Chapter - 6

Methods for the Analysis of Nitrite

OVERVIEW

Nitrogen makes up 78% of the atmosphere as gaseous molecular nitrogen, but most plants can use it only in the fixed forms of nitrate and ammonium. Nitrate and nitrite are inorganic ions occurring naturally as part of the nitrogen cycle. A nitrite is either a salt or an ester of nitrous acid. Nitrite is used predominantly as a food preservative, especially in cured meats. Nitrate is the more stable of the two forms of nitrogen but can be reduced by microbial action to nitrite, which is the more toxic form.

The nitrogen cycle is composed of four processes. Three of the processes—fixation, ammonification, and nitrification—convert gaseous nitrogen into usable chemical forms. The fourth process, denitrification, converts fixed nitrogen back to the unusable gaseous nitrogen state. Nitrification is the process in which ammonia is oxidized to nitrite and nitrate, yielding energy for decomposer organisms. Two groups of microorganisms are involved in nitrification. *Nitrosomonas* oxidizes ammonia to nitrite and water. Subsequently, *Nitrobacter* oxidizes the nitrite ions to nitrate [1].

In inorganic chemistry, nitrates are salts of nitrous acid, HNO₂. They contain the nitrite ion NO₂⁻. Nitrites of the alkali and alkaline earth metals can be synthesized by reacting a mixture of nitrogen monoxide (NO) and nitrogen dioxide (NO₂) with the corresponding metal hydroxide solution, as well as through the thermal decomposition of the corresponding nitrates. Other nitrites are available through the reduction of the corresponding nitrates. The two canonical structures of NO₂⁻ which contribute to the resonance hybrid are:
Sodium nitrite is used for the "curing of meat" because it prevents bacterial growth and, in a reaction with the meat's myoglobin, gives the product a desirable dark red color. Because of the toxicity of nitrite (lethal dose of nitrite for humans is about 22 mg per kg body weight), the maximum allowed nitrite concentration in meat products is 200 ppm [2]. Under certain conditions, especially during cooking, nitrites in meat can react with degradation products of amino acids, forming nitrosamines, which are known carcinogens.

In organic chemistry, nitrites are esters of nitrous acid and contain the nitrosooxoy functional group. They possess the general formula RONO, where R is an aryl or alkyl group. Amyl nitrite is used in medicine for the treatment of heart diseases. Structure of the nitrosooxoy functional group is:

![Nitrosooxoy Functional Group]

**Toxicity**

Unless favorable conditions exist for reducing nitrate to nitrite in the gut (i.e., high pH, proper intestinal microbial flora), ingested nitrate (NO$_3^-$) is metabolized and excreted without producing apparent adverse effects. The effects of nitrite (NO$_2^-$) are the same whether nitrite-containing compounds are ingested or inhaled, or nitrite is produced in vivo from nitrate [3].

Acute acquired methemoglobinemia is the most important adverse health effect caused by excessive nitrate exposure. Nitrate concentrations (as NO$_3^-$) >45 mg/L may cause methemoglobinemia in infants (Blue Baby Syndrome) [4]. The toxicity of nitrate in humans is a result of the reduction of nitrate (NO$_3^-$) to nitrite (NO$_2^-$). The principal mechanism of nitrite toxicity is the oxidation of the ferrous iron (Fe$^{2+}$) in deoxyhaemoglobin to the ferric (Fe$^{3+}$) valence state, producing methemoglobin. Methemoglobin cannot reversibly bind or transport circulating oxygen to tissues [3]. Thus, methemoglobin formation may lead to asphyxia. Normally, methemoglobin accounts for 1-2% of the globin in the body. A level greater than 3% is defined as methemoglobinemia. The most obvious symptom of methemoglobinemia is the appearance of a bluish tone on the skin, particularly around the eyes and mouth. If quickly discovered, this disease can be successfully treated with an injection of methylene blue, which changes methemoglobin...
back to haemoglobin. The condition is extremely serious if it’s not treated: death takes place when 70% of the body's haemoglobin has been converted to methaemoglobin [5].

Hypotension, shock, and cardiac arrhythmias can occur in cases of severe methaemoglobinemia. Severe methaemoglobinemia might lead to metabolic acidosis. A chocolate-brown or slate-gray central cyanosis (involving the trunk and proximal portions of the limbs, as well as the distal extremities, mucous membranes, and lips) is one of the hallmarks of methaemoglobinemia. This cyanosis is due to the dark chocolate-brown colour of methemoglobin itself and can become noticeable at a concentration of 10–15% of total haemoglobin [3].

Concern has been expressed about the cancer-causing potential of nitrates and nitrites used as preservatives and color-enhancing agents in meats. Nitrates can react with amino acids to form nitrosamines, which have been reported to cause cancer in animals [3,6]. Nitrosamines are produced from excess nitrite during frying and in the stomach [7]. The toxicological significance of nitrosation reactions in vivo and in the natural environment is the subject of much current concern and research. A slight increase in stomach cancer incidence was seen in a group of 1,756 male workers at a nitrate fertilizer plant. An increased incidence of stomach cancer has been observed in one group of workers with occupational exposures to nitrate fertilizer [8].

Environmental effects
The growth of macrophytes (aquatic plant) and phytoplankton (algae) is stimulated principally by nutrients such as phosphorus and nitrogen. Nutrient-stimulated primary production is of most concern in lakes and estuaries, because primary production in flowing water is thought to be controlled by physical factors, such as light penetration, timing of flow, and type of substrate available, instead of by nutrients [4].

*Freshwater system impacts:* Generally, phosphorus is the limiting nutrient in freshwater aquatic systems. That is, if all the phosphorous is used, plant growth will cease, no matter the amount of nitrogen available.

Many bodies of freshwater are currently experiencing influxes of nitrogen and phosphorus from outside sources. The increasing concentration of available phosphorus allows plants to assimilate more nitrogen before the phosphorus is depleted. Thus, if sufficient phosphorus is available, high concentrations of nitrates will lead to phytoplankton (algae) and macrophyte (aquatic plant) production [4].

*Estuarine system impacts:* In contrast to freshwater, nitrogen is the primary limiting nutrient in the seaward portions of most estuarine systems. Thus, nitrogen levels control the rate
of primary production. If a nitrogen limited system is supplied with high levels of nitrogen, significant increases in phytoplankton (algae) and macrophyte (larger aquatic plants) production may occur. [4]

Excessive aquatic plant production may negatively impact fresh water and estuarine environments in the following ways [4]:

1. Algal mats, decaying algal clumps, odors, and discoloration of the water will interfere with recreational and aesthetic water uses.
2. Extensive growth of rooted aquatic macrophytes will interfere with navigation, aeration, and channel capacity.
3. Dead macrophytes and phytoplankton settle to the bottom of a water body, stimulating microbial breakdown processes that require oxygen. Eventually, dissolved oxygen will be depleted.
4. Aquatic life uses may be hampered when the entire water body experiences daily fluctuations in dissolved oxygen levels as a result of nightly plant respiration. Extreme oxygen depletion can lead to death of desirable fish species.
5. Siliceous diatoms and filamentous algae may clog water treatment plant filters and result in reduced time between backwashing (process of reversing water flow through the water filter in order to remove debris).
6. Toxic algae (occurrence of "red tide") have been associated with eutrophication in coastal regions and may result in paralytic shellfish poisoning.
7. Algal blooms shade submersed aquatic vegetation, reducing or eliminating photosynthesis and productivity.

**Sources of nitrate/nitrite in the environment**

Nitrate (NO\textsubscript{3}) and nitrite (NO\textsubscript{2}) are naturally occurring inorganic ions, which are part of the nitrogen cycle. Microbial action in soil or water decomposes wastes containing organic nitrogen first into ammonia, which is then oxidized to nitrite and nitrate. Because nitrite is easily oxidized to nitrate, nitrate is the compound predominantly found in groundwater and surface waters. Nitrate-containing compounds in the soil are generally soluble and readily migrate with groundwater [3]. Maximum admissible concentration of nitrate and nitrite in drinking water is 50 and 0.1 mg L\textsuperscript{-1}, respectively [9]. As per United States Environmental Protection Agency (USEPA) the present maximum contamination level of nitrite in drinking water is 1μg mL\textsuperscript{-1} [10].
“Methods for the Analysis of Some Elements of Environmental Interest”
A Thesis Submitted to the University of Mysore for the Award of Degree of Ph.D. in Chemistry

Agriculture: Primary agricultural sources of nitrate include livestock excrement (from barnyards, pastures, rangeland, feedlots, and uncontrolled manure storage areas); nitrogenous fertilizers; irrigation return flows; and decomposing plant debris [4].

Residential and Urban: Primary residential sources of nitrate include nitrogenous fertilizer used on lawn and garden, leaky on-site wastewater disposal/septic systems, sewage treatment system outfalls, sewage treatment bypass outfalls, and human and domestic pet excreta [4].

Others: The combustion of fossil fuels, industrial and agricultural discharges of nitrogen-containing gases, aerosols, and air-borne particles contribute to the atmospheric nitrogen load. Evidence suggests that the atmospheric deposition of nitrogen in water bodies (directly and via rainfall) constitutes a large portion of total nitrogenous inputs to estuarine and marine systems and a somewhat lesser portion of total nitrogen inputs to freshwater systems. Additional nitrate sources include excreta both from wild animals in the surrounding watersheds, excreta from wildfowl congregating on the water body and boats that discharge raw sewage overboard [4].

Industries that use nitrates in manufacturing may release nitrate in the effluent water. Nitrate is used in the following processes: meat curing, production of fertilizer, explosives, glass, heat-transfer fluid, and heat-storage medium for solar-heating applications. Additional nitrates may be contributed by sewage treatment systems and sewage treatment bypass outfalls (during high flow periods). Estuaries may be particularly susceptible to nutrient enrichment from offshore sewage pipe outfalls [4].

Although vegetables are seldom a source of acute toxicity, they account for >70% of the nitrates in a typical human diet. Cauliflower, spinach, collard greens, broccoli, and root vegetables have a naturally greater nitrate content than other plant foods do. The remainder of the nitrate in a typical diet comes from drinking water (about 21%) and from meat and meat products (about 6%) in which sodium nitrate is used as a preservative and color-enhancing agent [3].

Accidental exposures to nitrites in chemical laboratories and ingestion in suicide attempts have been described. Deliberate abuse of volatile nitrites (amyl, butyl and isobutyl nitrites) as psychedelics or aphrodisiacs frequently occurs. These agents are known by street names such as “snappers,” “poppers,” “Locker Room,” and “Rush” [3].

Nitrate or nitrite exposure also can occur from certain medications. Infants and children are especially susceptible to nitrate exposure through topical silver nitrate used in burn therapy. Other medications implicated in cases of nitrate or nitrite toxicity are quinone derivatives (antimalarials), nitroglycerine, bismuth subnitrite (antidiarrheal), ammonium nitrate (diuretic),
amyl and sodium nitrites (antidotes for cyanide and hydrogen sulphide poisoning) and isosorbide dinitrate/tetranitrates (vasodilators used in coronary artery disease therapy) [3].

Sodium nitrite used as an anticonvulsive agent in cooling fluids, ammonium nitrate found in cold packs, and nitrous gases used in arc welding are other possible sources of exposure. An ethyl nitrite folk remedy called “sweet spirits of nitre” has caused fatalities. Serious poisoning and death have occurred when sodium nitrate was mistaken for table salt and ingested with food [3].

Nitrate and nitrite are ubiquitous within environmental, food, industrial and physiological systems, and while our understanding of their role within such matrices has increased, a substantial degree of uncertainty and speculation remains. These ions have been profitably exploited through the ages to further the development of various societies but there is no doubt that our affection for them has waned in recent years. Our incessant use of, and indeed reliance upon, these versatile agents combined with revelations of their potential toxicity have raised numerous concerns [11]. These problems have been widely recognized, and as a consequence, statutory frameworks aimed at controlling their level within wider environment and within food products have been imposed in most industrialized countries [7]. Our need and desire to monitor these ions are unquestionable, yet their utility can pose a significant challenge to the analytical community. In this chapter, the author has presented the spectrophotometric methods developed for the determination of nitrite and their application.

LITERATURE LOOK AT

The classical Griess method for nitrite determination is very well known and very widely used. However, some of the more recent methods which are also based upon the formation of azo dyes may be better. The modified Griess method based on the reaction of nitrite with primary aromatic amines in an acidic medium to form a diazonium salt, the salt is then coupled with a suitable aromatic compound to yield an azo dye. Sulphanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) system is the standard or official method for the determination of nitrite [6,12]. The use of several chromogenic reagents prior to 1986 for the spectrophotometric determination of nitrite has been well discussed by Marczenko [13]. Recently, Moorcraft et al. [14] admirably reviewed the strategies employed to facilitate the detection, determination and monitoring of nitrate and/or nitrite in a variety of sample matrices, which gives 180 references. In the following paragraphs, the author presents various spectrophotometric methods employed for the determination of nitrite in recent years.
Many spectrophotometric methods based on modified Griess method have been reported. Balasubramanian and Vijayanthimala [15] have proposed a spectrophotometric method for determining NO$_2^-$ based on the diazotization of acidified p-nitroaniline with NO$_2^-$ and coupling the product with resorcinol in an alkaline medium. The method was applied to the determination of NO$_2^-$ in air after trapping in an alkaline solution and in a water sample. Beer's law was obeyed for 0-25 μg NO$_2^-$. The molar absorption coefficient of the coloured reaction product at 555 nm was $3.8 \times 10^4$ L mol$^{-1}$ cm$^{-1}$. There are many other diazotizing and coupling reagents that have been used, viz. p-aminoacetophenone and N-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) [16,17], p-aminophenylmercaptoacetic acid and NEDA ($\epsilon = 4.65 \times 10^4$) [18], p-rosaniline hydrochloride and phloroglucinol [19], p-aminophenylmercaptoacetic acid and chromotropic acid [20], 3-nitroaniline and NEDA ($\epsilon = 4.9 \times 10^4$ L mol$^{-1}$ cm$^{-1}$) [21], o-nitroaniline and 1-aminonaphthalene-2-sulphonic acid ($\epsilon = 5.46 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 550 nm in aqueous medium and $\epsilon = 4.83 \times 10^5$ at 530 nm on extraction with isopentyl alcohol) [22], sulphadiazine and NEDA ($\epsilon = 6.9 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 545 nm) [23], p-aminophenylmercaptoacetic acid and α-napthol ($\epsilon = 3.49 \times 10^4$ L mol$^{-1}$ cm$^{-1}$) [24], p-nitroaniline and dibenzoylmethane ($\epsilon = 3.6 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 520 nm) [25], p-amino-benzenesulfonic acid and H-acid ($\epsilon = 3.1 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 520 nm) [26], p-nitroaniline and phloroglucinol [27], imipramine hydrochloride and 3-methyl-2-benzothiazolinone hydrazono hydrochloride ($\epsilon = 0.49 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 640 nm) [28], sulphathiazole and NEDA ($\epsilon = 4.61 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 546 nm) [29], 4-aminoazobenzene and resorcinol [30], p-nitroaniline and acetyl acetone ($\epsilon = 3.2 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 490 nm) [31], p-aminoacetophenone and citrazinic acid ($\epsilon = 2.9 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 495 nm) [32], dapsone and iminodibenzyl ($\epsilon = 7.5 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 570 nm) [33], dapsone and phloroglucinol ($\epsilon = 4.28 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 425 nm) [34], p-amino-benzenesulfonic acid and N,N-dimethylbenzeneamine ($\epsilon = 4.38 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 508 nm) [35], 4-trifluoromethylanilinium ion and NEDA (micro-phase sorbent extraction) [36], p-nitroaniline and citrazinic acid ($\epsilon = 2.8 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 530 nm) [37], p-nitroaniline and ethyl acetate [38], sulphanilamide and ethylacetocacetate ($\epsilon = 1.22 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 356 nm, Fe$^{3+}$ interferes severely) [38], p-nitroaniline in the presence of diphenylamine in micellar media ($\epsilon = 1.425 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 500 nm) [39], p-nitroaniline in the presence of diphenylamine in acidic media and micelle-mediated extraction [40], 4-amino benzene sulphonic acid and NEDA ($\epsilon = 5.02 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 545 nm) [41], p-nitroaniline and ethoxyethylene-maleic acid ($\epsilon = 5.04 \times 10^4$ at 439 nm) [42] and p-nitroaniline and ethyl cyanoacetate ($\epsilon = 1.21 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 465 nm) [42]. Very recently, Ozmen et al. [43] have synthesized the azo dye 4-(1-methyl-1-mesitylcylobutane-
3-yl)-2-(p-N,N-dimethylazobenzene)-1,3-thiazole by the reaction of 4-(1-methyl-1-mesitylcyclobutane-3-yl)-2-aminothiazole and N,N-di-methyl aniline with nitrite in acidic medium. The dye showed an absorption maximum at 482 nm ($\varepsilon = 2.03 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$). Beer's law was obeyed over the concentration range 0.05 to 2.00 $\mu$g nitrite per mL analyte. The method was applied to the determination of nitrite in tap and lake water.

Several authors [44–50] have proposed flow-injection analysis for determining nitrite, through formation of azo dyes. Recently, determination of nitrate and nitrite in dairy samples by sequential-injection spectrophotometric method using an in-line cadmium-reducing column has been described [51], which is based on the Griess reaction.

Some indirect spectrophotometric methods for the determination of nitrite based on bleaching action of dyes or organic compounds with nitrite have been proposed. Kawakami and Igarashi [52] have described the reaction of nitrite with 5,10,15,20-tetrakis (4-aminophenyl)porphine, which produces a large spectral change due to diazotization of the porphyrin. The decrease in absorbance was observed at 434 nm with increasing nitrite concentration ($\varepsilon = 2.63 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$). This method is time consuming, requires 30 min heating, and suffers serious interference by Fe(III). This method was applied to the determination of nitrite in rainwater, tap water, and spring water. Zatar et al. [53] have oxidized the phosphomolybdenum blue complex by the addition of nitrite and this was caused a reduction in intensity of the blue colour of the complex. The decrease in absorbance of the blue coloured complex was directly proportional to the amount of nitrite added. The absorbance of the phosphomolybdenum blue complex was monitored spectrophotometrically at 814 nm ($\varepsilon = 1.1 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$) and related to the concentration of nitrite present. Some other reagents used for the determination of nitrite based on decolourizing the dyes are: Xylene Cyanol FF [54], Neutral Red ($\varepsilon = 2.5 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 530 nm) [55], Pararosaniline [56], Phenoasfranine ($\varepsilon = 3.7 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 520 nm) [57], Safranin O [58], Acridine Red (the reaction time was 20 min at 525 nm) [59] and Azure I [60]. Very recently, a spectrophotometric method for the determination of nitrite by its bleaching effect on peroxovanadate complex has been reported [61].

Complex formation reactions are also the basis for the spectrophotometric determination of nitrite. Balasubramanian and Sivagami [62] have used barbituric acid with nitrite in acid solution to form violuric acid, which readily forms a coloured complex with Co(II) in the presence of NH$_3$-NH$_4$Cl buffer of pH 9.5. The complex had maximum absorption at 335 nm ($\varepsilon = 1.3 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$). The method was used for the determination of nitrite in air after
sampling in alkali and for the determination of nitrite in water. Zhang et al. [63] have synthesized 5,7-dihydroxy-4-imino-2-oxochroman and employed it for the nitrite determination. Nitrite and 5,7-dihydroxy-4-imino-2-oxochroman form a complex in acid medium, which was extracted into a solvent mixture of BuOH and AcOEt for spectrophotometric determination at 361 nm. The apparent molar absorption coefficient was $2.25 \times 10^4$ L mol$^{-1}$ cm$^{-1}$. Nitrite was also determined by spectrophotometry of complexes with morin at 460 nm [64]. A new test method for nitrite as nitrosoalkylresorcinolate was proposed by Johannes et al. [65]. The method was based on the easy nitrosation of alkylresorcinols (R) with nitrous acid formed from nitrite in acidic solutions, and on the subsequent formation of a coloured complex with a transition metal cation. The main oil shale originated phenols, 5-methylresorcinol and 2,5-dimethylresorcinol, were applied as R and the cobalt, nickel, copper and iron(II) cations as Me. Zhao et al. [66] have described a method for the determination of nitrite with 3,3',5,5'-tetramethylbenzidine (TMB). In a medium of HAc-NaAc (pH = 3.6), TMB was oxidized by nitrite, forming a TMB-TMB imine complex with its absorption maximum at 370 nm and an apparent molar absorption coefficient of $4.69 \times 10^4$ L mol$^{-1}$ cm$^{-1}$.

A number of other organic reagents have found application in the determination of nitrite, namely 2-methoxyethanol [67], salbutamol sulphate ($\varepsilon = 1.8 \times 10^3$ L mol$^{-1}$ cm$^{-1}$ at 410 nm) [68], naphthalene-2, 7-diol [69], Rhodamine 6G ($\varepsilon = 1.2 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 445 nm) [70], 1-naphthylamine [71], 4-iodo-N,N-dimethylaniline [72], 2,7-dihydroxynaphthalene [73], rhodamine 3G (based on diazo reaction) [74], N-phenylanthranilic acid [75], nuclear fast red [76] and tris-1-10 phenanthroline-Fe(III) complex ($\varepsilon = 2.1 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ at 512 nm) [77]. Recently, Chatterjee et al. [78] have proposed the reaction of nitrite with acidified KI to liberate iodine which oxidizes leuco crystal violet (LCV) to crystal violet having absorption maxima at 590 nm ($\varepsilon = 1.54 \times 10^6$ L mol$^{-1}$ cm$^{-1}$) forms the bases of this method. Aydm et al. [79] have developed a method for the spectrophotometric determination of nitrite in water based on the reaction of nitrite with barbituric acid in acidic solution to give the nitroso derivative, violuric acid with an absorption maximum at 310 nm ($\varepsilon = 1.533 \times 10^4$ L mol$^{-1}$ cm$^{-1}$).

Several spectrophotometric methods (some of these coupled with flow-injection system) for nitrite are based on its catalytic effect on the oxidation of different dyes by potassium bromate or other oxidants under acidic conditions. Mention may be made of Alizarin Green [80], Brilliant Cresyl Blue [81], Fuschin Red [82], chlorophosphonazo [83], Janus Green [84], Bromopyrogallol Red [85], Metanil yellow [86], litchi Chinese red [87], quinaldine red [88], pyronine G [89], carminic acid [90], Naphthol Green B [91], Indigo Carmine [92], Neutral Red [93], Victoria Blue
B [94], Crystal Violet [95], Catechol Violet [96], acridine orange [97], Galloceyanine [98], Bromocresol Purple [99], pyrogallol red [100], Victoria Green [101], pyronine B [102], xylene orange [103], methyl red [104], pyrogallol sulfonephthalein [105], hydrogen peroxide-methyl orange system [106], and methylene blue [107]. The aforementioned methods rely upon measuring the decrease in absorbance of the mentioned dyes at respective absorption maximum (λ_{max} nm). These methods are sensitive, but suffer from careful control of the temperature, acidity and reaction time, and these procedures are significantly influenced by a large number of ions, particularly Fe(II), Fe(III), Ag(I), SO_{3}^2−, Br−, I−, SCN−, and V(V). Under the same theme, the determination of nitrite based on its catalytic effect on the oxidation of organic compounds by various oxidants under acidic conditions, e.g., 2,2′-azinobis-(3-ethylbenzothia zoline -6-sulphonic acid) [108], chlorpromazine-hydrogenperoxide[109], prochlorperazine-bromate [110], molybdsilicic acid blue [111] and chlorpromazine with nitric acid [112].

Some other techniques recently adapted for nitrite determination include, flow injection analysis–flame atomic absorption spectrometry system [113], chemiluminescence [114], chemiluminescence microflow injection analysis [115], electrochemical (using modified aligned carbon nanotubes electrode) [116], amperometric [117], electrocatalytic [118], high-performance liquid chromatography [119], ion-chromatography [120], spectrofluorimetry [121], kinetic spectrofluorimetric [122], flow analysis–vapor phase generation–Fourier transform infrared (FA–VPG–FTIR) spectrometry [123] and capillary zone electrophoresis [124].

The present chapter deals with the investigation of two spectrophotometric methods for the determination of nitrite. The first method (Section 6A) is based on diazo-coupling reaction using 4-aminoazobenzene and acetyl acetone. The second one (Section 6B) is an indirect method based on decrease in absorbance of the dye, Thionin by nitrite.
Section - 6A

A Sensitive Spectrophotometric Method for the Determination of Nitrite Using 4-Amino Azobenzene and Acetyl Acetone

6A.1. INTRODUCTION
Acetyl acetone (AA), chemically pentane-2, 4-dione, CH₃-CO-CH₂-CO-CH₃, was prepared by means of the Claisen condensation between ethyl acetate and acetone [125]. It is a colourless liquid. It has been extensively used in the synthesis of metal chelates and also in the spectrophotometric determination of some metals. AA was recommended as a reagent for the photometric determination of copper [126] and titanium(III) [127]. Satary and Hladky [128] have reported the systematic study of the solvent extraction of metal β-diketonates. Many authors [129-132] have utilized AA as a chelating agent for the separation of many metals. AA was used as a spectrophotometric reagent for the determination of formaldehyde [133-135], alditols in biological samples [136] and pharmaceutically important sulpha drugs [137], heptaminol [138] and some chemotherapeutics in dosage forms [139].

6A.2. EXPERIMENTAL

6A.2.1. Instrumentation
ANALYTIC JENA AG model SPECORD-50 and ELICO model SL-171 digital spectrophotometers with 1.0 cm matched quartz cells were used for all absorbance measurements.

6A.2.2. Reagents
All chemicals used were of analytical reagent grade and doubly distilled water was used throughout.

*Standard solution of nitrite (1000 µg mL⁻¹)* was prepared by dissolving 0.150 g of the dried sodium nitrite in 100 mL water. A pellet of sodium hydroxide and a small amount of chloroform were added to prevent the liberation of nitrous acid and to inhibit the bacterial growth, respectively.

*4-Amino azobenzene solution (AAB), (0.04%)* was prepared by dissolving 100 mg of AAB in 50 mL of acetone, and diluting up to 250 mL with distilled water.
Acetyl acetone (AA), (10%, v/v) was prepared by diluting 10 mL of AA to 100 mL with methanol.

Other solutions prepared: sulphuric acid (1 M), potassium bromide (10%), sodium hydroxide (4 M), sodium carbonate (1%) and EDTA (0.2 M).

6A.2.3. General procedure for the determination of nitrite
Aliquots of the standard solution of nitrite (0 – 9 μg) were transferred to a series of 10 mL calibrated flasks containing 1 mL each of the AAB (0.04%), sulphuric acid (1 M) and potassium bromide (10%) solutions. The contents of the flasks were shaken thoroughly and 1 mL of 10% AA was added. After 2 min, the resulting solution was made alkaline by adding 2 mL of 4 M sodium hydroxide and diluted to the mark with distilled water, and mixed well. The absorbance of the formed bisazo dye was measured at 500 nm against the reagent blank. The calibration graph (Fig. 6A.1) was plotted and it was used to establish the nitrite concentration in samples of unknown concentration.

Fig. 6A.1. Beer’s law plot of NO₂⁻ - AAB - AA system
6A.2.4. Determination of nitrite in water samples
An aliquot (≤ 3 mL) of natural water sample containing not more than 10 μg of nitrite was treated with 0.5 mL of 1 M sodium hydroxide and 0.5 mL of 0.2 M EDTA. The solution was mixed and centrifuged to remove any formed precipitate. The centrifugate was transferred to 10 mL calibrated flask, and analyzed for nitrite by the proposed procedure (6A.2.3).

6A.2.5. Determination of nitrite in soil sample
A known mass of the soil sample was transferred into 50 mL beaker and extracted triply with 5 mL of 1% sodium carbonate. The extract was filtered, neutralized and the filtrate was diluted with distilled water up to 25 mL. A volume of ≤ 3 mL of the made up solution was taken and nitrite was determined according to the procedure for the water samples.

6A.3. RESULTS AND DISCUSSION
The present investigation is based on the diazotization of 4-amino azobenzene (AAB) with nitrite in an acid medium and subsequent coupling with acetyl acetone (AA) under alkaline conditions to produce bisazo dye.

6A.3.1. Choice of diazotizing component
Primary aromatic amines such as p-aminoacetophenone, p-aminobenzoic acid, sulphanilic acid, o-nitroaniline and 4-aminoazobenzene were tested for the diazotization. Of these, a good sensitivity was achieved by the use of 4-aminoazobenzene for diazotization reaction.

6A.3.2. Nature of the species responsible for colour
AAB undergoes diazotization on treatment with nitrite in presence of dilute sulphuric acid at room temperature (27 ± 3 °C). The use of bromide ion to enhance the rate of diazotization reaction is well known and this concept has been used to complete the reaction instantaneously [140]. The formed diazonium couples with AA under aqueous alkaline condition to give bisazo dye; 4-(2, 4-pentanedione-3-azo)azobenzene, which exhibits a maximum absorption at 500 nm. The proposed reaction pathway is presented in Scheme 6A.1.
Scheme 6A.1. Proposed diazo-coupling reaction

6A.3.3. Absorption spectra

A known amount of nitrite [0.5 µg mL⁻¹] was taken and the general procedure for the determination of nitrite [Section 6A.2.3] was followed for the formation of bisazo dye, and the absorbance of the bisazo dye was measured for wavelength of maximum absorption ($\lambda_{\text{max}}$). Absorption spectra (Fig 6A.2) show that, under alkaline condition, AAB has $\lambda_{\text{max}}$ at 390 nm,

![Absorption spectra](image)

**Fig 6A.2.** Absorption spectra: (a) Reagent blank vs. Distilled water; (b) Bisazo dye, [NO₂⁻] = 0.5 µg mL⁻¹ vs. Reagent blank
while the formed bisazo dye had $\lambda_{\text{max}}$ at 500 nm. As nitrite concentration was increased, more of AAB was diazotized resulting in decrease in absorbance at 390 nm, while corresponding amount of bisazo dye formed was shown by increase in absorbance at 500 nm.

6A.3.4. Optimization of experimental parameters

Effects of acidity, concentrations of AAB, AA and sodium hydroxide on the reaction system were studied with 0.5 $\mu$g mL$^{-1}$ of nitrite and the results of which are discussed below.

6A.3.4.1. Effect of acid concentration on diazotization

Sulphuric acid was found to be the best suited for the reaction system among other mineral acids. An acid concentration of 0.19 – 0.31 M sulphuric acid in an overall volume of 4 mL was found to be optimum for diazotization. Beyond this range, a slight decrease in the absorbance was observed. Hence, an acidity of 0.25 M was maintained by placing 1.0 mL of 1 M sulphuric acid for diazotization. Effect of acid concentration on diazotization is shown in Fig. 6A.3.

![Graph showing effect of sulphuric acid concentration on diazotization](image)

**Fig. 6A.3.** Effect of sulphuric acid (1 M) concentration on diazotization

6A.3.4.2. Effect of AAB concentration on diazotization

It was observed that 1 mL of 0.04% solution of AAB was required for completion of diazotization. There was a decrease in the absorbance at lower concentrations, whereas at higher
concentrations, no change in the absorbance was found. Effect of AAB concentration on diazotization is presented in Fig. 6A.4.

![Figure 6A.4](image)

**Fig. 6A.4.** Effect of AAB (0.04%) concentration on diazotization

### 6.4.3.4.3. Effect of AA concentration on coupling reaction

The study of effect of coupling agent revealed that 1 mL of 10% solution of AA was found to be sufficient for complete coupling reaction. Higher concentrations of AA had no effect on the absorbance values, but a decrease in the absorbance at lower concentrations was observed. Effect of AA concentration on coupling reaction is depicted in Fig. 6A.5.

![Figure 6A.5](image)

**Fig. 6A.5.** Effect of AA (10%) concentration on coupling reaction
6A.3.4.4. Effect of sodium hydroxide concentration

Maximum absorbance of the bisazo dye was observed in the range of 1.5 – 3.0 mL of 4 M sodium hydroxide. A change in the concentration range of sodium hydroxide causes a decrease in the absorbance values. Hence, 2.0 mL of 4 M sodium hydroxide was taken for the subsequent studies. Effect of sodium hydroxide concentration is shown in Fig. 6A.6.

![Graph showing effect of NaOH concentration](image)

**Fig. 6A.6.** Effect of NaOH (4 M) concentration

6A.3.4.5. Effects of temperature and time

The diazotization was carried out at room temperature (27 ± 3°C). No variation in the absorbance values of the bisazo dye was observed in the temperature range 5 – 40 °C. The maximum colour intensity of the bisazo dye was observed within 3 min after dilution. The dye was stable up to 9 h.

6A.3.5. Interference studies

The influence of various potential interferents on the determination of nitrite by the proposed procedure was examined. The tolerance limits of interfering species were established at those concentrations that do not cause more than ± 2% error in absorbance values of nitrite at 0.5 μg mL⁻¹ level. The metal ions forming hydroxides in alkaline medium were masked with EDTA. Sulphite did not interfere up to 25 μg mL⁻¹. The interference up to 100 μg mL⁻¹ of sulphite was overcome by the addition of 1 mL of 1% HCHO. Sulphide interfere seriously at 5 μg mL⁻¹ level, but at higher concentrations (up to 30 μg mL⁻¹) interference was overcome by precipitating it as
ZnS, by adding 1 mL of 1000 µg mL⁻¹ Zn²⁺ solution. The tolerance limits of various foreign ions likely to interfere during the analysis of nitrite in water and soil samples are listed in Table 6.1.

Table 6.1. Tolerance limits of diverse ions on the determination of 0.5 µg mL⁻¹ nitrite

<table>
<thead>
<tr>
<th>Interferents</th>
<th>Tolerance limit (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA, Na⁺, K⁺, HCHO</td>
<td>≥ 4500</td>
</tr>
<tr>
<td>F⁻, I⁻</td>
<td>≥ 2400</td>
</tr>
<tr>
<td>Mg²⁺, CO₃²⁻, NO₃⁻, Oxalate</td>
<td>≥ 1300</td>
</tr>
<tr>
<td>Zn²⁺, Al³⁺, Cr⁶⁺, Citrate, Acetate</td>
<td>≥ 1100</td>
</tr>
<tr>
<td>SO₄²⁻, PO₄³⁻, Tartrate</td>
<td>≥ 800</td>
</tr>
<tr>
<td>Mn²⁺, Cd²⁺, As³⁺, Bi³⁺</td>
<td>≥ 200</td>
</tr>
<tr>
<td>Hg²⁺, Fe³⁺, Cu²⁺, Co²⁺, MoO₄²⁻, VO₃⁻</td>
<td>≥ 30</td>
</tr>
<tr>
<td>Sulphite</td>
<td>25</td>
</tr>
<tr>
<td>Sulphite³</td>
<td>100</td>
</tr>
<tr>
<td>Sulphide</td>
<td>5</td>
</tr>
<tr>
<td>Sulphide⁴</td>
<td>30</td>
</tr>
</tbody>
</table>

³ Treated with 1 mL of 1% HCHO solution prior to nitrite determination.
⁴ Treated with 1 mL of 1000 µg mL⁻¹ Zn²⁺ solution, centrifuged and centrifugate was used for the analysis.

6A.3.6. Optical and Analytical Characteristics

Calibration graph exhibited linearity (r = 0.9998) for the nitrite amount ranging from 0.1 to 0.9 µg mL⁻¹. The calibration graph had a slope and intercept of 0.8779 and 0.0085, respectively

\[ y = (0.8779)x + 0.0085 \]; where ‘x’ is the concentration of nitrite in µg mL⁻¹.

The detection limit (DL = 3.3 σ /S) and quantitation limit (QL = 10 σ /S) [where ‘σ’ is the standard deviation of the blank n = 10 and ‘S’ is the slope of the calibration curve] of nitrite determination were 14 and 41 ng mL⁻¹, respectively. The molar absorption coefficient and Sandell’s sensitivity of the coloured system were found to be $4.2 \times 10^4$ L mol⁻¹ cm⁻¹ and
1.1 ng cm\(^{-2}\), respectively. The precision and accuracy of the method were established by the analysis of the standard solutions containing 3, 5 and 7 μg of nitrites in the final volume of 10 mL using the recommended procedure. Ten replicate determinations of each concentration gave relative standard deviations (RSD) of 0.1, 0.07 and 0.05%, respectively.

### 6A.3.7. Applications

The proposed method was applied to the determination of nitrite in real water and soil samples (6A.2.4 & 6A.2.5). Freshly collected water samples from various sources were filtered through Whatman No. 41 filter paper, stored at 5 °C to retard bacterial growth, and analyzed within 24 h.

#### Table 6.2. Determination of nitrite in water and soil samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount taken (mL)</th>
<th>NO(_2) added (μg mL(^{-1}))</th>
<th>Proposed method</th>
<th>Standard method [6,12]</th>
<th>F-test(^a)</th>
<th>t-test(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NO(_2) found (μg mL(^{-1}))</td>
<td>Recovery (%)</td>
<td>RSD (%)</td>
<td>NO(_2) found (μg mL(^{-1}))</td>
</tr>
<tr>
<td>Rain water</td>
<td>2.0</td>
<td>---</td>
<td>0.177 ± 0.004</td>
<td>---</td>
<td>2.3</td>
<td>0.183 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>---</td>
<td>0.182 ± 0.003</td>
<td>---</td>
<td>1.6</td>
<td>0.178 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>---</td>
<td>0.186 ± 0.002</td>
<td>---</td>
<td>1.1</td>
<td>0.187 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean = 0.182</td>
<td>---</td>
<td>---</td>
<td>Mean = 0.183</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.4</td>
<td>0.580 ± 0.006</td>
<td>99.7</td>
<td>1.0</td>
<td>0.582 ± 0.007</td>
</tr>
<tr>
<td>Lake water</td>
<td>0.50</td>
<td>---</td>
<td>0.147 ± 0.003</td>
<td>---</td>
<td>2.0</td>
<td>0.142 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>---</td>
<td>0.143 ± 0.002</td>
<td>---</td>
<td>1.4</td>
<td>0.145 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>---</td>
<td>0.148 ± 0.001</td>
<td>---</td>
<td>0.7</td>
<td>0.147 ± 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean = 0.146</td>
<td>---</td>
<td>---</td>
<td>Mean = 0.145</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.4</td>
<td>0.543 ± 0.005</td>
<td>99.5</td>
<td>0.9</td>
<td>0.541 ± 0.006</td>
</tr>
<tr>
<td>Soil</td>
<td>(g)</td>
<td>(μg g(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td>(μg g(^{-1}))</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>---</td>
<td>2.15 ± 0.05</td>
<td>---</td>
<td>2.3</td>
<td>2.14 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>---</td>
<td>2.13 ± 0.04</td>
<td>---</td>
<td>1.8</td>
<td>2.15 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>---</td>
<td>2.15 ± 0.03</td>
<td>---</td>
<td>1.4</td>
<td>2.16 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean = 2.14</td>
<td>---</td>
<td>---</td>
<td>Mean = 2.15</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>2.0</td>
<td>4.13 ± 0.02</td>
<td>99.8</td>
<td>0.5</td>
<td>4.14 ± 0.03</td>
</tr>
</tbody>
</table>

\(^a\) Mean value ± Standard deviation.

\(^b\) Tabulated F for (4,4) degrees of freedom at \(P\) (0.95) is 6.39.

\(^c\) Tabulated t for 8 degrees of freedom at \(P\) (0.95) is 2.306.
The method was applied directly to the determination of nitrite as the concentration of common pollutants is usually far below the tolerance levels for interfering agents. Similarly, the manured garden soil sample was analyzed. The results obtained were in very good agreement with those obtained with the standard method involving sulfanilamide-N-(1-naphthyl)ethylenediamine dihydrochloride ($\lambda_{\text{max}} = 543 \text{ nm}$) [6,12]. The results obtained are presented in the Table 6.2.

6A.3.8. Statistical evaluation of the results

The results of the proposed and standard methods were subjected to statistical analysis by F- and Student’s t-tests. At 95% confidence level, the calculated F- and t- values did not exceed the theoretical values, which showed no significant difference between the accuracy and precision of the proposed and standard method. The reliability of the procedures was confirmed by standard additions.

6A.4. CONCLUSIONS

Although a variety of methods are available for the determination of nitrite by spectrophotometry, the proposed method offers the advantage of simplicity, sensitivity, and rapidity over the many existing methods (Table 6.5). The diazotization was carried out at room temperature, cooling to 0 – 5 °C was not necessary. High stability of the bisazo dye (up to 9 h), inexpensive reagents and elimination of extraction steps makes the method versatile. High tolerance limit for large number of associated ions is an added advantage of the method. Statistical analysis of the results confirms the higher precision and accuracy of the proposed method. The application of the proposed method for determination of trace amounts of nitrite in water and soil samples demonstrated the utility of the method.
An Indirect Spectrophotometric Method for the
Determination of Nitrite with Thionin

6B.1. INTRODUCTION
Thionin (C.I. 52000) is a metachromic cationic dye. It is chemically 3,7-diamino-5-
phenothiazinium acetate (Lautha’s Violet), and widely used as a biological stain; especially in
histology. It has the following structural formula:

![Thionin Structural Formula]

[3,7-diamino-5-phenothiazinium acetate]

Thionin has been used as a spectrophotometric reagent for the determination of vanadium
[141,142], selenium [143] and gold [144]. It has also been used in the kinetic spectrophotometric
determination of nitrite [145], kinetic spectrophotometric determination of molybdenum(VI)
[146], catalytic spectrophotometric determination of manganese [147], catalytic spectrophotometric
determination of nitrite based on catalytic oxidation of thionin by potassium bromate [148], kinetic spectrophotometric determination of selenium(IV) [149], flow-injection
catalytic spectrophotometric determination of selenium(IV) [150], determination of hydrazine
[151] and fluoride [152].

The above literature survey on thionin revealed that a simple visible-spectrophotometric
determination of nitrite using thionin has not been reported in the literature. A kinetic
spectrophotometric [145] and a catalytic spectrophotometric [148] methods made use of thionin
have been found in the literature. In this section, the author has described the research studies on a
visible-spectrophotometric method for the determination of nitrite. The method is based on the
reaction of nitrite with primary amino group of a cationic dye thionin in sulphuric acid medium. A decrease in colour intensity of the dye is observed at 600 nm due to diazotization, followed by deamination. This decrease in absorbance is directly proportional to nitrite concentration. The rate of diazotization is enhanced by the addition of bromide ion, so that the reaction completes almost instantaneously. This method has been applied to the determination of nitrite in water and soil samples.

6B.2. EXPERIMENTAL

6B.2.1. Instrumentation

Instruments used for the absorbance measurements are described in Section 6A.2.1.

6B.2.2. Reagents

All chemicals used were of analytical reagent grade and doubly distilled water was used throughout.

*Standard solution of nitrite (1000 µg mL⁻¹)* preparation is described in Section 6A.2.2.

*Thionin (TN) (Eastman Kodak Co., USA, CI 52000) (0.005%)* was prepared by dissolving 50 mg of thionin in 4 M H₂SO₄ and diluting to 1000 mL with water. The dye solution was stable for 30 days.

*Other solutions prepared:* Sulphuric acid (4 M), potassium bromide (1%), sodium hypophosphite (1%), sodium hydroxide (1 M), sodium carbonate (1%) and EDTA (0.2 M).

6B.2.3. General procedure for the determination of nitrite

Aliquots (0.1–2.0 mL) of standard nitrite solution containing 0.25–5.0 µg of nitrite were transferred to a series of 10 mL calibrated flasks and the overall volume was adjusted to 4 mL with distilled water. Volumes of 0.75 mL of 0.005% thionin in 4 M H₂SO₄ and 0.5 mL of 1% potassium bromide were added to each flask followed by the addition of 0.5 mL of 1% sodium hypophosphite. The contents were diluted to the mark with distilled water. The reagent blank was prepared in the same way, excluding the analyte. The absorbance of the reagent blank and the decolourized dye solutions was measured at 600 nm against distilled water. The amount of nitrite present in the samples solution was computed from the calibration graph (Fig. 6B.1).
6B.2.3. Determination of nitrite in water and soil samples

A detailed procedure for the analysis of nitrite in water and soil samples is explained in Section 6A.2.4 and 6A.2.5, respectively. The sample solutions were then analyzed for nitrite content by following the above general procedure (Section 6B.2.3).

6B.3. RESULTS AND DISCUSSION

Thionin (CI 52000) is a metachromic cationic dye has a maximum absorption at 600 nm. Nitrite decolourizes the violet colour of the dye in sulphuric acid medium due to diazotization of the primary amino group in the dye. Addition of the catalyst, bromide ion completes the diazotization reaction almost instantaneously [140]. This was confirmed by the stability in the absorbance of unreacted thionin, which was stable for more than a day. Addition of sodium hypophosphite causes deamination. As a result, a decrease in absorbance was observed (Fig. 6B.2) at 600 nm, which is directly proportional to the nitrite concentration. The proposed reaction pathway is presented in Scheme 6B.1.
6B.3.1. Absorption spectra
The reagent blank was prepared as explained in the general procedure (Section 6B.2.3) and scanned against water in the wavelength range 500 - 700 nm. The dye, TN showed maximum absorption at 600 nm in the acid medium. The absorbance of the dye with different concentrations of nitrite [0.1 and 0.3 \( \mu \text{g mL}^{-1} \)] was recorded against water after following the general procedure (Section 6B.2.3). The absorption spectra of the dye without and with nitrite are shown in Fig. 6B.2. The absorption spectra revealed that there is a linear decrease in absorbance of the dye, TN with increasing nitrite concentration. The bleaching of violet colour of thionin was probably due to diazotization of the primary amino group followed by deamination.
Fig 6B.2. Absorption spectra: a) Reagent blank vs. distilled water; b) Same as ‘a’ with 1 µg of NO₂; c) Same as ‘a’ with 3 µg of NO₂.

6B.3.2. Optimization of experimental parameters
To develop a quantitative method based on this reaction, preliminary studies were carried out to determine the most effective and optimum experimental conditions with 2.5 µg of nitrite in the final volume of 10 mL.

6B.3.2.1. Effect of the acid concentration
The effect of hydrochloric acid and sulphuric acid on decolourization of TN with nitrite was studied separately. Sulphuric acid was found to be the most effective acid (in terms of sensitivity) compared to hydrochloric acid for this method. The studies revealed that the decrease in absorbance values of the dye was optimum in an overall sulphuric acid concentration between 0.2 and 0.4 M. That is, overall acidity of the reaction can vary from 0.2–0.4 M sulphuric acid, and the acidity was maintained at 0.3 M, which was achieved by the addition of 0.75 mL of 4 M H₂SO₄ in a final volume of 10 mL. The result of the effect of sulphuric acid on the absorbance of the dye, thionin is depicted in Fig. 6B.3.
6B.3.2.2. Effect of the reagents concentration

A preliminary study was carried out to fix the maximum and minimum limits of the absorbance values of thionin and thionin with nitrite, respectively. Under the optimum experimental conditions, the concentration of thionin to produce an absorbance value of 0.606 (upper limit) at 600 nm was selected and thus used in the further investigations. This was achieved by placing 0.75 mL of 0.005% TN in 4 M H₂SO₄ in a final volume of 10 mL reaction mixture.

It was found that 0.5 mL each of 1% potassium bromide and 1% sodium hypophosphite were sufficient for instant completion of diazotization and deamination, respectively. Any little excess of these reagents had no effect on the absorbance of the system.

6B.3.2.2. Effects of the time and temperature

The time required for the diazotization was investigated. Under the optimum conditions, the reaction was completed almost instantaneously in the presence of bromide ion. The absorbance values were independent of temperature up to 55 °C. The experiment was performed at room temperature (27 ± 3 °C). The colour of the unreacted dye was found to be stable for more than a day.
6B.3.3. Interference studies

The possibilities of interferences of the common foreign ions were studied to evaluate the suitability of the method for determining nitrite in water and soil samples. An error of less than ±2% in the absorbance values in the recovery of 0.25 μg mL⁻¹ of nitrite was considered to be tolerable. The tolerance limit of the various foreign ions studied is listed in Table 6.3. Sulphite (5 μg mL⁻¹) and sulphide (10 μg mL⁻¹) were interfere seriously. However, higher concentration of sulphite (30 μg mL⁻¹) could be tolerated by the addition of 1 mL of 1% HCHO to the sample prior to nitrite determination. The interference of sulphide (up to 50 μg mL⁻¹) was overcome by the addition of 1 mL of 1000 μg mL⁻¹ Zn(II) solution to the sample. The precipitated ZnS was removed and the clear supernatant liquid was taken for the analysis.

Table 6.3. Tolerance limits of interfering ions on the determination of 0.25 μg mL⁻¹ of nitrite.

<table>
<thead>
<tr>
<th>Interferents</th>
<th>Tolerance limit (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>5000</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>4000</td>
</tr>
<tr>
<td>Zn²⁺, Mn²⁺, SO₄²⁻</td>
<td>2500</td>
</tr>
<tr>
<td>Cu²⁺, Cd²⁺, Co²⁺, Ni²⁺, Mg²⁺, Cr³⁺, Al³⁺, Fe²⁺</td>
<td>1000 – 1100</td>
</tr>
<tr>
<td>PO₄³⁻, CO₃⁻, Cl⁻, acetate, oxalate, tartrate, citrate</td>
<td>800 – 900</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>700ᵃ</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>400</td>
</tr>
<tr>
<td>I⁻, IO₃⁻, VO₃⁻, Cr₂O₇⁻</td>
<td>50 -100</td>
</tr>
<tr>
<td>Sulphite</td>
<td>5, 30ᵇ</td>
</tr>
<tr>
<td>Sulphide</td>
<td>20, 50ᶜ</td>
</tr>
</tbody>
</table>

ᵃ Treated with 1 mL of 2% K₂SO₄ and centrifuged. Centrifugate was used for the analysis.
ᵇ Treated with 1 mL of 1% HCHO solution prior to nitrite determination.
ᶜ Treated with 1 mL of 1000 μg mL⁻¹ Zn²⁺ solution. Centrifuged and the centrifugate was used for the analysis.
6B.3.4. Optical and analytical characteristics

The system obeyed Beer’s law in the range 0.025 - 0.5 μg mL\(^{-1}\) of nitrite at 600 nm. The calibration graph exhibited a straight line with the slope, intercept and correlation coefficient of -0.8641, 0.602 and -0.9998, respectively [\(y = (-0.8641)x + 0.602\), where ‘\(y\)’ is the absorbance, \(x\) (μg mL\(^{-1}\)) is the concentration of nitrite]. The negative value of correlation coefficient (-0.9998) confirms the linear decrease in absorbance with increased concentration of nitrite. The detection limit (DL = 3.3 \(\sigma\) /S) and quantitation limit (QL = 10 \(\sigma\) /S) [where ‘\(\sigma\)’ is the standard deviation of the blank \(n = 10\) and ‘S’ is the slope of the calibration curve] of nitrite determination were 7 and 21 ng mL\(^{-1}\), respectively. The molar absorption coefficient and Sandell’s sensitivity of the method were found to be \(4.1 \times 10^{4}\) L mol\(^{-1}\) cm\(^{-1}\) and 1.1 ng cm\(^{2}\), respectively. The precision of the method has been established at 1.0, 2.0 and 3.0 μg of nitrites in the final volume of 10 mL, which gave relative standard deviations (RSD) (\(n = 10\)) of 0.08, 0.06 and 0.03 %, respectively.

6B.3.5. Applications

In order to assess the suitability of the developed method, it was employed to determine nitrite in water and soil samples. The analysis of nitrite in the samples is described in Section 6A.3.7. A parallel determination was carried out to validate the proposed procedure with the standard method involving sulfanilamide-N-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) (\(\lambda_{\text{max}} = 543\) nm) [6,12]. The results are statistically evaluated. The calculated values of F- and t-tests of the results of proposed and standard methods were found to be less than theoretical values at 95% confidence level. This indicates that the developed method is accurate and comparable to the standard method. The reliability of both the methods was checked by standard additions. The low RSD values at different levels are also reflect the precision of the method. The results obtained are presented in the Table 6.4.
Table 6.4. Determination of nitrite in water and soil samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount taken (mL)</th>
<th>NO$_2^-$ added (µg mL$^{-1}$)</th>
<th>NO$_2^-$ found$^\text{a}$ (µg mL$^{-1}$)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>NO$_2^-$ found$^\text{a}$ (µg mL$^{-1}$)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>F-test$^\text{b}$</th>
<th>t-test$^\text{c}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake water</td>
<td>0.5</td>
<td>---</td>
<td>0.175 ± 0.003</td>
<td>--</td>
<td>1.7</td>
<td>0.177 ± 0.004</td>
<td>--</td>
<td>2.2</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>---</td>
<td>0.178 ± 0.002</td>
<td>--</td>
<td>1.1</td>
<td>0.179 ± 0.003</td>
<td>--</td>
<td>1.6</td>
<td>2.2</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>---</td>
<td>0.177 ± 0.001</td>
<td>--</td>
<td>0.6</td>
<td>0.176 ± 0.001</td>
<td>--</td>
<td>0.6</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Average</td>
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<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.2</td>
<td>0.376 ± 0.005</td>
<td>99.7</td>
<td>1.3</td>
<td>0.375 ± 0.006</td>
<td>99.5</td>
<td>1.6</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>River water</td>
<td>1.0</td>
<td>---</td>
<td>0.122 ± 0.002</td>
<td>--</td>
<td>1.6</td>
<td>0.123 ± 0.003</td>
<td>--</td>
<td>2.4</td>
<td>2.3</td>
<td>0.6</td>
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<tr>
<td></td>
<td>1.5</td>
<td>---</td>
<td>0.124 ± 0.001</td>
<td>--</td>
<td>0.8</td>
<td>0.125 ± 0.002</td>
<td>--</td>
<td>1.6</td>
<td>4.0</td>
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<tr>
<td></td>
<td>2.0</td>
<td>---</td>
<td>0.123 ± 0.001</td>
<td>--</td>
<td>0.8</td>
<td>0.124 ± 0.001</td>
<td>--</td>
<td>0.8</td>
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<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td></td>
<td>0.123</td>
<td>--</td>
<td>--</td>
<td>0.124</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.2</td>
<td>0.322 ± 0.003</td>
<td>99.7</td>
<td>0.9</td>
<td>0.323 ± 0.004</td>
<td>99.7</td>
<td>1.2</td>
<td>1.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Soil</td>
<td>1.0</td>
<td>---</td>
<td>1.52 ± 0.03</td>
<td>--</td>
<td>2.0</td>
<td>1.50 ± 0.04</td>
<td>--</td>
<td>2.7</td>
<td>1.8</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>---</td>
<td>1.53 ± 0.02</td>
<td>--</td>
<td>1.3</td>
<td>1.54 ± 0.03</td>
<td>--</td>
<td>1.9</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>---</td>
<td>1.52 ± 0.02</td>
<td>--</td>
<td>1.3</td>
<td>1.54 ± 0.02</td>
<td>--</td>
<td>1.3</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td></td>
<td>1.52</td>
<td>--</td>
<td>--</td>
<td>1.53</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>2.0</td>
<td>3.50 ± 0.05</td>
<td>99.4</td>
<td>1.4</td>
<td>3.51 ± 0.06</td>
<td>99.4</td>
<td>1.7</td>
<td>1.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

$^a$ Mean value ± Standard deviation (n = 5).

$^b$ Tabulated F for (4,4) degrees of freedom at P (0.95) is 6.39.

$^c$ Tabulated t for 8 degrees of freedom at P (0.95) is 2.306.

6B.4. CONCLUSIONS

The interaction of nitrite with primary amino group of thionin to form diazonium salt is almost specific, which provides the determination of nitrite with good sensitivity. Temperature independence, elimination of extraction steps, stability of the unreacted dye (> a day), high tolerance limit for a large number of ions and low cost of the reagents used are advantages of the method. The method is simple, rapid, reliable, and accurate, and shows excellent reproducibility.

The sensitivity and simplicity the proposed methods are compared with many existing methods in Table 6.5.
### Table 6.5. Comparison of molar absorption coefficient (ε) of the proposed methods with some reported methods

<table>
<thead>
<tr>
<th>Reagent/s</th>
<th>Reference</th>
<th>$\lambda_{max}$ (nm)</th>
<th>$\varepsilon$ (L mol$^{-1}$ cm$^{-1}$)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-aminoazobenzene + Acetyl acetone</td>
<td>Proposed methods</td>
<td>500</td>
<td>$4.2 \times 10^4$</td>
<td>Simple, sensitive, rapid, selective, product is bisazo dye.</td>
</tr>
<tr>
<td>Thionin</td>
<td></td>
<td>600</td>
<td>$4.1 \times 10^4$</td>
<td>Simple, sensitive and rapid.</td>
</tr>
<tr>
<td>Sulfanilamide + N-(1-naphthyl) ethylenediamine dihydrochloride</td>
<td>[6,12]</td>
<td>543</td>
<td>$4.0 \times 10^4$</td>
<td>Sensitive to pH variation, requires 10 min for full colour development and the product can be carcinogenic.</td>
</tr>
<tr>
<td>$p$-aminophenylmercaptoacetic acid + $\alpha$-naphthol</td>
<td>[24]</td>
<td></td>
<td>$3.49 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>$p$-nitroaniline + dibenzoyl methane</td>
<td>[25]</td>
<td>520</td>
<td>$3.6 \times 10^4$</td>
<td>Less sensitive, SO$_2$ and sulphide interfere.</td>
</tr>
<tr>
<td>$p$-aminobenzenesulfonic acid + H-acid</td>
<td>[26]</td>
<td>520</td>
<td>$3.1 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>Imipramine hydrochloride + 3-methyl-2-benzothiazolone hydrazine hydrochloride</td>
<td>[28]</td>
<td>640</td>
<td>$0.49 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>$p$-nitroaniline + acetyl acetone</td>
<td>[31]</td>
<td>490</td>
<td>$3.2 \times 10^4$</td>
<td>Less sensitive, Cu$^{2+}$, Fe$^{3+}$, Co$^{2+}$ and Hg$^{2+}$ interfere.</td>
</tr>
<tr>
<td>$p$-aminacetophenone + Citrazinic acid</td>
<td>[32]</td>
<td>495</td>
<td>$2.9 \times 10^4$</td>
<td>Less sensitive, Fe$^{3+}$ and Cu$^{2+}$ interfere.</td>
</tr>
<tr>
<td>$p$-nitroaniline + citrazinic acid</td>
<td>[37]</td>
<td>530</td>
<td>$2.8 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>Sulphanimide + ethylacetocetate</td>
<td>[38]</td>
<td>356</td>
<td>$1.22 \times 10^4$</td>
<td>Fe$^{3+}$ interferes.</td>
</tr>
<tr>
<td>$p$-nitroaniline + diphenylamine</td>
<td>[39]</td>
<td>500</td>
<td>$1.425 \times 10^4$</td>
<td>Less sensitive, Micellar Media (Triton X-100) required, Fe$^{3+}$ interfere.</td>
</tr>
<tr>
<td>$p$-nitoaniline + ethoxyethylenemaleic acid</td>
<td>[42]</td>
<td>439</td>
<td>$5.04 \times 10^4$</td>
<td>Sensitive, but diazotization requires cooling (0 – 5 °C).</td>
</tr>
<tr>
<td>$p$-nitroaniline + ethyl cyanacetate</td>
<td>[42]</td>
<td>465</td>
<td>$1.21 \times 10^4$</td>
<td>Less sensitive, diazotization requires cooling (0 – 5 °C).</td>
</tr>
<tr>
<td>4-(1-methyl-1-mesitylcyclobutane-3-yl)-2-aminothiazole + N,N-di-methyl aniline</td>
<td>[43]</td>
<td>482</td>
<td>$2.03 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>5, 10, 15, 20-tetrakis (4-aminophenyl) porphine</td>
<td>[52]</td>
<td>434</td>
<td>$2.63 \times 10^4$</td>
<td>Highly sensitive, requires 30 min heating, Fe$^{3+}$ interfere.</td>
</tr>
<tr>
<td>Phosphomolybdenum blue</td>
<td>[53]</td>
<td>814</td>
<td>$1.1 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>Neutral red</td>
<td>[55]</td>
<td>530</td>
<td>$2.5 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>Phenoasfranine</td>
<td>[57]</td>
<td>520</td>
<td>$3.7 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>Salbutamol sulphate</td>
<td>[68]</td>
<td>410</td>
<td>$1.8 \times 10^4$</td>
<td>Very less sensitive.</td>
</tr>
<tr>
<td>Rhodamine 6G</td>
<td>[70]</td>
<td>445</td>
<td>$1.2 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
<tr>
<td>Barbituric acid</td>
<td>[79]</td>
<td>310</td>
<td>$1.533 \times 10^4$</td>
<td>Less sensitive.</td>
</tr>
</tbody>
</table>
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