ADDIS ABABA UNIVERSITY
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Application of an Official Spectrophotometric Method for Nitrite Determination in Tap and River Water Samples

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Graduate Project

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Dedicated

To my son

Nahom.

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Abbreviations and Acronyms

AACC	Addis Ababa City Council
APHA	American Public Health Association
AWWARF	American Water Works Association Research Foundation
ECETOC	European Chemical Industry, Ecology and Toxicology Center
EPA	Environmental Protection Agency
Hb	Hemoglobin
NEDA	N-(1- napthyl) Ethylendiamine Dihydrochloride
PAH's	Polycyclic Aromatic Hydrocarbons
RSD	Relative Standard Deviation
PCB's	Polychlorinated Biphenyl's
SULPHA	Sulphanilamine
USEPA	United States Environmental Protection Agency
UV-Vis	Ultraviolet–Visible
WHO	World Health Organization

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An internationally known test method for nitrite was applied to determine the anion in tap and river water samples. The method is based on the reaction of nitrite with sulphanilamide in acidic medium to form diazonium ion, which is then coupled with N-(1-napthyl)-ethylendiamine dihydrochloride to form an azo dye, showing absorption maximum at 540 nm. The method obeys Beer's law in the concentration range 0.25 -1.5 µgmL^{-1}of nitrite. The optimum reaction conditions and other analytical parameters were evaluated. The method was successfully applied to the determination of nitrite in tap water and river water samples.

1 Introduction

There are many chemicals that may occur in drinking water, however, only a few are of immediate health concerns in any given circumstances. The priority given in both monitoring and remedial action for chemical contaminants in drinking water should be managed to ensure that scarce resources are not unnecessarily directed towards those of little or no health concern. Among these chemicals high level of nitrate/nitrite that can arise from the excessive application of fertilizers, leaching of industrial waste water, from food and metallurgical activities and from other organic wastes enter into the surface and ground water, the result is increases the level of these chemicals in drinking water. The primary health hazard from drinking water with nitrate-nitrogen occurs when nitrate is transformed to nitrite in the digestive system. The nitrite oxidizes iron in the hemoglobin of the red blood cells to form methaemoglobin, which lacks the oxygen-carrying ability of hemoglobin. This creates the condition known as methaemoglobinaemia (sometimes referred to as "blue baby syndrome"), in which blood lacks the ability to carry sufficient oxygen to the individual body cells causing the veins and skin to appear blue [1].

Most humans over one year of age have the ability to rapidly convert methaemoglobin back to oxyhaemoglobin; hence, the total amount of methaemoglobin within the red blood cells remains low in spite of relatively high levels of nitrate/nitrite uptake. However, in infants under six months of age, the enzyme systems for reducing methaemoglobin to oxyhaemoglobin are incompletely developed and methaemoglobinaemia can occur. This also may happen in older individuals who have genetically impaired enzyme systems for metabolizing methaemoglobin. Nitrites may also react in the stomach with secondary or tertiary amines and amides present in foods such as cheese or meat to form nitroso compounds '*nitrosamines*" which are potentially carcinogens [2, 3].
Many countries, especially developed countries regulate the level of nitrite in their diet through government standards, however in many developing countries such standards are variables and are often less strict than the developed nations.

1.1 Nitrate and Nitrite

Nitrate and nitrite are naturally occurring ions that are part of the nitrogen cycle. Nitrate is used mainly in inorganic fertilizers, and sodium nitrite is used as a food preservative, especially in cured meats. The nitrate concentration in groundwater and surface water is normally low but can reach high levels as a result of leaching or runoff from agricultural land or contamination from human or animal wastes as a consequence of the oxidation of ammonia and similar sources. Anaerobic conditions may result in the formation and persistence of nitrite. Chloramination may give rise to the formation of nitrite within the distribution system if the formation of chloramine is not sufficiently controlled. [4].

1.1.1 Physicochemical properties

Property	Nitrate	Nitrite
Acid	conjugate base of strong acid HNO_3; $p^{Ka} = 5\times10^{-2}$	conjugate base of weak acid HNO_2; $p^{Ka} = 2.5\times10^{3}$
Salts	very soluble in water	very soluble in water
Reactivity	unreactive	reactive; oxidizes antioxidant, $Fe^{2+}$of hemoglobin (HB) to Fe^{3+} and primary amines

Table 1 The physico-chemical properties of nitrate and nitrite.

1.1.2 Major uses and sources of nitrate and nitrite in drinking-water

Nitrate is used mainly in inorganic fertilizers. It is also used as an oxidizing agent and in the production of explosives, and purified potassium nitrate is used for glass making. Sometimes Nitrate is also added to food to serve as a reservoir for nitrite. Nitrate can reach both surface and ground water as a consequence of agricultural activity (including excess application of inorganic nitrogenous fertilizers and manures), from wastewater treatment and from the oxidation of nitrogenous waste products in human and animal excreta, including septic tanks. Nitrite can also be formed chemically in distribution pipes by *nitrosamines* bacteria during stagnation of nitrate-containing and oxygen-poor drinking-water in galvanized steel pipes or if chloramination is used to provide a residual disinfectant and the process is not sufficiently well controlled [5].

1.1.3 Environmental fates

In soil, fertilizers containing inorganic nitrogen and wastes containing organic nitrogen are first decomposed to give ammonia, which is then oxidized to nitrite and nitrate. The nitrate is taken up by plants during their growth and used in the synthesis of organic nitrogenous compounds. Surplus nitrate readily moves with the groundwater [6].

In surface water, nitrification and denitrification may also occur, depending on the temperature and pH. The uptake of low water tables, the amount of rain water, the presence of other organic materials and other nitrate by plants, however, is responsible for most of the nitrate reduction in surface water [7].

Nitrification

$NH_4^+ + 3O_2 \rightarrow 4H^+ + 2NO_2^- + 2H_2O$.(using *nitrosomonas* bacteria). **(1)**

$2NO_2^- + O_2 \rightarrow 2NO_3^-$ (using *nitrobacter* bacteria)

Denitrification

$4NO_3^- + 5\{CH_2O\} + 4H^+ \rightarrow 2N_2\,(g) + 5CO_2\,(g) + 7H_2O$ (using *denitrifying bacteria*). **(2)**

1.2 Nitrite (NO_2^-)

The nitrite ion is an important intermediate in biological nitrogen cycle, like during the biodegradation of nitrogenous wastes and in effluents from various industries. It has found numerous applications ranging from dye manufacture to food preservation [8].

Nitrite is widely recognized as nitrate's partner in many aspects and can be considered to be the more dangerous of the two in terms of the potential effects of excessive concentrations on the health of humans and aquatic organisms. Justification for the latter statement arises from its primary role in methaemoglobinemia (commonly characterized in human health terms as ''blue baby'' syndrome) and its somewhat contentious implication in various cancers. Nitrite does not however feature in the top reaches of any list of priority pollutants. Those positions are normally reserved for species such as polycyclic aromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's), lead, mercury etc. that present an immediate or bioaccumulative health threat. The transient nature of environmental nitrite normally ensures its presence is maintained at concentrations (typically below 0.01 mgL^{-1}) that are insufficient to present any immediate threat to physiological or environmental well-being. Problems arise however where the environmental conditions are such that the ion is allowed to accumulate and the ecotoxic effects become

pronounced. The principal legislative concerns obviously relate to anthropogenic inputs of the ion (or compounds leading to its biogeneration) and these alone require the implementation of monitoring programmes. However, the ambiguity of nitrite and the various biogeochemical processes that can promote its generation means that the monitoring process could also be exploited to provide a quick assessment of the status of a particular ecosystem [8, 9].

The conversion of nitrate to the more reactive nitrite has long offered a more unwilling solution to the difficulties associated with direct determination. It could be envisaged therefore that the existence of an extensive knowledge base dedicated to the determination of nitrite would therefore serve as an ideal source from which field methodologies could emerge.

1.2.1 Environmental and physiological significances

Few chemicals have managed to arouse and sustain the level of scientific and public attention achieved by nitrate and nitrite. Legislative controls on maximum permissible levels vary from one country to another though there is international recognition of the potential health hazards posed by the presence of these agents within the environment. A recommended level of 50 mgL^{-1} nitrate and 3 mgL^{-1} nitrite within potable water has been specified by the World Health Organization [10, 11]. The lower limit set on nitrite reflects the fact that it is nitrite that is the causative agent for clinical manifestations previously attributable to both anions and, as such, serves to justify the bias, at least in part, towards nitrite. Although ubiquitous in nature, the reactivity of nitrite normally ensures its presence at trace concentrations (below 0.1 mgL^{-1} for drinking water – the current EU limit). Ingestion of the anion however has been proclaimed to lead to a number of detrimental effects on the health of mammals [12, 13], macro-invertebrates [14, 15], most aquatic organisms [16] and in a wide range of health issues [17, 18].

However many are contentious as the associations between disease incidence and levels of nitrite within drinking water are by their nature ambiguous and controversial [14, 19, 20]. The principal and clinically undisputed effect of nitrite poisoning however is methaemoglobinemia [21]. This arises through nitrite induced oxidation of the ferrous iron (Fe^{2+}) in haemeproteins to the ferric (Fe^{3+}) state and results in diminished oxygen carrying capacity by the hemoglobin molecule. This tends to be magnified in infants (‘‘Blue baby’’ syndrome) [22] due to the fact that they have 60% less NADH–*cytochrome b5 reductase* compared to adults, and are subsequently more susceptible to agents that cause methaemoglobinemia [23].

Epidemiological studies on human cases of methaemoglobinemia have indicated that the incidence is relatively low within the general population [24]. This can be attributed in large part to the increasing standardization of potable water. The main problem however arises where drinking water is derived from bore holes or surface waters within agriculturally intensive areas. Anaerobic conditions, in particular, tend to promote the accumulation of nitrite and while the significance in terms of human health can be serious, more often than not it can be devastating for aquatic life.

Nitrite accumulation is a common problem in intensive fish culture and is mediated both by abiotic (oxygen, temperature, pH, salinity) and biotic (predominantly microbial) factors [25]. Fish farming has increased markedly in recent years across the globe and often represents a considerable income source to rural communities. Sustainable fish culture however is dependent upon the selection of production sites that possess good water exchange characteristics where strong currents (i.e. from tidal flushing) can enable the efficient dispersal of waste and replenish the site with well oxygenated water [25]. In situations where natural food sources are low, the external input of nutrients through supplementation can however significantly alter the local water chemistry [25].

The accumulation of organic waste leads to an increase in sedimentary microbial activity resulting in decreased oxygen levels and an increase in ammonia and nitrite concentrations within the overlying water [26, 27]. Such nutrient enrichment will obviously effect plant and animal populations and, if left unchecked, can lead to “site souring’’ in which the microbial by-products directly impact on the production of fish stock. The problem is accelerated in warmer regions/summer months where increases in temperature can increase gill permeability towards nitrite and hence markedly increase its biotoxicity [15, 28].

Water courses receiving nitrogenous inputs from other anthropogenic sources such as agricultural run off or sewage discharge can also be subject to considerable increases in the concentration of both ammonia and nitrite. It has been established that low oxygen concentrations, high ammonia and high pH (typically in the range pH 7–8) can promote the transient build up of nitrite through the inhibition of the *Nitrobacter sp*. microbes that are responsible for the oxidation of nitrite to nitrate [26, 27] Water exchange will obviously be critical and with it, the morphology of the sediment layer. Coarse gravel and sandy sediments facilitate greater flow penetration and are less susceptible to nitrite accumulation than finer grain/muddy layers [26].

1.2.2 Sampling strategies

The reactive nature of nitrite poses a considerable problem in its detection and the ease with which sample deterioration can occur is a prime motivation in the acquisition of onsite data. Losses are typically attributed to air oxidation and/or bacterial conversion. In general, neutral or alkaline samples are preferred as acidic conditions tend to accelerate losses through the generation of nitrous acid which can be prone to volatilization, decomposition or participate in electrophilic (nitrosation/nitration) reactions with activated aromatics present within humic materials. Should medium (several days) to long term storage of the samples be required then refrigeration or freezing is advised. The latter generally indicate the need for the addition of bactericidal agents such as mercury salts. It has also been shown that both nitrate and nitrite are stable to autoclave conditions [29]. Much of the early work on the detection of nitrite originated from concerns over its use as a food preservative [30].

In such situations, sample clean up procedures such as filtration; centrifugation and the use of Carrez Reagents can be readily applied. Fortunately, the difficulties posed by persistent material in food do not pose the same level of interference for environmental samples though heterogeneous mixtures will often require filtration (typically using a sub micron antibacterial filter than can be used in conjunction with a syringe) [26, 30, 31].

It is clear from the previous section on the environmental significance of nitrite that the site of collection and the conditions under which sampling was conducted will be of prime importance in the interpretation of any potential ecotoxicological hazard. While samples derived from point sources such as tap water or pore water are relatively straightforward, the sampling of water courses presents a problem in that the nitrite profile will vary considerably with depth and current flow [25, 34, 35]. Tidal flushing within deep water will obviously lead to a lower nitrite concentration being recorded than a sample extracted from the upper most layer of a mud sediment where microbial activity will be higher and the immediate water layer is less mobile [26].

Any aqueous sample must therefore be reconciled with the spatial position, depth and current flow at which it was collected. Additional factors such as temperature, salinity, dissolved oxygen, pH and date/period of sampling will also be required to provide a complete profile of the sample but also to place the nitrite concentration within context [25, 33, 34]. It may well be stated that higher

concentrations of nitrite are the result of seasonal fluctuations (increased temperature and light) rather than from anthropogenic sources [26].

1.2.3 Detection and determination methodologies

Several reports have been published on the common methods for the determination of nitrite, including chromatography [35], electrochemical detection [36], capillary electrophoresis [37] and spectrophotometric [38, 39]. In some of these methods, selectivity is very poor, some demand expensive and complicated instruments or reagents and others require time consuming separation procedures while the spectroscopic technique is by far the most widely used method for determination of nitrite due to its excellent limit of detection obtained.

1.3 Spectrophotometric (Colorimetric) method

Photometric determination of inorganic substances with organic reagents are most frequently based on reactions which yield products that can absorb or emit radiations within the frequency range of electronic spectra. The absorption or emission of ultraviolet and visible radiation is thus measured in spectrophotometric or colorimetric application of organic reagents [40]. The traditional term colorimetric is used in analytical chemistry to denote the methods in which the amount of a given colored substance in the solution is determined by comparing the color either with solutions of known concentrations or with the other standards having the same color. The colors are compared either visually (i.e., visual colorimetry) or with the use of photocell (i.e., photoelectric colorimetric).

Nowadays, spectrophotometric methods are more commonly used than the colorimetric procedures, which are based on the measurement of the absorbance of monochromatic light passing through the solution containing the substance to be determined.

This instrument is called spectrophotometer and the reactions of inorganic substances with the reagents which yield colored product suited for colorimetric or spectrophotometric (color-producing) reactions. If the thickness of absorbing layer **l** and the absorption coefficient **ε** for the substance being determined at a given wave length are known and the absorbance **A** is measured, it is possible to use the Beer's law in order to determine the concentration of absorbing species **C** . In routine work calibration graph is usually used to read this concentration [41].

$$A = \varepsilon Cl \quad \text{..... Beer's Law} \qquad (3)$$

The desirable properties of organic reagents in colorimetric reactions are sufficient stability and resistance to aerial oxidation or photometric decomposition. The same should hold for the reaction products. Moreover in the absorption spectrum of a product there must be a characteristic intensive absorption band, at sufficient distance from that of reagent or substance being determined.

It is possible to increase the selectivity of photometric determination of a given substance by selecting a suitable wave length at which the absorption coefficient of a substance being determined is high, and those interfering substances present is negligible, and also by adjustment of pH. The pH and the presence of other components in solution affect the equilibrium concentration of the colored species. If these effects are neglected serious errors in photometric measurements (determinations) may arise.

There is also a salt effect caused by the presence of a large amount of electrolytes. This salt effect is due to the electrostatic forces of the ions causing deformation of the colored spacious and consequently a change in the absorption spectrum [42].

Another source of photometric errors is due to the effect of change in the concentration of the solution on the value of the refractive index and thus the amount of light scattered from the beam passing through the curette. Usually at high concentration there is a rapid change in refractive index that causes deviation of Beer's law at high concentration.

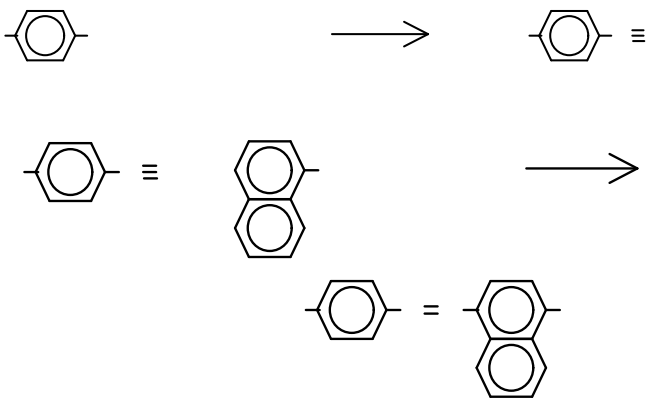
The accuracy and precisions of photometric measurement depend on both on types of instrument used and the chemical reactions chosen [43].

The Griess assay has been the main method of nitrite analysis for over a century [44] and in that time a substantial amount of experimental data on its application has been addressed and extensively reviewed [8,9]. The basic approach relies upon the acid induced diazotisation of an indicator amine by sample nitrite and its subsequent coupling with a secondary species to form an azo chromophore, The longevity of the technique has resulted largely from the selectivity of the reaction and the resolutive clarity of the absorption maximum (typically ranging between 500–600 nm). The underlying principle has remained largely unchanged since its inception with the

majority of the modifications involving the manipulation of the component amines in order to optimize analytical signal for specific applications [8, 9, 45, 46].

Alternatives to the azo methodologies have however been investigated and include the nitrosation/nitration of phenolic derivatives (310–400 nm) [47] and N-phenylanthranilic acid (410 nm) [48]. These offer little in the way of improved detection limits but offer a degree of procedural simplicity over the traditional Griess protocol through the removal of the secondary coupling step. The main drawback to these systems lies in the yellowness of the absorption maxima and the subsequent acquisition of an analytical signal that is sufficiently distinct from the background sample at trace levels. The main requirement in both the azo and nitrosation approaches is the presence of inactivated aromatic indicator (typically a phenol or aniline derivative) under acidic conditions.

The aim of this project is to apply an official spectrophotometeric method for the determination of nitrite in natural water samples. The basic principle of determination of nitrite in this project is based on the formation of purple azo dye, (Gress reaction) which can be measured at 540nm (Swedish standard).this purple azo dye is formed through the following reactions; upon the addition of the acidic reagent sulphanilamide (SULPHA) to a solution (or sample) containing nitrite, a diazo compound is formed. This compound then reacts to form apurple azo dye upon the addition of the second reagent N-(1-napthyl)-ethylendiamine dihydrochloride (NEDA) (equation 2).



(4)

The intensity of the formed purple azo dye is proportional to the concentration of (NO_2^-) present in the sample [48, 49, 50].

The sampling sites used to apply the official method were selected Based on their potential sources of pollution. In these sites agricultural activities, industrial activities and extensive irrigation are observed and also influence of the polluted river are clearly seen on the receiving land, which might possibly reflect on the product cultivated on such a land.

1.4 The pollution status of Akaki Rivers

Addis Ababa is a typical city in the developing countries where the rate of urbanization and availability of waste removal facility are not equal [52]. Surface water bodies receive the major parts of waste produced by the residents and various factories. The main surface water system in the city includes AKaki River and various reservoirs [53]. Akaki River, which has two main branches (Big Akaki and Little Akaki), is the most polluted surface water system in the city [53, 54].

The most important sources of pollution for these rivers are industrial and domestic, For example, the western part of Addis Ababa is an industrial estate along Little Akaki; each factory thus pours a lot of chemical pollutants, which affect the gardens down the stream. Little Akaki goes through highly populated areas. There, the river is used as toilets and for washing. The area is formed from very dense shantytowns a biggest problem comes from garbage. Little Akaki is used there as a public dump for thousands of inhabitants. In fact, and according to many authors [52, 56] the whole stream down to Kaliti is subject to all kind of waste disposals. In some localities sludge water and human feces and other wastes are dumped to these rivers. Big Akaki also crosses the highly populated region in the eastern part of the city where it receives any kinds of wastes from the residents [51-54]. Little Akaki is being used to get rid of industrial wastes. If we consider the down stream we can observe different sources of pollution, cement, leather, and beverage production and municipal wastes. Exposure to these wastes which contain toxic components such as trace metal ions is of great concern, as it poses not only health risks to humans but also potentially unacceptable ecological risks to plants which are produced by intensive agricultural practices along the river sides, animals and microorganisms. The pollution of a river would have its influence on the receiving land, which might possibly reflect on the product cultivated on such

land, since vegetables are grown on the embracement along Little Akaki and Big Akaki rivers within Addis Ababa town and the neighboring town such as Akaki, Alem Gena, and Sebeta [55-57].

2 Objectives

2.1 General Objective

- The objective of this project is to determine the concentration of nitrite in tap and river water samples using spectrophotometric method.

2.2 Specific Objectives

- To validate a batch mode analysis procedure for nitrite determination using UV-Vis spectrophotometric method
- To determine the level of nitrite in tap water samples
- To determine the level of nitrite in river water samples
- To evaluate reaction parameters and determine the nitrite level in a new laboratory environment.

3 Experimental

3.1 Apparatus

A Beckman DU-65 spectrophotometer was used to record the absorption spectra. The pH of water samples was measured with the help of HANNA PH 300 series BENCH-TOP P^H meters.

3.2 Reagents and Solutions

All chemicals used were of analytical reagent grade (Merck) or chemically pure grade and deionized water was used throughout.

***Nitrite standard stock solution*:** (250 μgmL^{-1}) was prepared by dissolving 37.5 mg dried (4 h at 105^0C) sodium nitrite (Merck) and few drops of sodium hydroxide pellets to prevent liberation of nitrous acid and 1 mL chloroform added to prevent bacterial conversion of nitrite to nitrate in 100 mL in deionized water.

Working standard nitrite solution **:** (5 μgmL^{-1}) was prepared by diluting 5 mL standard stock solution to 250 mL in deionized water.

Nitrite calibration solutions **:** were prepared by diluting appropriate concentration of working solution.

Phosphoric acid stock solution**:** (10% v/v) was prepared by diluting 100 mL of phosphoric acid 85% (Merck) in 1L deionized water.

Working phosphoric acid solution**:** (8% v/v) was prepared by diluting 160 mL stock solution into 200 mL deionized water.

Sulphanilamide solution **(*REAGENT I*) :** (0.6% m/v) was prepared by dissolving 600 mg of sulphanilamide (Merck) in 100 mL of 8% (v/v) phosphoric acid working solution while heating the solution to 75-80^{0}C using water bath.

N-(1-napthyl) –ethylendiamine dihydrochloride **(*REAGENT II*):** (0.02% m/v) was prepared by dissolving 20 mg NEDA (Merck) to 100 mL in deionized water.

EDTA solution**:** 10% (m/v) was prepared from the disodium salt of EDTA.

1 M NaOH was used for adjusting the pH of the solution.

3.3 Sample Collection, Treatment and Storage

3.3.1 Tap water samples

Tap water samples were collected in polyethylene plastic containers (bottles) by taking the samples directly from the tap after allowing the water to run for at least 20 minutes. The location within "Arat kilo" campus compound was selected to collect the tap water samples. Three samples as replicates were collected from three locations (near digital library, around student cafeteria and near "New Saba' student dormitory) and were mixed so as to take representative samples. Because of no preservation techniques are recommended by official method for tap water samples; analysis was done within 48 hours after collection to obtain reliable nitrite concentration [58]. For this short term preservation samples were stored at 4^{0}C in a refrigerator.

3.3.2 River water samples

Samples were collected in polyethylene plastic containers (bottles) at different points upstream and downstream both at little Akaki and Big Akaki rivers. The corresponding upstream and down stream aliquots were then mixed to obtain representative samples. In the laboratory, the contents of bottles were divided into equal parts. Half part was reserved for physico-chemical analysis and the rest was treated for nitrite analysis. Mercury (II) chloride (4 $mgmL^{-1}$ per 100 mL of sample) was added as a preservative and stored at $0^{o}C$ [58]. Before analysis, river water samples were filtered through Whatman No. 41 filter paper.

3.4 Procedures

3.4.1 General procedure for determination of nitrite

An aliquot of sample containing 0.25 - 1.5 μgmL^{-1} of nitrite (when diluted to 25 mL) was transferred to a series of 25 mL volumetric flasks. To these solutions 5 mL of *reagent (I)* and 5 mL of *reagent (II)* were added and the content were adjusted to 25 mL using deionized water and mixed well. After allowing the solution to 30-120 min in the dark for complete color development, the absorbance of the colored azo dye was measured at 540 nm against reagent blank.

3.4.2 Determination of nitrite in real samples (tap and river water)

10 mL of water sample (containing no more than 1.5 μgmL^{-1} nitrite) was treated with 1 mL of 10% (m/v) EDTA and 1mL of 1 M NaOH. The solution was mixed and centrifuged to remove any precipitate formed. Then the centrifuge was transferred to 25 mL standard flask and directly used for color development by following the procedure described above. The concentration of nitrite was calculated from calibration graph.

4 Results and Discussion

4.1 Spectral Studies

Absorbance was evaluated in visible range. A standard nitrite solution scanned in the range 400 - 700 nm against reagent blank showed a wave length 540 nm which is identical with that recorded in APHA test method [58].

4.2 Reaction Parameters Evaluation

The condition for the determination of nitrite was evaluated by studying the influence of different parameters such as reagents concentration and the time required for complete color development. A 1 μgmL^{-1} of NO_2^- solution in 25 mL final volume was used for the evaluation of the above parameters.

4.2.1 The concentration of reagent I

4.2.1.1 Phosphoric acid

The effect of acidity on the sensitivity of measurement was studied by changing the concentration of phosphoric acid from 2% (v/v) - 12% (v/v) .The experimental conditions for the other reagents is 0.4% (m/v) ,0.01% (m/v) and 1 μgmL^{-1} of SULPHA, NEDA and NO_2^-, respectively.

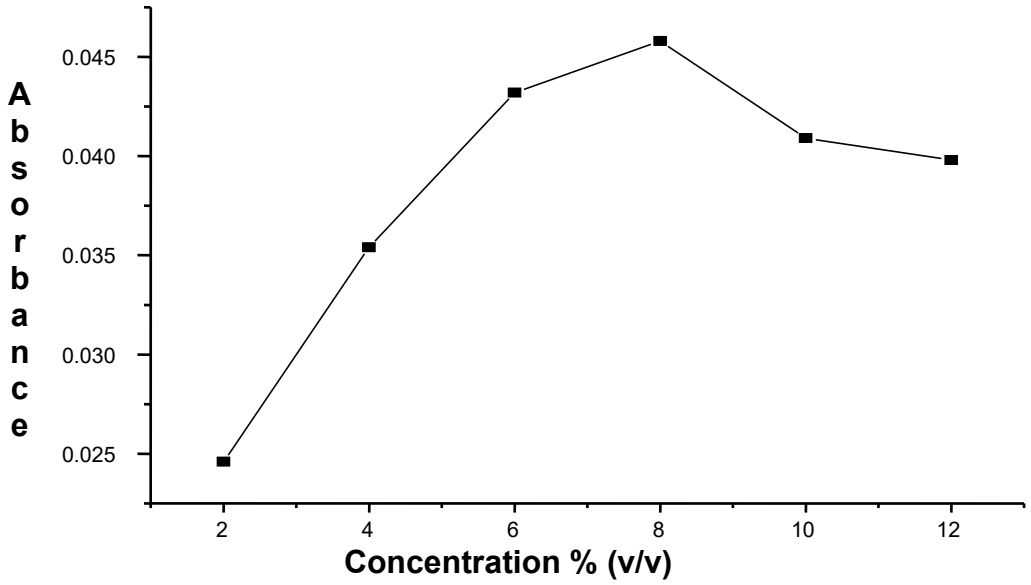


Figure 1 Effect of phosphoric acid concentration on the absorbance (peak)

The results of figure 1 show that the system is very sensitive to the change in pH of the medium. The absorbance of the solution increases with the concentration of phosphoric acid from 2% (v/v) to 8% (v/v) whereas greater than 8% (v/v) decreased the sensitivity.

4.2.1.2 Sulphanilamide

The effect of concentration of sulphanilamide was investigated by changing the concentration of sulfanilamide from 0.2% (m/v) to 1.2% (m/v). To see the effect of sulphanilamide concentrations of various amount of sulphanilamide were dissolved in 8% (v/v) phosphoric acid solutions in 100 mL volumetric flask. Then 5 mL of each solution was used directly for color development by following procedure described above. The experimental conditions for other reagents are 8%, 0.01% (m/v) and 1 μgmL^{-1} of phosphoric acid, NEDA and NO_2^-, respectively.

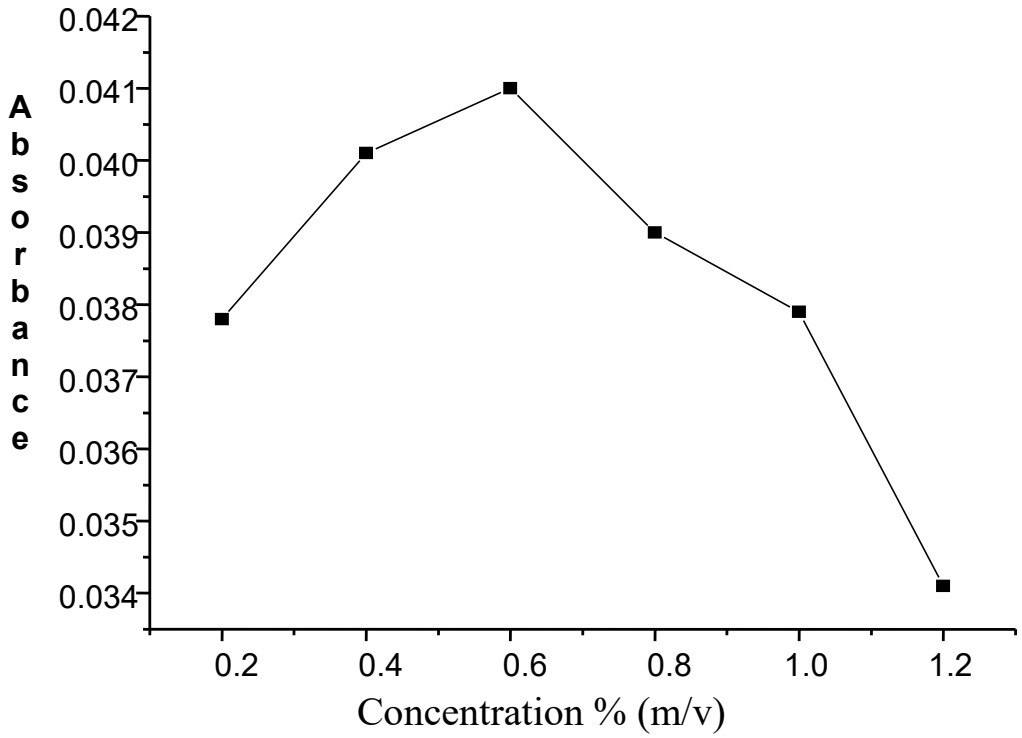


Figure 2 Effect of sulphanilamide concentration on the absorbance (peak)

The results of the above figure 2 also show that the average peak height at 0.6% (m/v) was most favorable for further studies.

4.2.2 The concentration of reagent

4.2.2.1 N-(1-naphtyl) ethylendiamine dihydrochloride (NEDA)

The effect of varying concentration of the coupling reagent was studied by using the above optimized concentrations of other reagents and various concentrations of NEDA ranging from 0.005% (m/v) to 0.05% (m/v), It was found that 0.02% (m/v) was found to be the optimum condition.

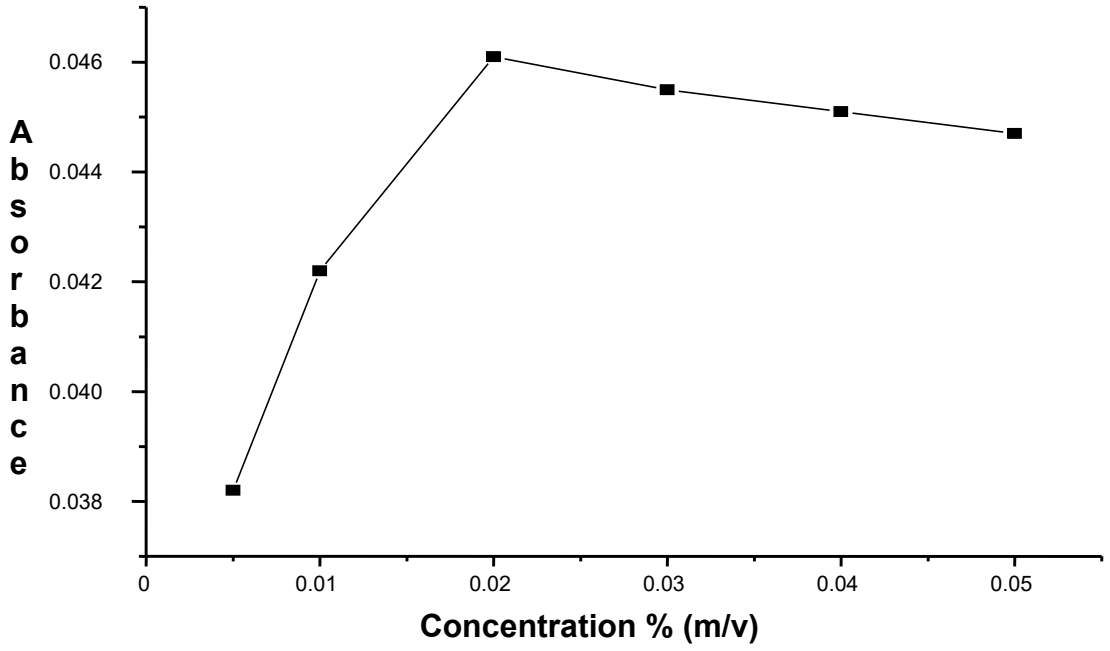


Figure 3 Effect of NEDA concentration on the absorbance (peak)

4.2.3 Time needed for complete color development

The effect of time required for complete color development, a solution was prepared according to the optimized concentration of reagents and directly applied for color development by following the procedure described above and allowed to stay in the dark from 10 min to 2 hrs for complete color development and the absorbance was taken.

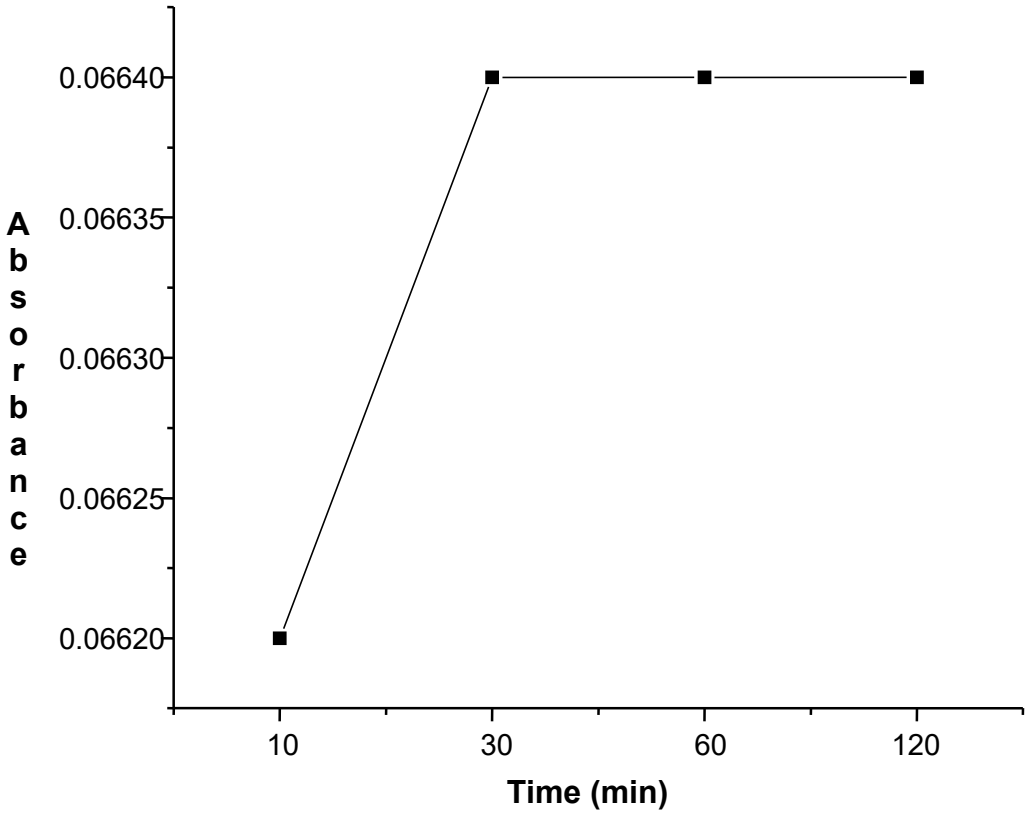


Figure 4 Effect of time required for complete color development

The results shows that for complete color development under the specified conditions the solution should be allowed to stay in the dark at least 30 minutes.

4.3 Calibration Curve Obtained

Calibration curve was run using standard solutions of nitrite employing all the optimized conditions. Each point on the curve obtained as the average of three determinations.

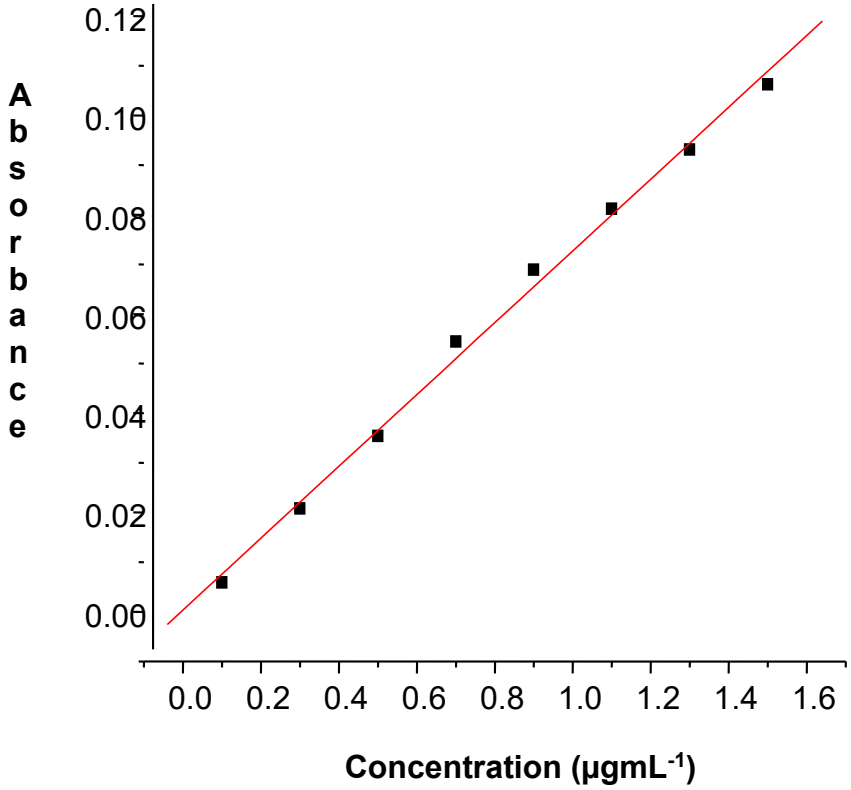


Figure 5 Calibration curve for NO_2^- standard solutions

Under the conditions evaluated above, in the *concentration range* of 0.25 to 1.5 µgmL^{-1} of nitrite a quite linear calibration curve was obtained with *linear correlation coefficient* (**R**) value **0.998.**

$\mathbf{A} = 0.07235537\ \mathbf{C} + 3.51964 \times 10^{-4}$

Where **A** is absorbance at 540 nm and **C** is concentration of nitrite in µgmL^{-1}.

The *detection limit* was **0.08** µgmL^{-1}, which was calculated by the standard deviation of 10 blank measurements multiplied by three and divided by the slope of the calibration curve.

The *relative standard deviation* (**RSD**) value for ten replicate measurements of 1 µgmL^{-1} NO_2^- solution was **2.46%.**

The *molar absorbitivity* (**ε**) was calculated from the calibration slope is **3.33 x 10^3** $Lmol^{-1}cm^{-1}$.

$$\varepsilon = \frac{\Delta A}{b\,\Delta C} \qquad (5)$$

Where **A** is absorbance ($Lmol\text{-}1cm^{-1}$), b is the length of the cell (cm) and C is molar concentration ($mol\ L^{-1}$).

4.4 Interferences Studies

The potential interference of different heavy metal ions that are possibly present in the river and tap water samples could adversely affect the azo method of determination of nitrite and it was evaluated by recording the absorbance both in the presence and in the absence of the interfering ions at 100% (w/w) with respect to 1 μgmL^{-1} NO_2^- concentration and measuring the percent error introduced due to the presence of these ions. Results obtained are shown in Table 2.

Interfering ions	Type of salt taken	Absorbance reading/ 25 mL [a]		(%) Relative Error
		Without interfering ion	With interfering ion	
Cu(II)	$CuCl_2$	0.07032 ± (0.002)	0.06959 ± (0.003)	-1.04
Co(II)	$CoCl_2$	0.07032 ± (0.001)	0.06959 ± (0.003)	-1.04
Zn(II)	$ZnCl_2$	0.07032 ± (0.002)	0.06956 ± (0.002)	-1.08
Hg(II)	$HgCl_2$	0.07032 ± (0.003)	0.07229 ± (0.003)	+2.80
Fe(III)	$FeCl_3$	0.07032 ± (0.002)	0.07181 ± (0.002)	+2.12
Fe(II)	$FeCl_2.4\ H_20$	0.07032 ± (0.001)	0.06854 ± (0.003)	-2.53

a mean ± SD (n=3)

Table 2 Effect of various ions on the determination of 1 μgmL^{-1} nitrite

As we can see from Table 2 none of them have a considerable effect on the determination of analyte in one to one ratios. Taking into account the result obtained, it can be concluded that the proposed application of the official method [58] is fairly selective for the determination of nitrite in tap water and river water samples.

4.5 Application in Real Samples

The present application official method was applied to the determination of nitrite in tap and river water samples.

4.5.1 Physico-chemical analysis

The sampling technique used to collect the river water samples was grap sampling, since grab sampling reflects the condition only at the point in time that the sample was collected, with respect to some unstable parameters like pH and temperature. The determination of such unstable parameters at the time of sampling is mandatory.

Temperature reading was made on site before sampling and the pH measurement was made in the laboratory after the samples collected at 11.00 a.m around the 4th week of October 2008 and transported to the laboratory. Mean values for temperature and pH measurements shown below.

Sample	pH	Temperature (^{0}C)	Time of Sampling (a.m)
Tap water	7.41	20	11.00
Little Akaki river	8.26	20.5	11.00
Big Akaki river	7.86	20	11.00

Table 3 Physico-chemical analysis result of tap and river water samples.

Results shown in Table 3 imply that the temperature measured at the time of sampling and pH reading taken after the sample was collected and transported to the laboratory were favorable for nitrite analysis.

4.5.2 Determination of nitrite in tap and river water samples

Samples	Nitrite found per 10 mL in μgmL^{-1}	% RSD
Tap water	N.D	_
Little Akaki river	2.45 ± 0.02	0.8
Big Akaki river	1.2 ± 0.02	1.6

mean ± SD (n=3)

Table 4 Results obtained in real sample analysis

Results of Table 4 show that tap water samples gave no test result because of the method detection limit but the nitrite in river water samples is determined with acceptable precision (0.8% - 1.6%).

The possible reason for large concentration of nitrite in Little Akaki River, unlike Big Akaki River, the western parts of Addis Ababa industrial states (like food and beverage factories) are along the Little Akaki River and each factory can pour a lot of chemicals that may contain nitrogenous organic compounds which will potentially converted to nitrate and nitrite by microbes [53, 54, 56].

4.6 Recovery Study

In order to check the accuracy of the method for the determination of nitrite, the recovery of nitrite was checked by spiking 0.5 μgmL^{-1} of nitrite standard solution into tap and river water sample solutions. The results obtained are given in Table 5.

Sample	Amount of NO_2^- added per 25 mL (μgmL^{-1})	Amount of NO_2^- found per 25 mL (μgmL^{-1}) [a]	(%) RSD	(%) Mean Recovery
Tapwater	0.5	0.49 ± (0.01)	2.04	98
Little Akaki	0.5	1.43 ± (0.02)	1.4	98
Big Akaki	0.5	0.92 ± (0.02)	2.2	94

a mean ± SD (n=3)

Table 5 Results of the recovery of 0.5 μgmL^{-1} (final concentration in 25 mL) of nitrite spiked in sample solutions.

Results of Table 5 show that the recoveries obtained in all samples are appreciable (94% - 98%). Hence it can be concluded that the method is valid to be applied in tap and river water samples.

The amount of reagents used and time needed for color development in the current application of an official method was compared with the values provided by the reference APHA method [58] and results are shown in Tables 6 and 7.

Reagents	Present application	APHA method	Difference (%)
SULPHA	0.05% (m/v)	0.04% (m/v)	+25
NEDA	0.002% (m/v)	0.004% (m/v)	-50
Phosphoric acid	0.77% (v/v)	0.38% (v/v)	+ 100

Table 6 Results of comparison of reagent concentrations used in the present application with the values provided by the APHA official method

Parameters	Present application	APHA method
Time needed for color development	30 min -120 min	10 min -120 min
Molar absorbitivity coefficient ($Lmol^{-1}cm^{-1}$)	3.33×10^3	4.0×10^4

Table 7 Results of comparison of time needed for complete color development and molar absorbitivity values with the APHA official method

Results of table 6 and 7 show that the amount of reagents used by the application of an official method are in good agreement for SULPHA and NEDA values with relative difference (+25% and -50%), respectively from the values provided by the APHA official method, whereas the value phosphoric acid used by the current application show large difference from the standard method by 100%.

The possible reason for such large difference of phosphoric acid evaluated in the present application from the values provided by the APHA official method is due to the pH effect of the medium and the reaction temperature which are not controlled in the present application. As it is reported in literatures [39,60, 63, 64] many of these methods quite often required a precise control of pH and reaction temperature during diazotization as well as in the coupling steps.

On the other hand, the value of molar absorbitivity coefficient calculated show 11 times smaller than the value provided by the APHA official method but the time needed for complete color development is nearly the same with those values provided by the APHA official method.

Analytical characteristics of the present application of an official method was also compared with those of the previously reported spectrophotometric methods that had been applied for the determination of nitrite in tap and river water samples, the result obtained are shown in Table 8.

Reagents	Linear range (μgmL^{-1})	Detection limits (μgmL^{-1})	RSD (%)	Reference
p- Nitroanilin +Diphenylamine	0.05 - 0.8	0.01	2.97 (n = 5)	60[a]
Thionin [3,7 diamino-5-phenothiazinium acetate]	0.025 - 0.5	0.007	0.8 (n = 10)	61 [b]
P-Amino phenyl mercaptoacetic acid +NEDA	0.02 - 0.80	0.01	0.61 (n = 9)	62[c]
SULPHA +NEDA	0.05 - 25	0.01	2.0 (n =10)	64[d]
SULPHA +NEDA	0.25 - 1.5	0.08	2.41 (n =10)	Present application

Table 8 Comparison of the present application of an official method with some of the reported methods

a for the determination of 0.3 μgmL^{-1} NO_2^-

b for the determination of 0.1 μgmL^{-1} NO_2^-

c for the determination of 0.4 μgmL^{-1} NO_2^-

d for the determination of 1 μgmL^{-1} NO_2^-

A comparison study shows that only the *precision* for the determination of nitrite in the present application of an official method is almost comparable with results obtained by other spectrophotometric methods whereas the linear range obtained is narrower and the *detection limit* also 8 - 10 times higher than reported values the other similar methods.

The possible reason for such differences evaluated in the present application might be either due to errors in the sample preparation procedures, pH effect of the medium or more importantly the decomposition of diazonium ion prior for coupling. As it is reported in many literatures [9, 39, 49,] one of the major draw back to the Griess azo method is the need to exercise control over the reaction conditions in order to prevent or at list minimize decomposition of the diazonium salt

prior to coupling. Controlling pH of the medium, the reaction temperature and Premixing of reagents will largely solve such differences found in the present application of an official method.

5. Conclusion

The APHA method for the determination of nitrite was validated for factors such as reagents concentration and the time needed for complete color development. *Precision* (**RSD**) obtained from replicate analysis (n =10) of 1 μgmL^{-1} NO_2^- standard solution was 2.46% and results obtained from recovery analysis was 94% and 96%. Therefore the *precision* and *accuracy* obtained in the present application of an official method appear to be satisfactory. But results obtained from various comparison studies show that the amount of reagents used by the present application and other *analytical performance characteristics* obtained by the present application are not comparable with the values provided by the official APHA standard method.

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