1.1 Introduction

Nitrogen is an element needed by all plants and animals to build protein. As aquatic plants and animals die, bacteria breakdown large protein molecules into ammonia. Ammonia is then oxidized by specialized bacteria to form nitrates and nitrites. These bacteria get energy for metabolism from oxidation. Nitrite is a characteristic pollutant. During the synthesis of nitric oxide from variety of cell types, nitrogen monoxide derives nitrite as a major oxidation product. NO is a free radical and has selective reactivity with other paramagnetic species, including ferrous or ferric iron in haem proteins and other radical species such as superoxide anion ($O_2^-$) resulting in production of nitrite [1-4]. Nitrate is a natural constituent of plants and is found in vegetables at varying levels depending on the amount of fertilizer applied and on other growing conditions. According to the World Health Organization (WHO), most adults ingest 20-70 milligrams of nitrate nitrogen per day with most of this coming from foods like lettuce, celery, beets, and spinach. When foods containing nitrate are eaten as part of a balanced diet the nitrate exposure is not thought to be harmful. High nitrate intake from food has been reported to associate with gastric cancer in some countries due to the formation of N-nitroso compounds in the stomach. Gastric cancer is the third offered leading cause of cancer death in men after lung and prostate cancers and is the fourth leading cause of cancer death in women worldwide [5-7]. Nitrate is an anion with a molecular weight of 62 g/mol. It does not significantly sorbs to aquifer materials under typical sub-surface conditions. Nitrate is non-volatile and is stable under aerobic groundwater conditions. However, under anaerobic conditions it can be converted to other oxides of nitrogen and to molecular nitrogen by the process of denitrification. Denitrification is the reduction of nitrate to nitrite then to NO, $N_2O$ and finally $N_2$ gas. It is almost always a microbial mediated redox process in groundwater and requires the presence of an electron donor (such as biodegradable organic carbon and/or sulphide minerals) [8]. Soil may be defined as the weathered superficial layer of the Earth’s crust that typically is made up of decomposed and partly decomposed parent rock material with associated organic matter in various stages of decomposition. Nutrients for healthy plant growth are divided into three categories: primary, secondary and micronutrients [9]. Nitrite is not a direct toxicant, but may be dangerous in the form of N-nitroso
compounds produced upon its interaction with proteins. In the recent years there has been an increasing concern about the role of nitrite, as important precursors in the formations of N-nitrosamines that possess tetratogenic and mutagenic activities and hence are potential carcinogens [10]. The potential harm to human health and the environment and compounds derived from nitrate is an issue that occasionally awakens media and public concern. Recent studies provide a new understanding of the role of nitrate and nitrite in our body and motivate revision of the long-held view that nitrate poses a health risk. Coincidently, national authorities are performing research, surveys and risk assessments to provide scientific support for the regulations laid down in the 1990s setting limits on nitrate in lettuce and spinach. In addition to the health factor, the economic (market unity) and environmental (agricultural contamination) aspects of the issue are being considered in this legislative work. In order to obtain data of appropriate and comparable quality in this study, a standardized method was needed for analyzing nitrate and nitrite in foodstuffs.

1.2 Occurrence of Nitrate and Nitrite

1.2.1 Nitrate and Nitrite in an Environment

Nitrate is an ubiquitous ion that is found every where: in water, soil, plants, food and the human body. Nitrogen is found in several different forms such as ammonia (NH₃), nitrates (NO₃), and nitrites (NO₂) in terrestrial and aquatic ecosystems. Nitrogen is essential for all living things: animals and plants. Nitrogen forms a part of the proteins and DNA that are found in cells. Animals get nitrogen by eating plants and other animals. Plants get nitrogen from water and from the soil. They get nitrogen by absorbing it in the form of nitrates and ammonium. Nitrates are the major source of nitrogen for aquatic plants. Nitrates are not utilized by aquatic organisms such as fish and aquatic insects, but nitrates are used by aquatic plants.

The nitrogen cycle explains the natural occurrence of nitrate and nitrite in the environment with biological reactions which includes six major process:

1. Assimilation of inorganic ammonia and nitrate by plants and micro-organisms to form organic nitrogen, e.g. amino acids, proteins and nucleic acids.
2. Heterotrophic conversion of organic nitrogen from one organism to another organism in different forms.

3. Ammonification, the decomposition of organic nitrogen to ammonia.

4. Nitrification, the oxidation of ammonia to nitrite and nitrate.

5. Denitrification, the bacterial reduction of nitrate to nitrogen oxides and molecular nitrogen under anoxic conditions.

6. Nitrogen fixation, the reduction of nitrogen gas to ammonia and organic nitrogen by various organisms [11-14].

1.2.2 Exogenous Sources of Nitrate and Nitrite

There are two sources of nitrate and nitrite in the body: exogenous and endogenous. Human exposure to nitrate is mainly from the exogenous source, while exposure to nitrite is mainly endogenous, through nitrate metabolism. The intake of exogenous nitrates and nitrites is mainly via food vegetables and meat products and drinking water.

1.2.2.1 Nitrate and Nitrite as Contaminant in Drinking Water

A contaminant is a substance that is either present in an environment where it does not belong or present at levels that might cause harmful effects to humans or environment [15]. Nitrate is a colorless, odourless, and tasteless compound that is present in some groundwater. Nitrate (NO₃) is a naturally occurring form of nitrogen found in soil. The formation of nitrates is an integral part of the nitrogen cycle in our environment. Plants use nitrates from the soil to satisfy nutrient requirements and may accumulate nitrate in their leaves and stems. Due to its high mobility, nitrate also can leach into groundwater. Nitrates are formed when microorganisms break down fertilizers, decaying plants, manures or other organic residues. Usually plants take up these nitrates, but sometimes rain or irrigation water can leach them into groundwater. Although nitrates occur naturally in soil and water, excess levels of nitrates can be considered to be a contaminant of ground and surface waters. In addition to animal waste, untreated human sewage can contribute to nitrate levels in surface and ground water. The source of excess nitrates can usually be traced to agricultural activities, human wastes, or industrial pollution. Sources of nitrates include wastewater treatment plants, runoff from fertilized
lawns and cropland, failing on-site septic systems, runoff from animal manure storage areas, industrial discharges that contain corrosion inhibitors and N-fixation from atmosphere by legumes, bacteria and lightning.

Water that is polluted with nitrogen-rich organic matter might show low nitrates. Decomposition of the organic matter lowers the dissolved oxygen level, which in turn slows the rate at which ammonia is oxidized to nitrite (NO\(_2\)) and then to nitrate (NO\(_3\)). Under such circumstances, it might be necessary to also monitor for nitrites and ammonia, which are considerably more toxic to aquatic life than nitrate. Nitrate can be expressed as either NO\(_3^-\) (nitrate) or NO\(_3\)-N (nitrate-nitrogen). Nitrate levels above the EPA Maximum Contaminant Level of 10mg NO\(_3\)-N per litre or 45 mg NO\(_3\)-N per litre may cause methemoglobinemia in infants. Nitrates are essential plant nutrients, but in excess amounts they can cause significant water quality problems. Nitrates in excess amounts can accelerate eutrophication, causing dramatic increases in aquatic plant growth and changes in the types of plants and animals that live in the stream. This, in turn, affects dissolved oxygen, temperature, and other indicators. Proper management of fertilizers, manures, and other nitrogen sources can minimize contamination of drinking water supplies [16-19].

1.2.2.2 Nitrate and Nitrite as a Natural Contaminant in Vegetables

A natural contaminant is a chemical present in the environment that is either a part of a food or natural growing conditions or that cannot be controlled by intervention [20]. Nitrate concentration in vegetables depends on the biological properties of the plant culture, lightning conditions, type of soil, temperature, humidity, frequency of plants in the field, plant maturity, vegetation period, harvesting time, size of vegetable unit, storage time and source of nitrogen [21, 22]. The nitrate ion has a low level of acute toxicity, but if transformed into nitrite, it may constitute a health problem. Reduction to nitrite may take place in the presence of bacteria or enzyme nitrate reductase, and in contact with metals. Nitrite is unstable at acidic pH values at which it can disproportionate to yield nitrate and nitrogen oxide and/or react with food components including amines, phenols and thiols [23]. It has been estimated that 5 to 8 % of the nitrate from the diet may be reduced to nitrite by the microflora in the oral cavity [24]. Nitrite in food is primarily considered to be a health problem because its
presence both in food and body may lead to the formation of carcinogenic nitrosamines [25-28] and the clinical symptom of methemoglobinemia [29].

An acceptable daily intake (ADI) of 0 to 3.7 mg kg\(^{-1}\) body weight for nitrate and of 0 to 0.06 mg kg\(^{-1}\) body weight for nitrite has been established by EU Scientific Committee for Food [30]. It has been estimated that vegetables constitute a major source of human exposure to nitrates contributing approximately 80 to 92 % of the average daily intake [31, 32].

1.2.2.3 Nitrate and Nitrite as a Contaminant in Soil

As a part of nitrogen cycle, there are many sources of nitrate addition to soil, but modern agricultural practices are primarily responsible for nitrate accumulation in soils. About 260 million tones of atmospheric nitrogen is being fixed every year globally. The global mean N use efficiency is estimated to be about 50 % [33, 34]. The remaining quantity of nitrogen is lost into the environment. Nitrates also come from the Earth. Soil contains organic matter, which contains nitrogen compounds. Just like the ammonia in water, these nitrogen compounds in the soil are converted by bacteria into nitrates which, being soluble in water and not retained by soils, gets leached into water bodies. Leaching of nitrate from agricultural land and from other sources to groundwater is a global phenomenon. Leaching of nitrates from agriculture land has been a major research focus in the past two decades [35]. When nitrogenous fertilizers are applied to soils, they are rapidly converted to nitrate form, which are readily available to plants, but are highly soluble and hence easily leachable. When quantity of externally added nitrogen exceeds the amount that crops can use, the excess nitrate leaches out from the root zone by water percolating through the soil profile and ultimately accumulates into the groundwater resulting in water pollution. Nitrogen fertilizers have been applied to yards, fields, golf courses to promote the growth of plants. Rainwater can wash nitrates in the fertilizer into streams and rivers or the nitrates can seep into ground water. This can also occur with animal waste and manure. Nitrates which act as nutrients from land sources end up in rivers and streams more quickly than other nutrients like phosphorus. This is because they dissolve in water more readily than phosphates, which have an attraction for soil particles. As a result, nitrates serve as a better indicator of the possibility of a source of sewage or manure pollution during dry weather. Nitrate enrichment of groundwater is a
function of many factors such as soil characteristics, particularly its hydraulic
conductivity, nature of crops grown, their rooting behavior, quantity of fertilizer use,
depth of aquifers, magnitude of rainfall/irrigation water received etc. [36]. Many studies
across the world such as China [37], Saudi Arabia [38], Morocco [39], Iran [40], Turkey
[41] and India [42, 43] have reported high levels of nitrates in groundwater, originating
mainly from intensive agriculture areas where use of nitrogenous fertilizers is very high.
Modern agricultural practices are the biggest contributor of nitrate pollution.

1.2.2.4 Nitrate and Nitrite as a Constituent in Pharmaceutical Preparations

Nitrate and nitrite intake can play a valuable role in reducing blood pressure for
those following the well-regarded Dietary Approaches to stop Hypertension (DASH)
diet. About 93% of human dietary nitrate comes from vegetables and saliva. As such, the
dietary provision of nitrates and nitrites from vegetables and fruit may contribute to the
blood pressure-lowering effects of the DASH diet. Consumption of nitrate and nitrite
considered as nutrients present in plant foods and thus beneficial to health as they may
lower the risk of developing cancer [44]. Amyl nitrite (amyl nitrite inhalant) is a rapidly
acting vasodilator administered by inhalation. Poppers are a class of chemicals called
alkyl nitrates. These are chemical compounds of structure R–ONO. In more formal terms,
they are alkyl esters of nitrous acid. Amyl nitrite, butyl nitrite, isobutyl nitrite, cyclohexyl
nitrite, pentyl nitrite etc under the class of alkyl nitrates called as poppers used as a
powerful drugs. First produced amyl nitrite in 1857 was used in the treatment of angina.
The vapour released from amyl nitrite causes veins and arteries to dilate resulting in the
blood flowing faster through the heart and brain. Nitrates open (dilate) the arteries to the
heart. This increases blood flow to the heart, relieving angina symptoms such as chest
pain or pressure. Few examples of nitrate drugs are isosorbide dinitrate, isosorbide
mononitrate and nitroglycerin. Nitroglycerin for angina is taken as a pill or a liquid spray.
Skin patches or paste are also available to prevent angina [45, 46].

1.2.3 Endogenous Sources of Nitrate and Nitrite

Nitrate in vegetables like spinach, lettuce and beets are converted to nitrite in the
mouth. Nitrite is used as a curing ingredient in meat to stabilize color and flavor and to
prevent rancidity. However, cured meats contribute less than five percent of total human
nitrite intake. Nitrites are produced endogenously through the oxidation of nitric oxide and through a reduction of nitrate by commensal bacteria in the mouth and gastrointestinal tract. The endogenous formation of nitrite mainly occurs via reduction of dietary nitrate through microbial action of saliva in the mouth.

\[
\text{NO}_3^- + 2 \text{e}^- + 2 \text{H}^+ \xrightarrow{\text{nitr}ate \text{ reductase}} \text{NO}_2^- + \text{H}_2\text{O}
\]

In plasma or other physiological fluids or buffers NO is oxidized almost completely to nitrite, where it remains stable for several hours [47, 48]. The oxidation of NO by molecular oxygen is second order with respect to NO:

\[
\begin{align*}
2\text{NO} + \text{O}_2 & \rightarrow 2\text{NO}_2 \\
2\text{NO} + 2\text{NO}_2 & \rightarrow 2\text{N}_2\text{O}_3 \\
2\text{N}_2\text{O}_3 + 2\text{H}_2\text{O} & \rightarrow 4\text{NO}_2^- + 4\text{H}^+
\end{align*}
\]

The \(\text{NO}_2^-\) derived from NO autoxidation is rapidly converted to \(\text{NO}_3^-\) via its oxidation by certain oxyhemoproteins (P-Fe\(^{2+}\)O\(_2\)) such as oxyhemoglobin or oxymyoglobin.

\[
\begin{align*}
2\text{P-Fe}^{2+}\text{O}_2 + 3\text{NO}_2^- + 2\text{H}^+ & \rightarrow 2\text{P-Fe}^{3+} + 3\text{NO}_3^- + \text{H}_2\text{O} \\
4\text{P-Fe}^{2+}\text{O}_2 + 4\text{NO}_2^- + 4\text{H}^+ & \rightarrow 4\text{P-Fe}^{3+} + 4\text{NO}_3^- + \text{O}_2 + 2\text{H}_2\text{O}
\end{align*}
\]

NO would react directly and very rapidly with oxyhemoproteins (P-Fe\(^{2+}\)O\(_2\)) to yield \(\text{NO}_3^-\) before it has an opportunity to autoxidize to \(\text{NO}_2^-\) as described above in reactions 1-3.

\[
\text{P-Fe}^{2+}\text{O}_2 + \text{NO} \rightarrow \text{P-Fe}^{3+} + \text{NO}_3^-
\]

Therefore detection and quantification of nitrite and nitrate provides an index of NO bioavailability or production [49]. The fate of nitrate and nitrite in environment is complex. It is plausible that many other biochemical reactions take place in the human body when nitrate and nitrite are consumed in the diet as a contaminant (drinking water), natural contaminant (vegetables) [50].

1.3 Effect of Nitrate and Nitrite Pollution on Human Health

In moderate amounts, nitrate is a harmless constituent of food and water. Human health is affected by high concentration of nitrate and nitrite. Nitrates can interfere with the ability of red blood cells to carry oxygen. Babies can turn blue when there is not
enough oxygen being transported by their blood. This "blue baby syndrome" (technically known as methemoglobinemia) is a serious condition that can cause brain damage or death. Hypotoxic signs may develop at about 20%, while death occurs at 50% methHb or higher. Drinking water and vegetables are the major sources of nitrate consumed by human. The stomach acid of an infant is not strong thus causes an increase in bacteria that can readily convert nitrate to nitrite (NO\textsubscript{2}). Pregnant women, adults with reduced stomach acidity, and people deficient in the enzyme that changes methemoglobin back to normal hemoglobin are all susceptible to nitrite-induced methemoglobinemia and may cause birth defects and miscarriages. Nitrates are colorless and odorless, so their presence cannot be determined without the use of special testing equipment.

Nitrite reacts in stomach with nitrosable compounds to form N-nitroso compounds. These compounds have been found to be carcinogenic. The US National Research Council found an association between high nitrate intake and gastric and esophageal cancer, bolder cancer and stomach cancer. High levels of nitrate were also linked with the infection with the diabetes, the occurring of some birth defects, and miscarriages. Healthy adults can consume fairly large amounts of nitrate with few known health effects. In fact, most of the nitrate we consume is from our diets, particularly from raw or cooked vegetables. This nitrate is readily absorbed and excreted in the urine. However, prolonged intake of high levels of nitrate is linked to gastric problems due to the formations of nitrosamines. N-nitrosamine compounds have been shown to cause cancer in test animals. Studies of people exposed to high levels of nitrate or nitrite have not provided convincing evidence of an increased risk of cancer [51-53].

1.4 Objectives and Statement of Work

The main objectives of the research work include-

4. Application of the developed methods for estimation of nitrate and nitrite in water and complex sample matrices such as soil, vegetables and pharmaceutical preparations.


1.5 Research Plan

The plan of research work included:

- Detailed literature survey of existing non-instrumental and instrumental methods used for estimation of nitrates and nitrates.

- Development and validation UV-VIS spectrophotometric method for simultaneous estimation of nitrate and nitrite by with respect to following points:
  i) Study of spectra and selection of wavelengths
  ii) Verification of Beer-Lambert law for both the ions
  iii) Optimization of the analysis conditions for various parameters such as wavelength of measurement, pH of buffering media, polarity of mobile phase, column conditions and mobile phase flow rate etc.
  iv) Analysis of laboratory mixtures by this method
  v) Validation of the method with respect to various parameters such as accuracy, precision, specificity, limit of detection and limit of quantization, linearity and range, ruggedness and robustness etc. as per the ICH guidelines
  vi) Analysis of complex samples

- Development and validation of analytical methods for simultaneous estimation of nitrate and nitrite by reversed phase-HPLC and ion chromatography methods with respect to following points:
  i) Selection of suitable stationary and mobile phases
  ii) Optimization of chromatographic parameters
  iii) Analysis of laboratory mixtures by these methods
  iv) Validation of the method with respect to various parameters such as accuracy, precision, specificity, limit of detection and limit of quantization, linearity and range, ruggedness and robustness etc. as per the ICH guidelines.
v) Analysis of water and complex environmental samples

- Comparative study of various developed methods with standard reference method with respect to the following parameters.
  i) Sensitivity
  ii) Selectivity.

- Statistical analysis of the data generated with respect to following statistical tests using computer aided software.
  i) Averages and Standard deviations
  ii) Student-t test

1.6 Significance of Study

Nitrate and nitrite gives short term and long term health hazards in human and other animals. Major exposure occurs through food and water which is very difficult to avoid. Hence, monitoring of these ions in various samples is very much important to decide the preventive strategies for protecting the common populations from their adverse effects. Spectrophotometric or colorimetric methods available for estimation of nitrates are relatively more simple, rapid, inexpensive and sensitive but less specific. Majority of the classical methods are much more time-consuming and laborious than ion chromatography, and sometimes require the use of expensive and toxic reagents. The main advantages of HPLC methods includes the short time needed for analyses, possibility of analysis of small volume samples, high sensitivity and selectivity, and most importantly- a possibility of simultaneous separation and determination of ions, which provides more detailed information of the sample studied. The selection of proper column, use of aqueous and non-toxic eluents, use of proper buffering media etc. are important for chromatographic method development. The chromatographic method may be used as a reference method for other methods.

Ion chromatography is a modern method of analysis and its applications have been extended to new analytes and samples. Basically, ion chromatography was designed for ion analyses in water, now a day it is possible to apply it for analysis of complex samples such as foods, drugs and biological samples due to development in sample pretreatment procedures and enhanced detection system. Ion chromatography is a method applied for
ion separation and estimation. It is also applied as a reference standard for evaluation of sensitivity, repeatability and efficiency of other routine methods used for analysis of ions. Generally, nitrate and nitrite contents are determined using ion chromatography with conductometric or UV detection. The sensitivity is less with these detectors for direct estimation of nitrates and nitrites. The sensitivity and selectivity of such estimation can be improved by post column and precolumn derivatization methods. e.g. Tri-iodates of nitrites for post column derivatization and 2,3-diaminonaphthalene for precolumn derivatization. The sensitivity increases up to few μg/L and also interference due to chlorides is reduced as chlorines are not oxidized by iodides.

1.7 Structure of the Thesis

The bibliographic references in the text are cited by numerals in the square brackets running consecutively through the text. The full list of the references is mentioned at the end of each chapter. The description of this thesis is based on the papers published in national and international journals. These research papers have been edited to provide uniform format and notation throughout the thesis. However, for practical reasons, the original organization of the references is maintained.

The thesis entitled 'Development and Validation of Analytical Methods for Simultaneous Estimation of Nitrate and Nitrite in Complex Sample Matrices' consists of six chapters and embodies an account of:

Chapter 1: Introduction

This chapter provides the introduction of the thesis and provides a brief overview of its context. The main objective of this chapter is putting light on the sources of nitrate and nitrite in environment and effect of nitrate and nitrite pollution on public health. The chemical reactions governed by nitrate and nitrite during its cycling through the environment are described. The significance of the work undertaken and its relevance is discussed. The objectives of the work undertaken are listed at the end of the chapter.

Chapter 2: Background of Study

This chapter includes literature survey on nitrate and nitrite pollution and its adverse health effects. It provides justification to the work and produces a substantiating
background to the study. A brief review of available methods for estimation of these ions with the advantages and limitations of these methods is described. It also covers the theoretical background of the instrumental methods used for estimation of nitrate and nitrite from complex sample matrices. The general steps involved in development and validation of an analytical method are described. The short summary concerning to the present probe in a relegated manner is presented as a background literature.

Chapter 3: Spectrophotometric Determination of Nitrate and Nitrite

This chapter describes the formation of azo dye using mosapride and metoclopramide reagents when coupled with ethyl acetoacetate. A set of optimum conditions for the determination of nitrate and nitrite using mosapride and metoclopramide are obtained from this work and applied for the spectrophotometric determination of nitrate and nitrite from the synthetic samples.

Chapter 4: Liquid Chromatographic Determination of Nitrate and Nitrite

This chapter comprises the development of HPLC method. A reversed phase HPLC method has been developed for the analysis of nitrite and nitrate in various samples. The study was focused on the selection and optimization of reversed phase HPLC conditions, the demonstration of method performance (sensitivity, accuracy and precision) and the investigation of interferences from common inorganic anions. The effect of mobile phase, pH and flow rate variation on nitrite and nitrate separation has been studied. The parameters were optimized for proper resolution of nitrite and nitrate. A set of optimum resolution conditions for the determination of nitrate and nitrite are obtained from this work and the same are applied for the determination of nitrate and nitrite from the synthetic samples and complex environmental sample matrices.

Chapter 5: Ion chromatographic Determination of Nitrate and Nitrite

This chapter deals with the development of ion chromatographic method. The Ion chromatography method was developed using potassium hydroxide as an eluent and was applied for the separation of nitrite and nitrate under study from real samples such as water and vegetables. By comparing the data obtained from a sample to that obtained from the known standard, sample ions were identified and quantitated. The data was
collected using a computer running chromatography software. The results obtained are discussed and justified. The results are compared with the results obtained by the previous researchers working in the same field.

Chapter 6: Summary and Conclusion
This chapter contains the summary of the whole thesis and the conclusions which are drawn from the comparison of methods developed for the estimation of nitrate and nitrite. Adequate numbers of appropriate references regarding the study are cited at the end of each chapter.
References:


