peak height was studied in the range of 0.5–1.5 ml min^{-1} . The peak height decreased with the increasing of the flow-rate. Taking into consideration

Table 1

Effect of foreign species on the simultaneous flow-through determination of nitrite, nitrate and in their mixtures

Foreign ions	Tolerance limit ($\mu\text{g ml}^{-1}$)
NO_3^- , Cl^- ^a , EDTA	10000
K^+ , Cd^{2+} , CH_3COO^- , Ca^{2+} , CO_3^{2-} , SO_4^{2-} , Na^+ , Mg^{2+}	5000
Al^{3+} , WO_4^{2-} , Ba^{2+} , Tartarate, PO_4^{3-}	4000
SO_3^{2-} , Mn^{2+} , CN^- , Hg^{2+} , SCN^-	2000
I^- , F^-	1000
S^{2-} ^a	400
Pb^{2+} , MoO_4^{2-} , Ni^{2+} , Zn^{2+}	200
Fe^{2+} , Co^{2+} , Cu^{2+} , Sn^{2+}	40
Fe^{3+} ^b , V^{5+} , Cr^{6+}	8

^a Can be masked up to 400 and 300 $\mu\text{g ml}^{-1}$ by the addition of 2 ml of 5 M HgCl_2 .

^b Can be masked up to 200 $\mu\text{g ml}^{-1}$ by the addition of 3 ml of 5 M EDTA.

Table 2
Comparison of basic characteristics between flow-through determination and direct determination

Basic characteristics	Flow-through determination of NO ₂ ⁻	Flow-through determination of NO ₃ ⁻	Direct determination of NO ₂ ⁻ with Dapsone + NEDA [12]
Slope of linear part (mg ml ⁻¹)	0.030	0.01630	0.0245
Detection limit (μg ml ⁻¹)	1.0	0.12	0.56
Determination limit (μg ml ⁻¹)	1.48	3.96	0.44
Repeatability (%)	0.65	1.84	1.5
Linear calibration range (mg ml ⁻¹)	1.46–36.0	3.6–46.0	0.40–74.0
Correlation coefficient	0.9992	0.9994	0.9994

the stability of the pump, peak shape and sampling time, the flow-rate of the reagent carrier solution was adjusted to 2 ml min⁻¹.

3.3.2. Sample solution

The influence of the sample volume on the absorbance was investigated by injecting different volumes (150–250 μl) of nitrite standard solutions into the system at 1 ml min⁻¹ flow-rate. The flow-through volume has a significant effect, yielding increased peak height and reproducibility with increasing of the flow-through volume up to 200 μl. After increasing the volume of the sample beyond 200 μl, the peak height gradually decrease. So, 200 μl of sample solution was selected for maximum peak height in present investigation.

3.3.3. Effect of color forming reagent concentration

The effect of concentration of 4,4'-methylene-bis-*m*-nitroaniline reagent in the carrier solution, on the peak height was investigated at flow-rate of 1.3 ml min⁻¹ by using 8 μg l⁻¹ nitrite solution. The concentrations of 4,4'-methylene-bis-*m*-nitroaniline reagent were varied over the range 0.1–1.0 M. Maximum peak height was obtained at concentration of 0.5 M of 4,4'-methylene-bis-*m*-nitroaniline as color developing reagent in the carrier solution for lower concentration levels of nitrite and nitrate in the sample solution.

3.3.4. Effect of reductor length on efficiency of reduction

In monitoring the effect of the length of reductor on the efficiency of the reduction, the magnitude of the active contact area of the reductor was changed through a change in the length of the reductor. We found that the reduction of nitrates to nitrites is maximum when a reductor with a length of more than 150 mm. Lengthening of the reductor beyond 200 mm led to greater broadening of the nitrate sample zone. So, reductor length of 200 mm was chosen for the present study.

3.3.5. Effect of HCl concentration and NH₄Cl flow rate

In addition, a study was performed for the simultaneous effect of HCl concentration and NH₄Cl flow rate for determination of nitrate and nitrite ions in various environmental samples. Under the optimal conditions for attaining the maximum sensitivity of the flow-through determinations of nitrates, following prior reduction to nitrites, were given as: λ_{max}, 495 nm; HCl, 1 M; HCl flow rate, 1.0 ml min⁻¹; NH₄Cl,

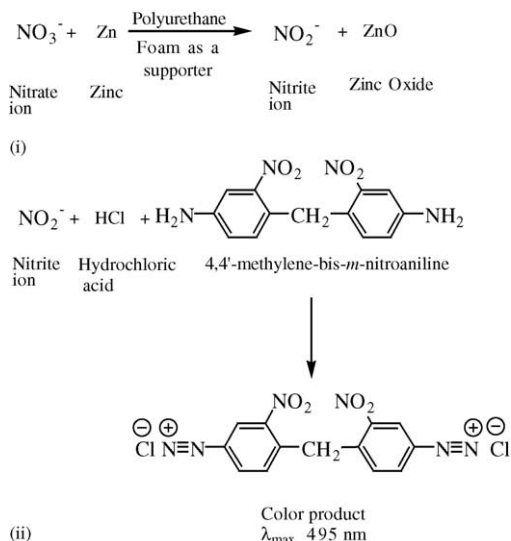
0.5 M; NH₄Cl flow rate, 2.0 ml min⁻¹; volume of sample injected, 200 μl; reaction coil length, 200 mm; reductor length, 200 mm). The basic characteristics of this determination are given in Table 2.

3.3.6. Tolerance of foreign ions

Several cations and anions, as potential interferences for different sample matrices, were studied in detail. Table 2 summarises the tolerance limits of interfering ions on the determination of 10 mg l⁻¹ of nitrite and 120 mg l⁻¹ of nitrate, respectively. Sulphate and chlorides can be masked up to 50 and 300 μg ml⁻¹ with 2 ml of 5 M mercuric chloride. Iron(III) can be masked up to 200 μg ml⁻¹ with 3 ml of 5 M EDTA. The tolerance limit was taken as the amount, which caused an error of ±5% at the peak height. Almost all the ions examined did not interfere with nitrites, nitrates and their mixtures in environmental and biological samples by simultaneous flow-through method.

3.4. Reaction sequence

Nitrate ion interact with zinc coated polyurethane foam in reductor column and converts into nitrite ion. This nitrite ion with the help of carrier solution flow-through the system to react with 4,4'-methylene-bis-*m*-nitroaniline in presence of



Scheme 2. (i) Reaction in reductor column; (ii) formation of color product.

Table 3
Analytical results of total amount of nitrite and nitrate in various environmental samples with 4,4'-methylene-bis-*m*-nitroaniline

Sample	Proposed method						Reported method[7,8,12-20]			
	Added ($\mu\text{g ml}^{-1}$)	Found ($\mu\text{g ml}^{-1}$)	Recovery (%)	S.D. ^d (%)	<i>f</i> -test ^e	<i>t</i> -test ^f	Added ($\mu\text{g ml}^{-1}$)	Found ($\mu\text{g ml}^{-1}$)	Recovery (%)	S.D. ^d (%)
Synthetic sample-I	0.2	0.197	98.5	0.860	2.4	0.9	0.2	0.194	97.5	0.665
Synthetic sample-I	0.5	0.496	99.2	0.912	1.2	0.7	0.5	0.492	98.4	0.716
Natural water sample-I	–	0.236	–	–	1.1	0.6	–	0.261	–	–
	0.1	0.334	98.0	0.590	–	–	0.1	0.3585	97.5	0.716
Natural water sample-II	–	0.391	–	–	–	–	–	0.329	–	–
	0.3	0.688	99.0	0.547	1.3	0.7	0.3	0.29	97.3	0.678
Soil sample-I	–	0.126	–	–	–	–	–	–	–	–
	0.2	0.324	99.0	0.730	1.3	0.7	0.3	0.292	97.3	0.476
Soil sample-II	–	0.280	–	–	–	–	–	–	–	–
	0.2	0.477	98.5	0.840	1.0	0.2	0.5	0.47	98.5	0.752
Urine ^a	0	0.158	–	–	–	–	–	0.114	–	–
	0.4	0.555	99.2	0.496	–	–	0.5	0.606	98.4	0.640
Human serum (hypertension) ^b	0	0.396	–	–	–	–	–	0.27	–	–
	0.4	0.791	98.7	0.810	–	–	0.4	1.198	98.0	0.910
Human serum (health) ^c	0	0.623	–	–	–	–	–	0.426	–	–
	0.4	1.02	99.5	0.930	–	–	1.5	1.906	98.6	0.894

^a Urine samples collected from S.V.R.R. Hospital.

^b Blood samples collected from S.V.R.R. Hospital.

^c Blood samples collected from SVIMS.

^d Mean \pm standard deviation ($n = 4$).

^e Tabulated *f*-test for (4,4) degree of freedom at $P(0.95)$ is 6.39.

^f Tabulated *t*-test values for 8 degrees of freedom at $P(0.95)$ is 2.36.

HCl to form stable diazonium chloride as shown in Scheme 2. Hence, it is not required to develop the color by the reaction with other coupling reagents.

3.5. Method evaluation

The proposed flow-through analyser was critically evaluated with regard to reproducibility, accuracy, sample interaction and sample frequency.

3.5.1. Reproducibility

To test the reproducibility of the flow-through analyser, four repetitive analysis cycles of each sample were run. A % S.D. in the range 0.49–0.93 was obtained as shown in Table 3.

3.5.2. Accuracy

The accuracy of the proposed flow-through analyser was evaluated by comparing the results with those obtained by the

Table 4
Comparison of spectrophotometric methods with present method for determination of nitrite and nitrate ions

Reagents	λ_{max} (nm)	Sensitivity ($l \text{ mol}^{-1} \text{ cm}^{-1}$)	Remarks	References
Sulphanilamide	550	4.0×10^4	Less sensitive	[7]
Sulphanilic acid	550	4.6×10^4	Less detection limit	[8]
Dapsone	540	7.2×10^4	SO ₂ and Fe(III) interfered and less sensitive than the proposed method	[12]
Sulphadiazine	545	6.9×10^4	Controlled heating is required	[13]
3-Nitroaniline	535	4.9×10^4	–	[14]
<i>p</i> -Aminobenzophenone	555	7.0×10^4	Cu ²⁺ , Fe ³⁺ , PO ₄ ³⁻ interfered seriously	[15]
<i>p</i> -Aminophenyl mercapto acetic acid	565	4.65×10^4	15 min is needed for colour development, Fe(III), Sn(II), iodide and sulphide interfered seriously	[16]
<i>p</i> -Rosanilinium chloride	560	8.33×10^4	30 min is needed for colour development and metal ions are interfered seriously	[17]
<i>p</i> -Aminoacetophenone	545	4.6×10^4	Cu ²⁺ , Fe ³⁺ , S ₂ ⁻ and SO ₂ interfered seriously	[18]
Pararosaniline	565	5.75×10^4	Fe ²⁺ , Cr(VI) and sulphide interfered seriously	[19]
<i>p</i> -Nitroaniline	545	6.4×10^4	Tedious extraction into iso amyl alcohol	[20]
4,4'-Methylene-bis- <i>m</i> -nitroaniline	495	7.5×10^4	Most sensitive, rapid, facile, non extractive, low cost reagent, common ions do not interfere	This work

direct determination of nitrite ion only. The results shown in Table 3 reveal the good correlation between the two methods indicative of present method is more sensitive than the reported methods in literature [7,8,12–20].

3.5.3. Effect of sample interaction

Samples interaction or the carry over effect was investigated by analysing a sample with a low analyte concentration followed by that of high concentration and again that of low concentration. A sample containing 6.0 mg l^{-1} was used to represent the low analyte concentration. Sample interaction was then calculated using the following reaction:

$$\text{Sample interaction} = \frac{K_3 - K_1}{K_2} \times 100\%$$

where K_1 is the sensitivity of the analyte containing 6.0 mg l^{-1} nitrite ions, K_2 the sensitivity of the analyte containing 30 mg l^{-1} nitrite ions and K_3 is the response of the analyte containing 6.0 mg l^{-1} nitrite ions. The calculated carry over effect was found to be 0.05%.

3.5.4. Sampling frequency

The experimental period for one complete analytical cycle was 240 s long giving an overall sampling rate of 15 samples/h.

3.6. Applications of the method for simultaneous determination of NO_2^- , NO_3^- and statistical comparison with reported method

The proposed method for the simultaneous determination of nitrates and nitrites has been employed for real samples of water, soil, urine and serum samples were presented in Table 3. The obtained results was compared with the reported methods [7,8,12–20] in terms of Student's *t*-test and *f*-test. The analytical data summarised in Table 3 suggest that the percentage of both ions recovery from water, soil and biological samples range from 98.0 to 99.5%, which is more reliable and precise than the other methods. The recovery percentage of both ions (nitrite and nitrate) under study presented in the Table 3 indicates that their order in various environmental systems under study are as follows:

- Biological samples > soil samples
- Soil samples > water samples
- Biological samples > water samples.

It is evident from the above data that the proposed method is facile, rapid and more sensitive (Table 4).

4. Conclusion

The method is facile, fast, reliable and highly sensitive for simultaneous determination of both nitrites, nitrates and

in their mixtures in real samples. The limit of detection of the proposed method is superior and compared with some previously reported methods [7,8,12–20]. The method has added advantage over reported method [7,8,12–20] owing to its:

- (i) Simplicity in synthesis of color forming reagent at ordinary laboratories.
- (ii) Synthesised organic reagent has distinct in terms of sensitivity, selectivity towards nitrite ion.
- (iii) Schlieren effect does not effect the color intensity, density, dynamic flow, temperature etc.
- (iv) Proposed method is sensitive due to the stabilisation of diazonium chloride having a λ_{max} of 5 nm indicative of no additional coupling reagents.

Freedom from reagent effect, temperature independence, less interference, more accuracy and avoidance of lengthy extraction steps which have to be placed among the most sensitive ones.

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