DETERMINATION OF NITRATE IN ENVIRONMENTAL MATRICES AND FOOD PRODUCTS BY REVERSED PHASE ION PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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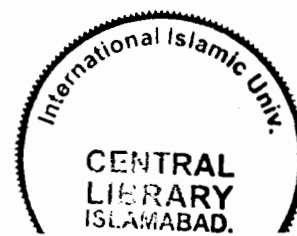
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DATA ENTERED

Environmental Matrices

بسم الله الرحمن الرحيم

Dedicated to

My Parents

"Whose blissful prayers enabled me to complete this uphill task"

Determination of Nitrate in Environmental Matrices and food products by reserved phase Ion Pair High Performance liquid chromatography

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(Acceptance by the Viva Voce Committee)

Title of the Thesis: <u>Determination of Nitrate in Environmental Matrices and Food Products by</u>

<u>Reversed Phase Ion Pair High Performance Liquid Chromatography</u>

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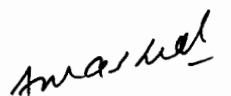
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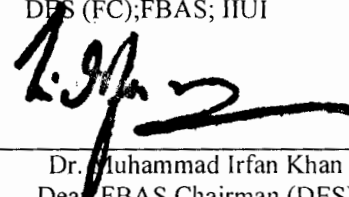DES(FC);FBAS; IIUI

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DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

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Date 15 June, 2012.

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FORWARDING SHEET

The thesis entitled Determination of Nitrate in Environmental matrices and food products by Reversed Phase Ion pair High performance Liquid Chromatography submitted by Adila Hilal in partial fulfillment of MS in Environmental Science has been completed under my guidance and supervision. I am satisfied with the quality of student's research work and allow her to submit this thesis, for further processes per IIU rules and regulations.

Dr. Tahira Sultana

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ABSTRACT

The potential harm to human health and environment caused by compounds derived from nitrate is an issue that occasionally awakens media and public concern. Recent studies provide a new understanding on the role of nitrate in the environment and its harmful effects. This motivates long-held view that nitrate poses a health risk. The present study aimed to determine nitrate concentration in environmental matrices and food products. A simple and sensitive Reversed Phase Ion Pair High Performance Liquid Chromatography (RP-IP- HPLC) method for the determination of nitrate has been developed.

The determination of nitrate concentration was carried out on HPLC (Waters) coupled with UV detector, using reversed phase chromatography technique. Investigations were performed using mobile phase comprising 80% tetra-ethyl ammonium chloride (6.0 mM) and 20 % pure acetonitrile (HPLC grade). 4.0 mM sodium dihydrogen phosphate was used as a buffering reagent. 0.5M phosphoric acid (as per requirement) was used to maintain pH of mobile phase at pH 3.0. Separation was done by reversed phase C18 column (150mm 4.5mm i.d., 5µm particle size, waters, Canada) with flow rate of 0.5mL/min at optimized wavelength of 210 nm in isocratic mode. Nitrate peak was eluted at 3.2 minutes.

 A total of ninety samples (water and food samples) were analyzed consisting of twenty three rainwater samples (from Rawalpindi, Islamabad and surrounding), thirty groundwater samples from Rawalpindi and Islamabad cities, twelve surface-water samples from three lakes (Rawal, Simly and Khanpur), twelve vegetables (spinach and cabbage) and thirteen meat samples (beef, mutton, chicken and fish). The results indicated that nitrate concentration in Rawalpindi ground-water was higher than Islamabad, possibly due to infiltration of Nala Lei and other waste drains in the nearby tube wells. On the other hand the lake samples had lower concentration of nitrate. Most of the rain water had nitrate concentration in the range of 0-0.5 mg/L. Rain water collected from Rawalpindi and some of its surrounding areas had high nitrate concentration particularly in the areas of high traffic load.

As nitrate in vegetables accumulates in different parts so in spinach nitrate concentration in stem and leaves were analyzed separately to compare nitrate

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concentration in these parts. Nitrate concentration in cabbage was lower than spinach which may be due to difference in physiology, fertilizer application and their uptake, sun light duration and intensity. Nitrate in spinach stem was higher as compared to its leaves.

There was some variation in concentration of nitrate in raw (61.5-669 mg/L) and cured meat products (84.1-558.5mg/L). Highest nitrate concentration was observed in the fish collected from Rawal dam. The study led to the realization that advocacy efforts for the awareness and education of the communities regarding the nitrate contamination, health hazards and ways to prevent drinking water and food products contamination as well as detailed monitoring and mitigation activities are highly recommended to safeguard the natives from the possible potential hazards.

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SYMBOLS AND ABBREVIATIONS

Symbol/Abb.	Description
Atm.	Atmosphere
Cl^-	Chloride ions
CO_2	Carbon dioxide
C	Cabbage
DO	Dissolved oxygen
EC	Electrical conductivity
Gw	Ground-water
H	Hydrogen
HNO_3	Nitric Acid
L	Litre
Lat.	Latitude
Long.	Longitude
M	Molarity
Meq	Milli equivalent
mS	Milli Siemens
N	Nitrogen
NaOH	Sodium hydroxide
NO_3^{-2}	Nitrate Ions
Org.	Organic
Ppm	parts per million
Ppb	parts per billion
Rw	Rain-water
Sl	Spinach Leave
SO_4	Sulphate ion
Ss	Spinach Stem
Std.	Standard
Sw	Surface-water
TDS	Total Dissolved Salts
%	Percent
^oC	Degree Celsius

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CHAPTER # 1
INTRODUCTION

1 INTRODUCTION

Pollution is one of the most important global issues that has attained a global attention, it effects all life aspects and is great threat to biodiversity. After industrial revolution the pollution problem arose and still getting worse day by day (Abu-Dayea, 2006).

Today there have been concerns at regional and global level over inorganic, organic anions and heavy metal pollution in environment. River basins where the intensive farming and industrial zones are located are the main sources of contamination, contributing pollution through industrial wastes, municipal, landfills and agriculture chemicals. These contaminants are polluting ground-water as well as drinking water. Therefore the correlation between these pollutants and their pollution sources could lead to suggest some ways to prevent their flow through environment (Neal & Harrow, 2000).

Anions like fluoride, chloride, nitrite, bromide, nitrate and sulfate are important water quality indicators. They are essential elements for aquatic and terrestrial biota. These ions can be present in rivers naturally as a result of dissolution of geological deposits, biological degradation of organic matter and seawater intrusion in coastal areas. These sources include surface runoff generated from urban and agricultural lands, excessive application of chemical fertilizers, waste discharges, industrial and septic tank effluents, landfill leakages and irrigation drainage (Galloway, 1998 ; Meybeck, 1998 Malmqvist and Rundle, 2002; Mayer *et al.*, 2002; Wakida, 2005).

Approximately 78% of earth's atmosphere contains nitrogen (Steven *et al.,* 2004), that is not useful for living organisms and most of the ecosystems have shortage of useable nitrogen. Global nitrogen cycle is badly affected by human activities like fossil fuel combustion, use of artificial nitrogen fertilizers and release of nitrogen in wastewater.

Nitrate ions exist in almost all environmental matrices; they are produced as a result of the oxidation of nitrogen by micro-organisms in plants, water and soil and to small extent by electrical discharges (lightning) in air.

Air pollutants like Sulfur dioxide (SO_2) and Nitrogen dioxide (NO_2) present in upper layers of troposphere when mix and transport over the land causes acid rain. They

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reach upper layers of the troposphere; react with oxygen and water to produce sulphuric acid (H_2SO_4) and nitric acid as shown in following reactions:

$$SO_2 + \tfrac{1}{2}\,O_2 + H_2O \longrightarrow 2H_2SO_4$$

$$2NO_2 + \tfrac{1}{2}\,O_2 + H_2O \longrightarrow 2HNO_3$$
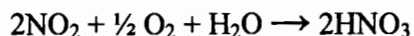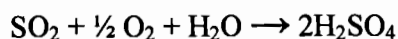
This acid rain can have a series of effects such as direct phytotoxicity , destruction of forests, acidification of lake water which effect water life, corrosion to metal structures and other exposed materials. (Pettergrew & Gordan, 2001).

Almost all surface-waters show a low nitrate concentration which is below permissible limits and may not cause any public health hazard. There exists a direct link between surface-water nitrate concentration and agriculture activities. Cropland and livestock contributions vary in different surface-waters for nitrate concentration. In the case of livestock, better waste management can minimize nitrate significantly, but cropland situation is slightly complex due to excessive nitrogen fertilizer use.

 Rainfall, decomposition of soil organic matter and nitrogen amendments (fertilizers, manures, etc.) are three main primary sources that contribute nitrate in surface waters. If the crops do not remove sufficient amount of soil nitrate, the excessive nitrate moves into surface-water after harvesting or when no growing roots are present there to intercept excessive nitrogen.

Nitrate-containing compounds present in the soil are generally soluble and mobile, so readily migrate into ground-water. Polluted water wells may also contain elevated concentration of nitrate due to insufficient sewage treatment plants. The World Health Organization (WHO) guideline of 50 mg/L of nitrate is protective for bottle-fed infants and population of different age groups (WHO, 2008). When nitrate concentration in drinking water exceeds 50 mg/L, water can substitute vegetables as the major source of total nitrate intake in population diet.

Percolating water take excess nitrate down the soil profile with leaching of nitrate which is more likely to occur in sandy soils, but it takes place in fine textured soils also (Jones & Schwab, 1993). In most of the studies, nitrate concentration in ground-water exceeded the permissible limit of 45 mg/L. Three fourth of world population lives in developing countries like Pakistan where an alarming threat of ground-water pollution by nitrate exists (Bijay *et al.,* 1994).

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The concentration of nitrate in vegetables depends on genetic factors, environmental variables (season, light, temperature, etc.) and agricultural practices (Maynard *et al.,* 1976).The nitrate concentration of vegetables is affected by a number of factors such as variety, fertilizer, soil conditions and maturity. Human exposure to nitrate is ten percent from cured meat and rest of ninety percent is from vegetables and other sources. Some studies claim that people normally consume more nitrates from their vegetable intake than from the cured meat. High concentrations of nitrate are observed in spinach, beet, radish, celery, and cabbage (Addiscot, 1991). However, most vegetables usually have low concentration of nitrate, with leafy vegetables clearly having the highest concentration (Prasad & Chetty, 2008).Very high concentration (over 5000 mg/g) of nitrate in leafy vegetables has been observed in different places such as Mainland China as well as various countries in Europe. The potential hazard of nitrate for consumers mainly associated with the generation of carcinogenic N-nitrosamines.WHO has recommended a minimum of 400 g intake of fruits and vegetables per day for the prevention of chronic diseases (WHO, 2004).

Nitrate concentration in vegetables has been studied in different parts of world. In India 30 to 270 mg/g nitrate is present in leafy vegetables and 31 to 2043 mg/g in roots and tubers (Usha *et al.,* 1993). In China, the concentration of nitrate in different vegetables (Chinese cabbage, fresh spinach, leek, frozen spinach, white cabbage and potatoes) are observed in range of 140.6–2762.5 mg/g, while the total intake of nitrate and nitrite was estimated to be 61 and 0.5 mg day^{-1} respectively (Annette & Soren, 1999). In Poland, maximum acceptable limit of nitrate concentration exceeded in 8.2% of radish and 65% of lettuce samples (Josef *et al.,* 1999). It has been documented that the nitrate concentration in greenhouse vegetables are greater than in field-grown vegetables.

Since ancient time it was common practice to preserved meat with salt to prevent its decomposition which may be due to such types of bacteria that are involved in the process of meat spoilage. Use of preservative salts for meat curing produced a pink color and special flavor in meat. This is the same effect of preservatives seen in cured meats today. In the beginning of twentieth century it was observed that nitrate present in some of the salt produces this special color and flavor. It was proved later on, that actually nitrate is changed to nitrite which changes the color of meat.

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Nitrates are usually mixed with meat binders in the form of sodium and potassium salts (ex: sodium nitrate sodium nitrite, potassium nitrate and potassium nitrite) (Hyytia *et al.*, 1997).

Nitrites give a color reaction in the meat and an appealing pink color to cooked products. With no nitrates/nitrites, meat products give brown or gray color. Nitrates normally undergo a chemical reaction and are converted to nitrites which react with the protein (myoglobin) of the meat and are converted to nitrosomyoglobin (bright red). On cooking nitrosomyoglobin converts into pink colored nitrosohaemochrome, this bright pink color is seen in cured meat such as bologna, wieners and ham.

Drinking water is second largest source of nitrate in the diet after vegetables (Knobeloch *et al.*, 2000). Risk of thyroid cancer is increased if average nitrate concentration in public water supplies is higher and with longer consumption of water exceeding 5 mg/L nitrate-N.

There are two sources of nitrate induction in the body, endogenous and exogenous. In humans, nitrates are produced by a series of biochemical reactions inside the body. The non-essential amino acid L-arginine is oxidized by molecular oxygen in the presence of the enzyme NO synthase to L-citruline and nitric oxide. Once the nitric oxide is formed, there are numerous reactions involving radicals, enzymes, oxyhaemoglobin, myoglobin, auto-oxidation, etc, which lead to the formation of nitrate (Leaft *et al.*, 1989). Nitrate is also formed by the oxidation of nitric oxide in the presence of oxy-haemoglobin.

1.1 Toxic Effects of Nitrate

The toxicity of nitrate to human beings is considered to be the result of its reduction to nitrite (Gulis *et al.*, 2002). In a study carried out at California a direct link between maternal preconception exposure to nitrate from drinking water and as well as an enhanced risk for encephaly was indicated (Coren *et al.*, 2001). An earlier Australian study also showed an association between nitrate and neural tube defects (Dorsch, 1984). In 1986 a study observed that in USA, greater number of ground-water samples showed higher concentration of nitrate than that of surface-water (WHO, 1996).

Met-haemoglobinemia is sometimes misdiagnosed as cause of sudden infant death syndrome, which contributes to the national infant death rate statistics in USA. In

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1950 a research listed 144 cases of infant met-haemoglobinemia with 14 deaths in one month period in Minnesota(Gupta *et al.,* 2001). Nitrate concentration in water and food commodities increase above permissible limits set by legislation, can cause serious threats of met-haemoglobinemia, pregnancy complication carcinogenic effects (WHO, 2008; Ward *et al.,* 2010). According to some studies a link among high nitrate ingestion, met-haemogloinemia and pathological changes in bronchi and lung parenchyma is seen (Gupta *et al.,* 2001).

Other health problems linked to nitrate toxicity include oral cancer (Badawi *et al.,* 1998) as nitrate can be reduced endogenously to nitrite with in human body. Nitrite can be further reduced to N-nitroso compounds, which are potential cancer causing chemicals. Reduced casein digestion (Jan *et al.,* 1998), multiple sclerosis (Govannoni *et al.,* 1997) cytogenesis effect in children (Aspasia *et al.,* 1996), non- Hodgkin's lymphoma (Michal *et al.,* 1998) and hypertrophy of thyroid (Van *et al.,* 1992) are some other health problems related with nitrate ingestion. Nitrate is a potential risk factor in environment for human health specifically, its contribution through drinking water and vegetables.

Nitrate is manufactured in mammals by the oxidation of nitric oxide which has potential for disinfecting the food (Benjamin, 2000).

1.2 Contextual frame work of Nitrate at international level

Moreno *et al.,* (2010) validated method for the determination of chloride, nitrate and sulphate in potable drinking water and non treated water. Neal *et al.,* (2007) determined bromide, chloride, Fluoride, nitrate and sulphate by Ion chromatography for rain fall, cloud water and stream waters in the plynlimon experimental catchment of midwales. Zuo *et al.,* (2006) developed a sensitive, simple, fast and accurate RP-IP HPLC, method for determination of nitrate and nitrite in atmospheric liquids and lakes waters. Michalski & Kurzyca (2006) described a method for simultaneous determination of nitrate and nitrite by high performance liquid chromatography in tap water, lettuce and apple tree leaves. Chou *et al.,* (2003) described a simple, sensitive rapid and precise HPLC method using an UV detector for the determination of nitrate and nitrite in vegetables.

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Connolly & Paul (2001) developed a simple ion interaction chromatography for fast determination of nitrate and nitrite at trace concentrations in water samples. Blanco *et al.*, (1995) described a method for simultaneous determination of nitrate and nitrite with HPLC in tap water, lettuce and apple tree leaves.

Sanderson *et al.*, (1991) compared HPLC with cadmium reduction-Gries method. Nitrate and nitrite were measured in fresh and cured meat that HPLC can perform indirect spectrophotometric detection in rain-water to detect and identify the nitrate source.

Radojevic, (1986) determined measurement of nitrate in urban rainwater collected in the U.K with reference to measurement in Japan and agreeing that nitrate is only a minor species in rain-water, being represent $0.012-0.181 \mu gmL$ (~1% of nitrate).

1.3 Contextual frame work of Nitrate at National level

In major cities of Pakistan like Islamabad, Karachi and Quetta etc. the problem of potable drinking water supply has become a serious problem. The situation is even worst in villages, which lack public water distribution system. The mixing of sanitary and sewage systems in the underground-water in Karachi is quite unsafe. About 900 out of 1100 water samples were collected from different pipelines, hotels and analyzed at Karachi Metropolitan Corporation laboratory were found unfit for human consumption because nitrate concentrations exceeds permissible levels. Users of this water were vulnerable to gastrointestinal troubles like diarrhea and other abdominal ailments (Gupta *et al.*, 2001).

Different studies carried out to identify the ground-water contamination of nitrate in different areas of Pakistan (Latif *et al.*, 1999), as the Punjab and Sindh are well irrigated by the Indus and its tributaries. Similar type of study was carried out in the rural areas of Rawalpindi and Islamabad as well (Tahir & Rasheed, 2008).

Use of fertilizers in Pakistan is very common which leads to high nitrate concentration in surface/ groundwater and in vegetables due to poor farming practices. Use of cured meat products like K&Ns, Menu and other brands are increasing.

Not much work done on nitrate in environmental samples is observed in Pakistan especially in rain and food commodity. Very few papers are available on nitrate concentration in environmental matrices by HPLC. Ali Bacha *et al.*, (2010)

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determined chemical characteristics of drinking water of Peshawar including nitrate. Karim *et al.,* (2008) developed HPLC method for nitrate determination in groundwater and vegetables. Tahir & Rasheed, (2008) determined nitrate in sixteen cities of Pakistan and concluded that <70% of the water samples have nitrate values in the range of 11-160 mg/L and reason is poor farming practices especially in Baluchistan and Punjab province.

Ashraf *et al.,* (2006) observed concentration of nitrate and nitrite in serum by Cd-Zn reduction Method. Sabahat & Saadat (2005) have determined nitrate concentration in ground-waters of twin cities and concluded need of further research in this regard as scarce of data. Uzaira *et al.,* (2002) conducted drinking water quality in Rawalpindi and Islamabad and Attock in terms of cation and anion concentration. Rauf *et al.,* (2002) determined water analysis of Rawal Lake and its surroundings and nitrate was determined by spectrophotometer. Kahlown *et al.,* (2001) observed comprehensive study of surface/groundwater quality in Pakistan, They determined nitrate in addition to other water quality parameter. Butt *et al.,* (2001) developed HPLC method for nitrate determination in groundwater and vegetables. The present study for the first time in Pakistan highlights the nitrate concentration in meat (raw and canned) and in rain-water of Rawalpindi/Islamabad and surrounding areas.

1.4 Analytical methods

There are different chromatographic and spectroscopic methods used for nitrate determination such as Spectrophotometry, Ion Chromatography (IC), Gas Chromatography (GC), HPLC, Capillary Electrophoresis (CE) and Polarography (Butt *et al.,* 2001; Jurtchenko, 2002).

Interference which has been seen in colorimetric determination of nitrite and nitrate encounters by dissolved organic matter (Okembgo, 1999). Fluorescence methods are good but the presence of some organic matter, like formaldehyde, in the sample may reduce or enhance the fluorescence intensity and lead to higher Standard Deviation (Buidt & Karsat, 1999). Capillary zone electrophoresis is matrix sensitive and has commonly associated problems (Okembgo, 1999). Nitrate analysis by enzymatic method is not only time consuming but its dissolved gas may increase Relative Standard Deviation by 20–30% (Girroti *et al.,* 1999). One of the most commonly used methods for determination of anions such as nitrates and nitrites is IC. It

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simultaneously determines a few anions and cations in a short time, high sensitivity and good reproducibility of results.

Ion chromatography has become a standard method for anions determination and cations in water, air and solid samples by American Society for Testing Materials (ASTM) in 1990. In 1993 European Committee for Standardization set up a working group, which after comparing twenty seven methods adopts HPLC method as European Standard for food analysis. Use of HPLC methods has gained more popularity in last decades, since they are more rapid, accurate and sensitive than spectrophotometric methods. The detection limits of HPLC methods are quite attractive for anions and other analytes , even at concentration level of µg/L. While other spectrophotometric methods can determine analytics only at mg/L concentration level (Butt *et al.,* 2001).

Nitrite and nitrate in food and water can be determined simultaneously by Ion pair chromatography (IPC) as an alternate IC method. It can be performed both in normal and reverse phase mode. It provides a great opportunity of efficient and stable packing material of column, smaller particle size which separate samples containing neutral and ionized solutes with minimum broadening and peak tailing. These are the few advantages of IPC over IC. Tetra alkyl ammonium salts are mostly used for ion-pairing reagent with negatively charged metal complexes and inorganic anion, such as nitrite and nitrate formed dynamic ion exchange and ion interaction mechanism with them. The ion pair model is better from normal phase as highly reproducible results, sharper peak shapes and reduced separation times, simultaneous separation of ionized and non-ionized analytes in one run (Snyder & Kirkland, 1976 ; Pool & Schuette, 1984).

1.5 HPLC

HPLC is a type of chromatography and is commonly used analytical technique. Chromatography is a separation technique which involves mass transfer between stationary phase and mobile phase. HPLC employs a mobile phase to separate the components of a mixture and the stationary phase can be a liquid or a solid and is an immobile packing material in the column. The compounds under study are first dissolved in appropriate solvent and then forced to flow through the column under high pressure where the mixture separates into its components. This separation

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depends upon the extent of interaction between solute components and stationary phase. The interaction of solute with mobile and stationary phase can be manipulated through proper selection of solvents and stationary phase. HPLC exhibits a high degree of versatility which is not found in any other mode of chromatography and it also has the ability of separating a wide range of chemical mixtures.

Mobile Phase

The nature and composition of element affects the separation in HPLC. Several properties of solvents like purity, detector compatibility, low viscosity, solubility of the sample and chemical inertness affects the selection of mobile phase. In case of size exclusion chromatography (SEC), the eluents have to dissolve polymers and have to suppress the possible interactions of the sample molecules with the surface of packing material. In case of normal phase (NP) chromatography the solvents are mainly non polar and in case of reverse phase (RP) chromatography eluent is usually a mixture of water and polar organic solvent like acetonitrile or methanol etc.

Based on mode of HPLC, various sorption forces can be included in retention process such as hydrophobic (non-specific) interactions which are operative in reverse phase separations. Dipole-dipole interactions are dominant in normal phase. Ionic interactions play major role in retention of compounds in ion-exchange chromatography. All these different interactions compete with each other. Analyte molecules compete with eluent molecules for sorption sites and the stronger analyte molecules interact with the surface. If the eluent interaction is weaker, the analyte molecules will be retained on the surface for longer time. In case of SEC the separation of mixture is on the basis of molecular size. Bigger the molecule the less will be its penetration into the sorbent pore space and less will be its retention time.

Stationary Phase

HPLC separations are based on surface interactions and are dependent on type of sorption sites. HPLC sorbents are small rigid porous particles and have high surface area. Main sorbent parameters are particle size and particle size distribution. Based on the type of ligand attached to the surface, the sorbent can be normal phase (-OH, -NH$_2$) or reverse phase (C-5, C-8, -CN, -NH$_2$) or anion (CH$_2$NR$_3$ + OH$^-$) or cation (R-SO$_3$ –H$^+$) exchangers.

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Classification of liquid chromatography can be done in different ways. Three modes can be distinguished on the basis of stationary phase and the separation process.

Sorption chromatography

The stationary phase is a sorbent like silica gel or any other silica based packing and the separation depends on repeated sorption-desorption steps. Under sorption chromatography two modes i.e. normal and reversed-phase chromatography can be defined based on relative polarity of stationary and mobile phase. In case of NP chromatography the stationary phase is polar in nature e.g. silica gel and the mobile phase is non-polar e.g. n-hexane. Polar samples are retained on the column surface for longer time then less polar molecules.

Ion-exchange chromatography

In this case the stationary bed has an ionically charged surface of opposite charge to sample ions. This method is used exclusively with ionic or ionizable samples. Stronger the charge on the sample stronger it will be attracted to ionic surface and longer will be retention time of eluent. The mobile phase in this case is an aqueous buffer and to control elution time pH and ionic strength are used.

Size exclusion chromatography

In this case column is packed with material having precisely controlled pore size. The sample is filtered with respect to solvent molecular size. Large sizes molecules are rapidly washed through column whereas smaller size molecules penetrate inside the porous packing material and elute later. This method is also called gel filtration or gel permeation chromatography.

Reversed-phase chromatography:

The mechanism of this method is converse of Normal Phase chromatography. Stationary phase is non-polar and mobile phase is polar liquid. More non-polar the material is the longer it will be retained in column. This type of chromatography is used for almost 90% of all chromatographic processes.

Components of HPLC system

HPLC process starts with injection of solute on the top of column. Each component elutes from the column as a narrow band/peak on the recorder. Depending on the detector used the detection of eluting components can be universal or selective. The response of detector to each component is displayed on the chart recorder or computer

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screen and is known as chromatograph. HPLC used in current investigation is shown in Figure 1.1. The components of HPLC are as follows:

Pumps

High pressure pumps are required for forcing the solvent through packed stationary phase. Small sized particles of column require high pressure. Small sized columns are more beneficial as compared to larger sized particles but they may not be employed in all separations. Higher resolution, faster analysis and increased sample load capacity are advantages offered by smaller size particles. Large sized particle packing requires lesser pressure. Another important feature of pumps is flow rate stability. For analytical chromatography, very stable flow rate is not essential. However, the SEC mode requires extremely stable flow rate. External electronic control is another important feature of pumps. This feature is desirable when electronically controlled gradients have to be run.

Mobile Phase Reservoir

A glass bottle reservoir is a common solvent reservoir. These are equipped with special caps, Teflon tube and filter for connecting to the pump inlet and purge gas (helium) which is used to remove dissolved air from pumps. For degassing aqueous solvents before helium purging, a vacuum can be applied for 5 -10 minutes.

Injector

Samples can be injected into HPLC in many ways. An injection valve is commonly used. In advanced LC systems, automatic sampling device is employed. In the case of liquid chromatography, liquid samples are directly injected while the solid samples are dissolved in appropriate samples. Injection of particulate substances cause blockage of injector and column so the samples should be filtered or centrifuged before injection.

Column

Columns are considered to be the central component of HPLC system. Usually columns are 5- 25 cm in length and have an internal diameter usually of 4.6 mm. As

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the analytes and mobile phase are pumped through the column, separation of components occurs.

Detector

In LC systems, optical detectors are commonly employed. Detectors pass a light beam through a flowing column effluent as it crosses a low volume (10 µl) flow cell. The variation in light intensity is caused due to UV absorption, fluorescence emission or change in refractive index by the sample particles passing through the cell. This variation is monitored as change in output voltage. This voltage change is recorded on a strip chart recorder and is regularly fed into data system to obtain retention time and peak area information. UV absorption detector is frequently used in LC system. Photo Diode Array UV detector (PAD), Refractive Index (RI), Fluorescence (FLU), Electrochemical (EC) detector are also used.

Data system

For collection, storage and analysis of chromatographic data, a computer integrator and other data processing equipments are often employed (2). Components of HPLC are shown in Figure 1.1.

HPLC is extensively functional for separations and purifications in a multiple areas including pharmaceuticals, biotechnology, environmental, polymer and food industry. HPLC has become a technique of choice for the analysis of a large range of compounds. Its main benefit over Gas chromatography (GC) is that the analytes need not to be volatile so macro-molecules are appropriate for HPLC analysis. HPLC is most frequently employed in performing a target compound analysis, where the compounds present in a mixture are known so reference standards can be used for determination of retention time. Quantitative analysis is frequently done with HPLC. Some common applications of HPLC are:

- Quantitative/qualitative analyses of amino acids, nucleic acids, proteins in physiological samples.

- Measuring amount of active drugs, synthetic by-products and degradation products in pharmaceuticals.

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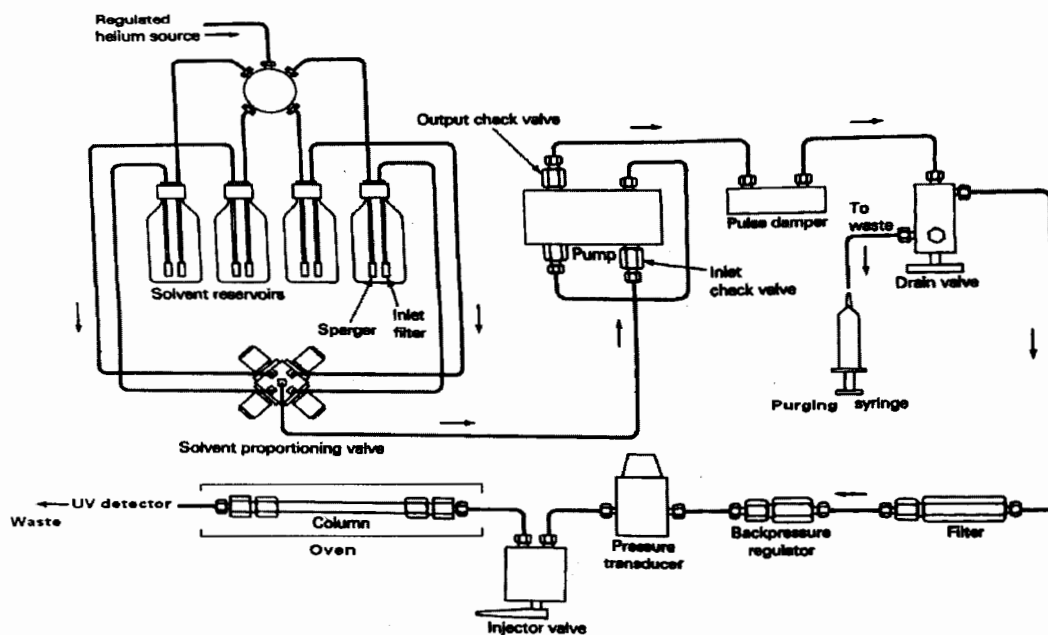
Figure 1.1 Components of High Performance Liquid Chromatography System (Topping, 2006)

- To gauge levels of hazardous compounds such as pesticides and insecticides.
- Monitoring environmental samples.
- Purifying compounds and impurities from mixtures (2).

1.6 Significance of study

The current investigation is focused on nitrate determination in environmental matrices and food products by RP-IP-HPLC. Nitrate is an important component of man's chemical environment and the major source of human exposure is from food and drinking water. The synthetic nitrogen fertilizers and livestock manure are being intensively used in modern agricultural practices, leading to increased nitrate concentration in vegetables and drinking water.

Nitrates and nitrites in food may be a causative factor for met-haemoglobinemia in babies, where due to the oxidation of ferrous iron in haemoglobin to ferric state, the ability of red blood corpuscles to carry oxygen is lost and the affected baby dies. Nitrate consumption leads to decreased ascorbate/nitrite ratio in gastric juice, which

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regulate the synthesis of potentially carcinogenic N-nitroso compounds and decreases the ratio leads to increased risk of gastric cancer (Craig *et al.,* 1999). Use of nitrates is going on since long but its toxic effects have been ignored for centuries, it has recently been observed. In the 1970s, the safety of meat products cured with nitrate was one of the most common discussed topics in newspaper. Under certain conditions which are yet to be understood, the natural breakdown of proteins (amines) is supposed to combine with nitrites to form nitrosamines. Nitrosamines are of many different types and some of them are proven to be carcinogens in nature.

Nitrate is a part of medicine which is being used in cardiovascular disease, where they reduce platelet aggregation and prevent angina attacks of symptomatic and silent types (Thomas, 2011). But continued consumption of nitrate is discouraged as it may causes tolerance (Wilfred Snodgrass, 1996). WHO has set maximum limit of 3.65 mg nitrate kg^{-1} of body weight per day (Arnold *et al.,* 1998). It is, therefore, very useful and innovative that some studies should be conducted on nitrate, its presence in environmental matrices and food products, its precise determination as well as its impact on environment.

1.7 Aims and objectives

The overall objectives of the research was to

- Develop a cost effective and rapid HPLC method for determination of nitrate in environmental matrices and food products
- Investigate nitrate concentration in rain-water and identify possible source types.
- Characterize the nitrate concentration in surface/ground-water to establish nitrate levels in drinking water sources.
- Provide more accurate data on the concentration of nitrate in fresh meats/ canned meat and leafy vegetables to enable dependable estimation of nitrate exposure to local population.

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CHAPTER # 2

METHODOLOGY

2 METHODOLOGY

2.1 Chemicals and Reagents

Tetra-ethyl ammonium chloride (TEACl) (98%), Sodium dihydrogen phosphate (NaH_2PO_4) (98%), Methanol (CH_3OH) (99.9 %), Phosphoric acid (H_3PO_4) (85 %), Diethyl ether ($C_4H_{10}O$) (98%), Sodium nitrate ($NaNO_3$) 99 % were purchased from Merck, (Steinheim, Germany) and Acetonitrile (C_2H_3N) (99.9%) HPLC grade was purchased from Fischer, UK. Ultra high quality water was supplied from milli-Q water purification system (Millipore, Bedford, Ma, USA). All samples and solvents were filtered through 0.45 µm filter. Wattmann filter paper (jwg-01, Kavon, USA) was used for filtration purpose. All glass wares of Pyrex (Germany) were used, and soaked in 10 % nitric acid for one night then washed and rinsed with deionized water. All chemicals used were of analytical standard.

2.2 Apparatus and Equipments

For pH adjustment, pH meter (HLSI-19156, Ambala, India) with maximum limit 1-14 was used; TDS/EC meter (CTS-406) was supplied from HL-Scientific, India. Filtration was done with (EW-34509) coleparmer, UK, filtration assembly. For centrifugation (DSC- 200A) centrifuge from digital system, USA was employed. The maximum limit for centrifuge was 6000 rpm, for sample blending Kenwood blender USA, was used. Separating funnel (6383 IL) for solvent-solvent extraction was purchased from Gilson USA. Ultra sonic water bath (A 104-45-230) used was supplied by Scientico inds, India.

A waters High Performance Liquid Chromatogram (Water Breeze System, Toronto, Canada), comprising of a Waters 1500 Series HPLC gradient/isocratic pump, and waters 2487 dual Wave length UV detector was used. The analytical column used was a reversed phase C-18(5µm particle size, 150mm. 4.5mm i.d. column. Waters). The injection loop was used of 10 µL, Waters. Breeze software was used to acquire process and interpret the data (annexure I).

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2.3 Sample Preparation

2.3.1 Standard Solution Preparation

2.3.1.1 Nitrate stock Solution Preparation

Stock solution of Sodium Nitrate ($NaNO_3$) of 1000 ppm was prepared by dissolving 0.137g of $NaNO_3$ in 100mL of double deionised distilled water. Working standards of different concentrations were prepared by dilution of stock solution. For surface water and ground water working standard solutions of (0.5, 1, 2, 5 and 10 mg/L) concentrations, for rain water (0.1, 1, 2, 5 and 10 mg/L) and (20, 40, 60, 80, 100, 150, 200, 300, 400 mg/L) concentration solutions were used for meat and vegetable sample analysis. Stock solution was prepared weekly from which respective dilutions were prepared on a daily basis. Six point calibration Curve was constructed by using Standard solutions ranging from 0.1 to 400 mg/L. All samples were analyzed by reverse phase HPLC method (Butt *et al.*, 2001; Zuo *et al.*, 2006).

2.3.1.2 Mobile phase preparation

 The mobile phase composition was optimized by mixing 80% TEACl (6.0 mM) and 20% pure acetonitrile (using 4.0 mM sodium di hydrogen phosphate as buffering reagent). 0.5M H_3PO_4 (as per requirement) was used to maintain pH at 3.0. Solution of 6.0 mM TEACl was prepared by dissolving 0.495 g TEACl in 500 ml double deionised distil water and 4.0 mM NaH_2PO_4 was prepared by dissolving 0.274 g in 500 ml double deionised distilled water. Mobile phase was filtered through 0.45µm filter paper. Double deionised distilled water was supplied by Millipore system.

2.4 Environmental Sample Collection and Preservation

2.4.1 Study Area

For nitrate determination rain-water, surface-water and ground-water were collected during the period from 15th July to 15th September 2010, from different areas of Islamabad and Rawalpindi and its surroundings. Ground-water samples were collected from tube well/boring in the jurisdiction of Rawalpindi and Islamabad, while surface-water samples were collected from Rawal, Simli and Khanpur dams. For determination of nitrate in food commodities, meat samples were bought from local markets. These samples were in raw form as well as local and branded cured

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meat products which included fish, mutton, beef and chicken. Fresh vegetables, spinach and cabbage were collected and bought from local markets, organic retailers as well as from farms. These collections were made during the month of July 2010 as well as some in October 2010 due to cabbage production season.

2.4.2 Ground-water sampling

Thirty ground-water samples were collected during the month of July, 2010 and October, 2010 from Rawalpindi and Islamabad area (Figure 2.1).The difference in sampling month was due to monsoon rain which ended in the month of October.

Ground-water samples were collected in polyethylene bottles, immediately stored at 4^0C in dark until used. Samples were filtered through 0.45μm membrane filters before HPLC analysis. Precautions have always been taken to minimize sample contamination (Morales *et al.,* 2000). Details of ground-water samples, collection date and source is given in table 2.1.

Table 2.1 Inventory of Ground Water Samples

Sample code	Sample name	Water	
Gw-1	H-9/2	Tube well,40 feet	July, 2010
Gw-2	I-8		
Gw-3	G-9/4	Boring	July, 2010
Gw-4	E-8		
Gw-5	F-9	Boring	July, 2010
Gw-6	I-8/2		
Gw-7	G-10/4	Tube well	July, 2010
Gw-8	G-10/3		
Gw-9	G-10/2	Tube well	July, 2010
Gw-10	I-10/2		
Gw-11	I-10/4	Tube well	July, 2010
Gw-12	F-10/1		
Gw-13	G-9/4	Tubewell150,300 feet	July, 2010
Gw-14	H-8/1		
Gw-15	G-9/2	Boring	July, 2010
Gw-16	Barma Chowk		
Gw-17	Margalla Town	Tube well	0ctober, 2010
Gw-18	Best Western		

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Gw-19	Rawal Dam Area	Tube well	October, 2010
Gw-20	Rawal Town	Tube well	October, 2010
Gw-21	College Road	Tube well	October, 2010
Gw-22	Muree Road	Tube well	October, 2010
Gw-23	SatelliteTown, Fire Brigade	Tube well	October, 2010
Gw-24	Rehmanabad	Tube well	October, 2010
Gw-25	Shamsabad	Tube well	October, 2010
Gw-26	Faizabad	Tube well	October, 2010
Gw-27	Harley street	Tube well	October, 2010
Gw-28	Gawal mandi	Tube well	October, 2010
Gw-29	Children park	Tube well	October, 2010
Gw-30	Liaqat Bagh	Tube well	October, 2010
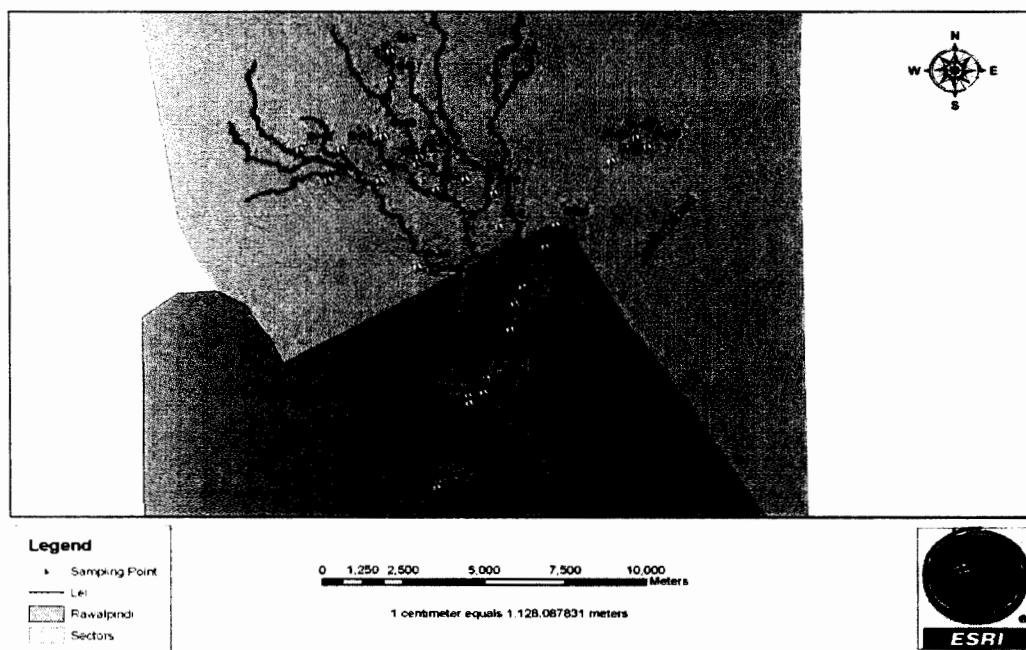
Figure 2.1 Sampling locations of groundwater

2.4.3 Surface-water sampling

Twelve surface-water samples were collected from Rawal Lake, Simli Dam and
Khanpur Dam randomly (Figure 2.2). Details of surface-water samples, collection
date and source is given in table 2.2.

*Determination of Nitrate in Environmental Matrices and Food Products by Reversed Phase
Ion pair High Performance Liquid Chromatography*

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Table 2.2 Inventory of Surface Water Samples

Sample codes	Sample name	Source	Month/year
Sw-1	H-9/2,	Nala-Lai	July, 2010
Sw-2	Entrance of Bari Imam	Rawal Lake	July, 2010
Sw-3	Korang River Entrance	Rawal Lake	July, 2010
Sw-4	Centre of lake	Rawal lake	July, 2010
Sw-5	Spillways	Rawal Lake	July, 2010
Sw-6	Rawal Lake (Old Picnic Point)	Rawal Lake	July, 2010
Sw-7	Simli Dam entrance	Simli Dam	August, 2010
Sw-8	Simli Dam Centre	Simli Dam	August, 2010
Sw-9	Simli Dam	Simli Dam	August, 2010
Sw-10	River-Harrow entrance	Khanpur Dam	August, 2010
Sw-11	Khanpur-Dam entrance	Khanpur Dam	August, 2010
Sw-12	Spillways	Khanpur Dam	August, 2010
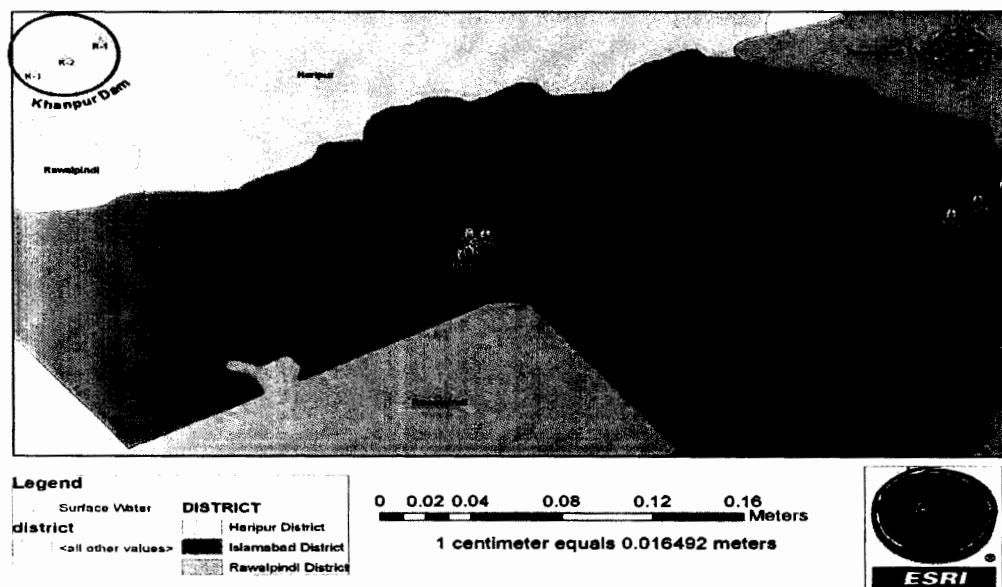
Figure 2.2 Sampling locations of surface-water

2.4.4 Rain-water Sampling

Twenty three rain collections were made through the course of monsoon in the month of July/ August, 2010. Figure 2.3 shows sampling locations. The rain samples were collected in stainless steel beakers. The beakers were placed tentatively on selected

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locations when precipitation was anticipated. Samples were capped in glass vials and duly labeled with date, location, and precipitation type (Pettergrew & Gordan, 2001). Rain-water samples were stored at 4°C in dark till used. Samples were filtered through 0.45μm membrane filters (Fischer scientific) before HPLC analysis. All sample containers, glass wares and filtration devices were thoroughly cleaned with 10% Nitric acid solution and finally with double deionised distilled water (Zuo *et al.,* 2006). Details of rain-water samples, collection date and source is given in table 2.3.

Table 2.3 Inventory of Rain Water Samples

Sample codes	Sample name	Source	
Rw-1	Attock	Near oil field	July, 2010
Rw-2	Hasanabdal	Road-site	July, 2010
Rw-3	Wah Cantt.	Factory site	July, 2010
Rw-4	Taxila	Road-site	July, 2010
Rw-5	Hawelian	Road site	July, 2010
Rw-6	Khanpur	Road site	July, 2010
Rw-7	Lalazar	Residential area	July, 2010
Rw-8	Kohinoor Mill	Residential area	July, 2010
Rw-9	Pirwadhai	Bus Stand	July, 2010
Rw-10	Raja Bazaar	Main Bazaar	July, 2010
Rw-11	Shamshabad	Commercial area	July, 2010
Rw-12	Tench Batta	Road-site	July, 2010
Rw-13	Waris Khan	Residential Area	July, 2010
Rw-14	Thanda Pani Nilore	Residential Area	July, 2010
Rw-15	I-9	Residential Area	July, 2010
Rw-16	I-10	Residential Area	July, 2010
Rw-17	PINSTECH	Premises	July, 2010
Rw-18	Al-Noor Colony	Residential Area	July, 2010
Rw-19	Karachi Company	Residential Area	July, 2010
Rw-20	Khandak	Road-site	July, 2010
Rw-21	Nilore	Road-site	July, 2010
Rw-22	H-10 Islamic University	Premises	July, 2010
Rw-23	H-10	Residential Area	July ,2010

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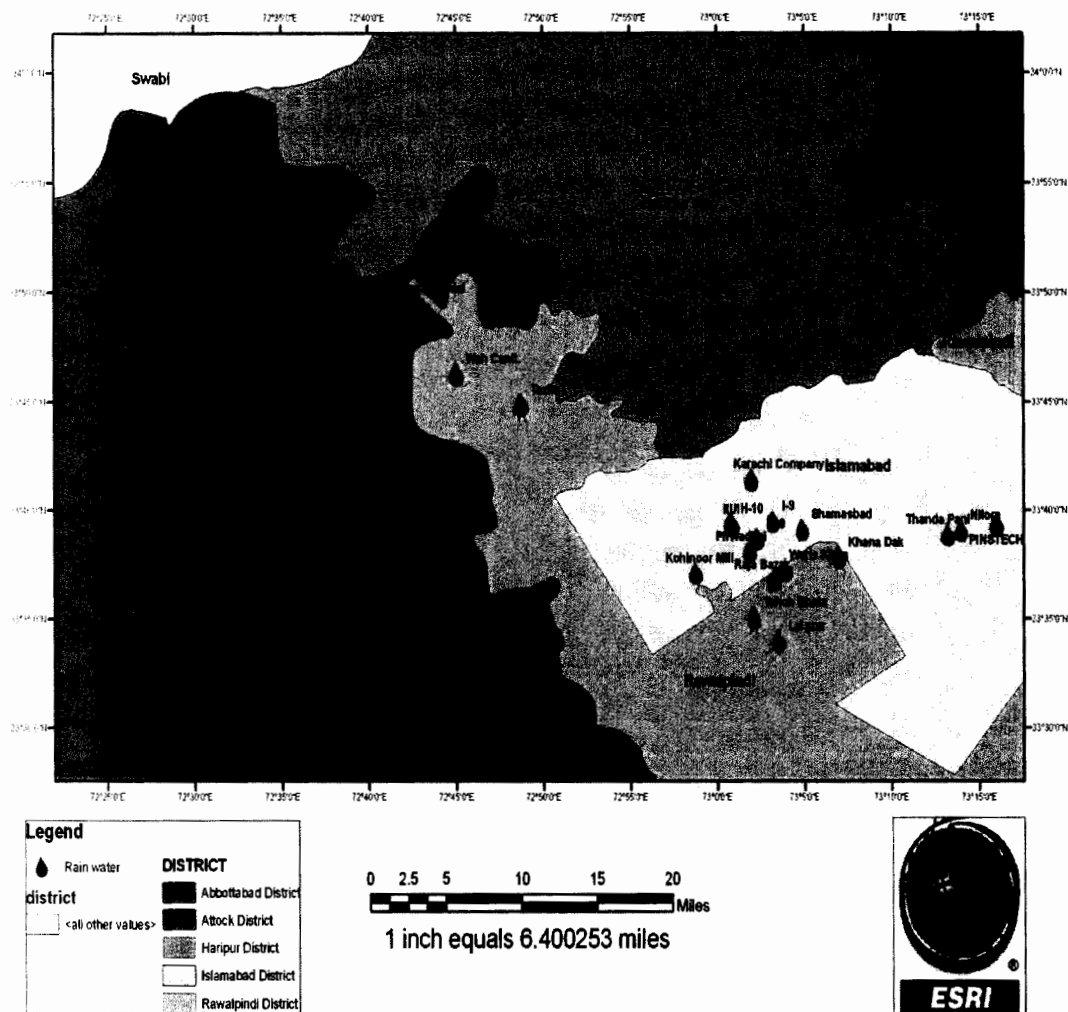
Figure 2.3 Sampling locations of rain-water

2.4.5 Vegetable and meat samples collection

A total of twelve fresh spinach and cabbage samples were collected from different local markets, organic food retailers and directly from farms. Prior to analysis, non-edible parts of each sample were removed. Sample collections were carried out according to the methods described by Radojevic *et al.*, (1986) and were kept in clean unused polyethylene bags and transported to the laboratory. A total of thirteen meat samples which includes cured and raw meat were collected from local markets and were frozen overnight at - 10°C until used (Sanderson *et al.,* 1991). Details of meat and vegetable samples along with collection date and source is given in table 2.4 and 2.5 respectively.

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Table 2.4 Inventory of Meat Samples

Sample codes	Sample name	Source	Month/year
M-1	Hot dogs	Metro-Islamabad	August, 2010
M-2	Quails	Metro-Islamabad	August, 2010
M-3	Canned hot dogs	Metro-Islamabad	August, 2010
M-4	Chicken bologna	Metro-Islamabad	August, 2010
M-5	K&Ns Kabab	Metro-Islamabad	September, 2010
M-6	Chunks	Metro-Islamabad	September, 2010
M-7	Nuggets	Metro-Islamabad	September , 2010
M-8	Beef (Raw)	Gondal Market	September, 2010
M-9	Chicken (Raw)	Islamabad market	September, 2010
M-10	Mutton (Raw)	Noshera Market	September, 2010
M-11	Fish	Rawal dam	September, 2010
M-12	Chicken	Chaksi Market	September, 2010
M-13	Chicken	Commercial Market	September, 2010

2.4.6 Extraction

2.4.6.1 Food Sample Extraction

2.4.6.1.1 Vegetable samples extraction

For spinach (stem and leaves separately) and cabbage extracts, slightly modified alkaline extraction method described by Sen & Donalds, 1978 was used. Vegetable samples were cleaned to remove visible soil followed by washing with tap water, thereafter with distilled water several times and sliced into nearly uniform sizes to facilitate drying at the same rate, immediately stored at -20°C before it is subjected to analysis.

Two grams of vegetable sample was blended in Kenwood blender for five minutes in 50 mL distilled water until homogenized. Homogenates were transferred into 100 mL volumetric flask and heated on a shaking water bath at 80°C for 20 minutes and diluted to 100 mL with distilled water. After cooling samples were centrifuged at 6000 rpm for 15 minutes in centrifuge. The supernatant was collected in another cleaned beaker and was filtered through 0.45µm filter paper. All samples were immediately analyzed within 1 hr after sample preparation.

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2.4.6.1.2 Meat Sample Extraction

2.4.6.1.2.1 Extraction of Meat Samples with Low Fat Content

For meat extracts, the method described by DIONEX (Application note 112) was followed with some modifications. 20 grams of each meat sample was blended in Kenwood blender for five minutes in 100 mL distilled water until homogenized. Homogenates were transferred into 100 mL volumetric flask and heated on hot plate for 10-15 minutes at 80-90^{0}C followed by cooling to room temperature. After cooling samples were centrifuged at 6000 rpm, for fifteen minutes in centrifuge. The supernatant was collected in another cleaned beaker and was filtered through wattmann 2, GFA/A and 0.45µm filter paper. This procedure was applicable for samples which had lower fat contents. Samples which had high fat contents were first solvent-solvent extracted and then filtered by the procedure described in following section

Table 2.5 Inventory of Vegetable Samples

Sample codes	Sample name	Source	Months/year
Sl-1	Attock	Farm	October, 2010
Sl-2	Hasanabdal	Farms	October, 2010
Sl-3	Khanpur	Farms	October, 2010
Sl-4	Attock	Farms	October, 2010
Sl-5	Shamshabad	Farms	October, 2010
Sl-6	Bermaa (Khana)	Farms	October, 2010
Sl-7	Wah Cantt.	Farms	October, 2010
Ss-1	Attock	Farms	October, 2010
Ss-2	Hasanabadal	Farms	October, 2010
Ss-3	Khanpur	Farms	October, 2010
Ss-4	Attock	Farms	October, 2010
Ss-5	Shamshabad	Farms	October, 2010
Ss-6	Barma (Khana)	Farms	October, 2010
Ss-7	Wah Cantt.	Farms	October, 2010
C-1	Attock	Farms	October, 2010
C-2	Hasanabadal	Farms	October, 2010

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C-3	Rawalpindi	Farms	October, 2010
C-4	Islamabad	Farms	October, 2010
C-5	Wah Cantt	Farms	October, 2010

2.4.6.1.2.2 Extraction of Meat Samples with High Fat Content with Solvent-Solvent Extraction

50 mL of sample was transferred to a separating funnel then 50mL of acetone and 50 mL of diethyl ether was added into it. The mixture was thoroughly shaken. The assembly was left to stand for one hour. Afterwards the clear bottom layer was collected in 100 mL beaker, while the upper layer which consists of fats was discarded. The samples were again filtered through wattmann filter paper No. 2 and GFA/A filters to remove fats and impurities from the solution.

2.5 Physico-chemical Characterization

2.5.1 Physical Parameters

pH, Electrical Conductivity (EC), Total Dissolved Salts (TDS) of water samples (rain, ground and surface-water) were measured with portable meters. Meters were calibrated with standard solution after every five measurements.

2.5.2 Chemical Parameter

An innovative and accurate method for determination of nitrate ions in environmental matrices and food products was developed. The analytical conditions including the concentration and pH effect of mobile phase, UV detector response at various wave lengths and flow rate were optimized to get extremely linear calibration curves (r > 0.999 in the range 0.1-400 mg/L (Figure 2.4).

To attain optimum condition different compositions of Tetra ethyl ammonium chloride and acetonitrile were tested at various pH ranges (3, 3.5 and 4) and different flow rates (0.5, 0.8 and 1) mL/min. The excellent results were achieved by using 80% tetra-ethyl ammonium chloride (6.0 mM) and 20% pure acetonitrile as mobile phase using 4.0 mM Sodium dihydrogen phosphate as a buffer. pH of the mobile was adjusted at 3.0 with the help of 0.5 M H_3PO_4 (as per requirement). Optimum conditions were attained at flow rate of 0.5mL/min on reversed phase C 18 column (150 mm 4.5mm i.d ; 5μm particle size,Waters).

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The total run time for one sample analysis was less than 5 minutes. The mobile phase with optimized composition and pH was run at different flow rates and the optimized

Name: Nitrate Time: 3.372 Fit Type: Linear (1st Order) R: 0.989827 R†: 0.979757 Equation: Y = 3.32e+0
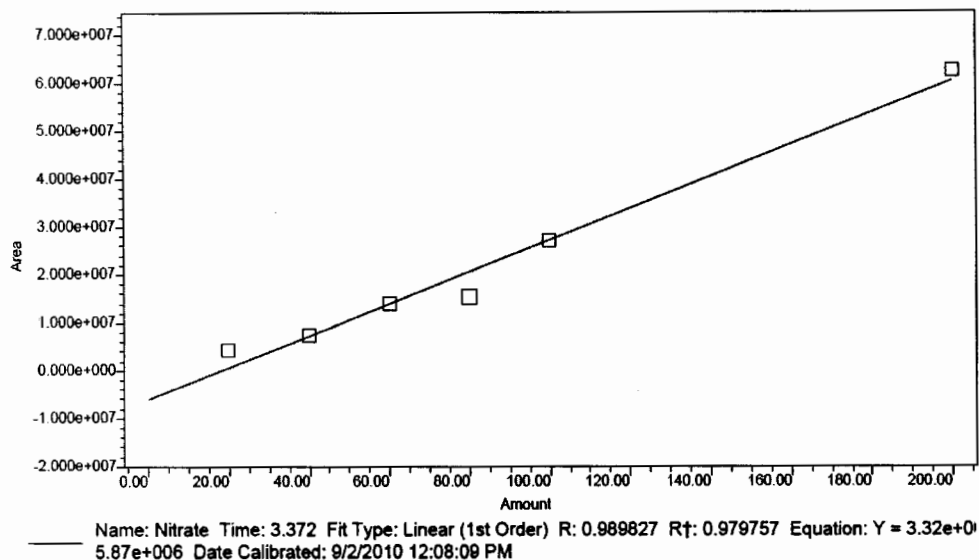5.87e+006 Date Calibrated: 9/2/2010 12:08:09 PM

Figure 2.4 A 6 point Calibration curve for nitrate analysis

flow rate was 0.5mL/min. Ambient column temperature was set for the operation of HPLC. In rain-water samples the concentration of nitrate is usually below 1.00 µg/L. While nitrate has a much broader concentration range in ground, surface-water, vegetables and meat products. Therefore, the calibration curve for the analysis for different environmental samples was prepared according to their expected concentration in these matrices. Samples with low nitrate concentration were spiked with known standard and nitrate value was obtained by subtracting it from standard value.

2.5.2.1 Qualitative and Quantitative analysis

The mobile phase was allowed to pass through the HPLC until a stable baseline signal was equiberated at flow rate of 0.5mL/min at optimized UV wavelength of 210 nm. The injection volume was 10 µL. When the injections of the standard solution gave reproducible retention time and peak areas, each sample solution was then injected for analysis. The peaks of the sample were identified by comparison with the peaks of the standards. The amount of nitrate in the test solutions were calculated from peak areas using regression equation of nitrate standard curves (analyzed by the Waters soft

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ware). If the curve of the peak areas was larger than that of the maximum amount from the standard curve, the test solutions were diluted to appropriate concentrations. The HPLC column was refreshed by passing a solution of 80% methanol and 20 % water (80:20) for at least 45 minutes with flow rate of 1.5 mL/min at the end of the day. Some of the best chromatograms of tested samples are presented in Annexure II. Standard solutions were run for the calibration curve. Qualitative analysis of each standard solution was evaluated by its retention time. The retention time for the standard solutions were 3.2 minutes. Overlapping peaks were obtained as shown in Figure 2.5.

 Linearity was addressed by preparing standard solutions of nitrate at 6 levels (20, 40, 60, 80, 100 and 200 mg/L), standards were injected in a random into the chromatograph. A linear regression of analyte concentration vs. peak response was performed. The coefficient of determination (r^2) for nitrate was 0.99. The upper concentration of the range is equivalent to 400 mg/L for nitrate sample.
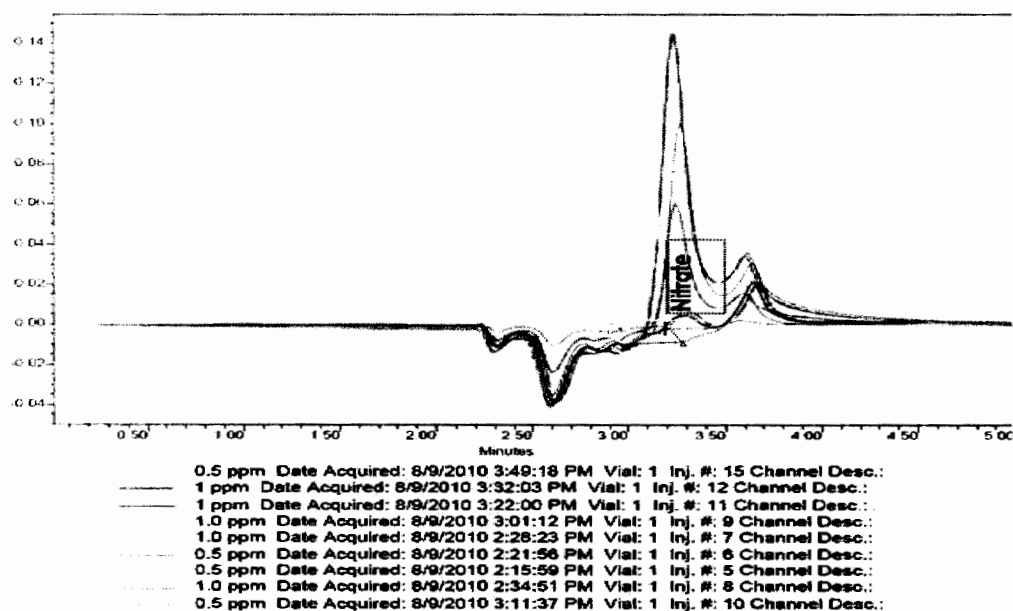

0.5 ppm Date Acquired: 8/9/2010 3:49:18 PM Vial: 1 Inj. #: 15 Channel Desc.:
1 ppm Date Acquired: 8/9/2010 3:32:03 PM Vial: 1 Inj. #: 12 Channel Desc.:
1 ppm Date Acquired: 8/9/2010 3:22:00 PM Vial: 1 Inj. #: 11 Channel Desc.:
1.0 ppm Date Acquired: 8/9/2010 3:01:12 PM Vial: 1 Inj. #: 9 Channel Desc.:
1.0 ppm Date Acquired: 8/9/2010 2:28:23 PM Vial: 1 Inj. #: 7 Channel Desc.:
0.5 ppm Date Acquired: 8/9/2010 2:21:56 PM Vial: 1 Inj. #: 6 Channel Desc.:
0.5 ppm Date Acquired: 8/9/2010 2:15:59 PM Vial: 1 Inj. #: 5 Channel Desc.:
1.0 ppm Date Acquired: 8/9/2010 2:34:51 PM Vial: 1 Inj. #: 8 Channel Desc.:
0.5 ppm Date Acquired: 8/9/2010 3:11:37 PM Vial: 1 Inj. #: 10 Channel Desc.:

Figure 2.5 Different Nitrate standard solutions peak

2.6 Recovery Experiments/Reproducibility Check

2.6.1 Reproducibility test

Three replicates of known standard and samples were run in each day to check the reproducibility precision values and were characterized by coefficient of variance. Recalibration was performed regularly.

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2.6.2 Recovery test

A series of various concentrations of (1, 5, 10, 50, 100 mg/L) standard solutions containing nitrate were spiked into environmental waters and food samples, each spiked concentration was analyzed in triplicate, including blank test to evaluate the average recoveries.

The difference between the total amount in the spiked samples and the amount in the non-spiked samples was used to calculate recoveries. Recovery experiment was repeated three times. For Nitrate samples, the recoveries were found between 98.0 % - 100.5 % which gives a proof for the accuracy of proposed method.

Average reproducibility for the 2 different levels of nitrate, 5 mg/L and 10 mg/L are shown in Table 2.6. Average percent reproducibility for the method was closer to 100%, indicating that the method was quite accurate.

Table 2.6 Results of Recovery Experiments for 5ppm & 10ppm Nitrate Levels

Amount injected	Peak Name	Channel Description	Retention Time (min)	Area	% Area	Height	Amount observed
5 mg/ L	Nitrate	210	3.329	960668	100.00	111460	5.494 mg/ L
10 mg/ L	Nitrate	210	3.328	2545171	100.00	147874	10.770 mg/L

2.7 Detection limit

The detection limit of Waters HPLC was 10 µg/L.

2.8 Statistical Analysis

ANOVA was applied to examine statistical significance of difference in mean physico-chemical (pH, EC, TDS and Nitrate values) of different Water Samples viz., surface, rain and ground water. Nitrate concentration in food commodities (meat and vegetables) were presented as mean. Statistica version.8 and Microsoft Excel was used for data analysis.

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CHAPTER # 3

RESULTS AND DISCUSSIONS

3 RESULTS AND DISCUSSIONS

The results and discussion section contains physio-chemical characterization of water samples, method development procedure of nitrate determination and assessment of nitrate concentration. Nitrate concentration in food samples is presented in a separate section.

3.1 Physico-chemical characterization of water samples

3.1.1 Physical Parameters

This section details about pH and EC (with calculated TDS) for all water samples to determine the source type chemistry and relate it with nitrate concentration. The results for pH and EC/TDS are presented for groundwater, rain and surface-water from Rawalpindi, Islamabad and surrounding areas. The Figures 3.1, 3.2, and 3.4 depict data for pH, EC and TDS, respectively, for all water samples.

3.1.1.1 pH

Overall, ground-water and surface-water were neutral to alkaline in nature while rain-water was neutral to slightly acidic. Figure 3.1 depicts that among water samples, highest mean pH was observed in surface water, than in ground water and minimum value in rain water. The ground-water of Islamabad was predominantly alkaline with pH in the range of 7.0 to 8.5, and thus is in the satisfactory limit prescribed by WHO value for pH i.e.6.5 to 8.5 (WHO, 2004). The highest value was observed in Margalla Town and Rawal Dam area and lowest in the residential sector of F-10/1. Lowest pH values were recorded at Liaqat Baagh and highest values in Faizabad and Harley Street ground-water. Most of the ground-water had pH range between (7.0-8.0). Ground-water in the Rawalpindi area had lower pH, Ground-water recharge by passing through sedimentary rocks dissolves limestone characterizing water supply as HCO_3 type (Munir *et al.*, 2011).

Surface-water pH was in the range of 7.6 to 8.0. Nala-Lai water had higher pH values than the lake waters. Slight alkalinity of surface-water might be due to dissolution of Margalla Hill limestone.

Rain-water at Rawalpindi had pH in the range of 6.22 to 7.0 with average of 6.88. Average Islamabad pH range was 6.87. Lowest pH was recorded from Shamshabad area whereas highest value of pH was found in Waris Khan Area. Lower pH values in Rawalpindi rain-water might be due to heavy traffic load which results in burning of

more fossil fuel. Lowest pH value at Islamabad was observed in Khana Dak. Most of the rain samples had pH in neutral range whereas, in surrounding area lowest pH values were observed in Hasanabdal, Attock and highest in Khanpur area. pH values of Hasanabdal and Attock area were slightly acidic in nature. Acidic nature of rain water is due to presence of oxides of carbon, nitrogen and sulphur in the atmosphere. As the concentration of these oxides increases the acidic nature of rain increases. Hasanabdal is close to Hattar Industrial area and wind direction may have a role in bringing pollutants of industrial area to this location, while the collection point of rain in Attock was near the bus stand where heavy traffic load is observed. Burning of fuel from nearby oil field may be another reason of low pH in this area. The lower value of pH was also observed in those areas of Rawalpindi and Islamabad where there were heavy traffic load. The area with large water bodies like Khanpur (Near Khanpur dam) and in Islamabad (near Rawal dam) had pH values of rain-water close to neutral. It has been observed that the rain chemistry is influenced by different factors such as erogenous sources (rock, soil and dust), agricultural sources (livestock and crop fertilizers), marine sources and biomass burning (fuel wood and forest fires)(Galy-Lacaux, *et al.,* 2009).
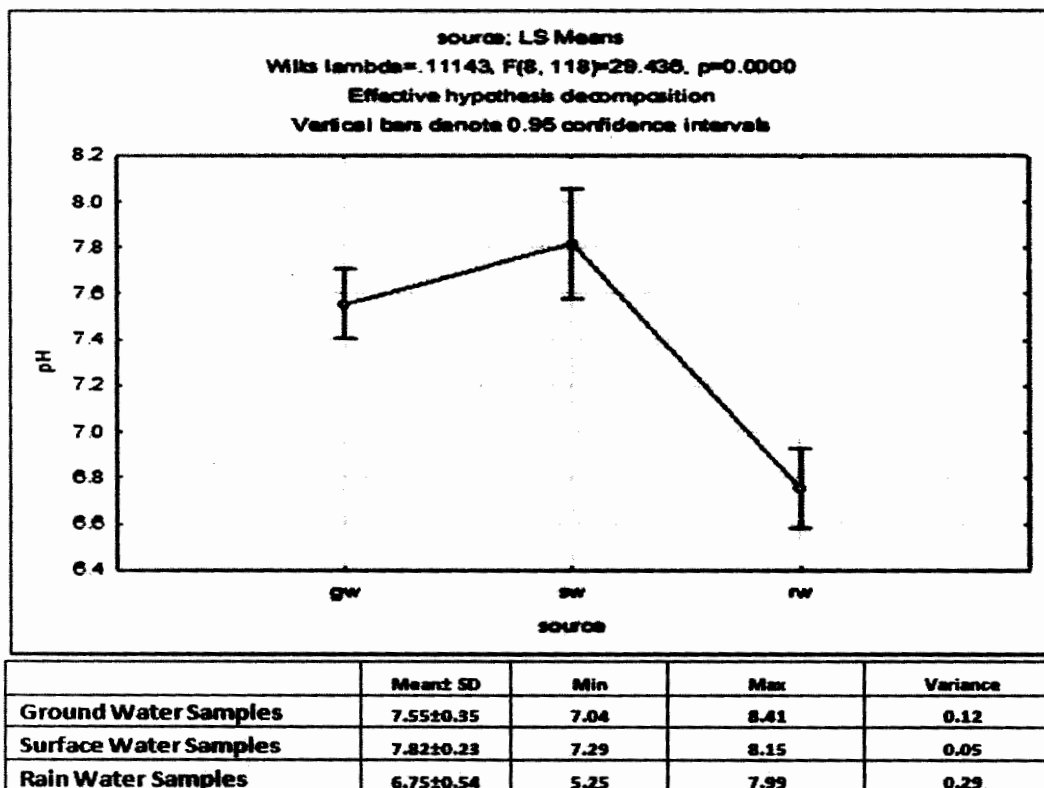

source: LS Means
Wilks lambda=.11143, $F_{(8, 118)}=29.436$, p=0.0000
Effective hypothesis decomposition
Vertical bars denote 0.95 confidence intervals

	Mean± SD	Min	Max	Variance
Ground Water Samples	7.55±0.35	7.04	8.41	0.12
Surface Water Samples	7.82±0.23	7.29	8.15	0.05
Rain Water Samples	6.75±0.54	5.25	7.99	0.29

Figure 3.1 pH of water samples

3.1.1.2 Electrical conductivity

Ground-water had highest EC values than surface-water. Rain-water had comparatively very lower values. In Islamabad lowest EC values were observed in Rawal town area and highest values in F-10/1 ground-water. Most of the Ground-water had EC ranges up to 800µS/cm.

The EC values of Rawalpindi ground-water were in the range of (227-1038 µS/cm) which was higher than Islamabad groundwater. Lowest EC values were observed in college road (Rawalpindi) and highest values in Gawal Mandi (Rawalpindi) ground-water. Most of the Ground-water had EC range between (0-600 µS/cm).

It is apparent from the graph that highest EC values were observed in ground waters than surface and rain water. EC of the Nala-Lai was significantly higher than lake waters. This is attributed to domestic /industrial waste input into Nala-lai. Chaudhary *et al.,* (2009) had observed that EC of surface-water compared to depth water profile o water bodies is season dependent. The EC of surface-water in hot and dry seasons is higher due to high evaporation rate from surface-water thus increasing salt contents, whereas in humid seasons the EC of surface-water is low due to very little evaporation. As the sample collection was carried out during monsoon season, so this may be the reason of low EC values of these lakes. High values of EC are related to enhanced concentration of soluble inorganic salts in ground-water of twin cities. Apparently, the reason for such high concentration of salts in ground-water is due to geographical location of study area across the rocky mountainous region namely Margalla Hills. Thus excessive soluble salts in ground-water of the area may be on an account of dissolution of minerals from local soil and bed rock; nevertheless, few samples with comparatively less EC values were of those sites which are farther from mountainous area.

Lowest EC values were observed in sector H-10 residential area and highest in PINSTECH colony rain-water at Islamabad. EC values of rain-water of surrounding area (Attock, Hasan abdal, Khan pur, Hawelian, Taxila, Wahcantt.) were in the range of 9.8-728 µS/cm. Lowest EC values were observed in Khanpur and highest EC values in Attock area due to presence of nearby oil fields.

Electrical Conductivity (EC) values of rain-water of Rawalpindi area were in the range of 43.0–173.4 µS/cm. Lowest EC values were recorded in rain-water collected from Kohinoor area, whereas highest EC value was found in Shamsabad area,due to

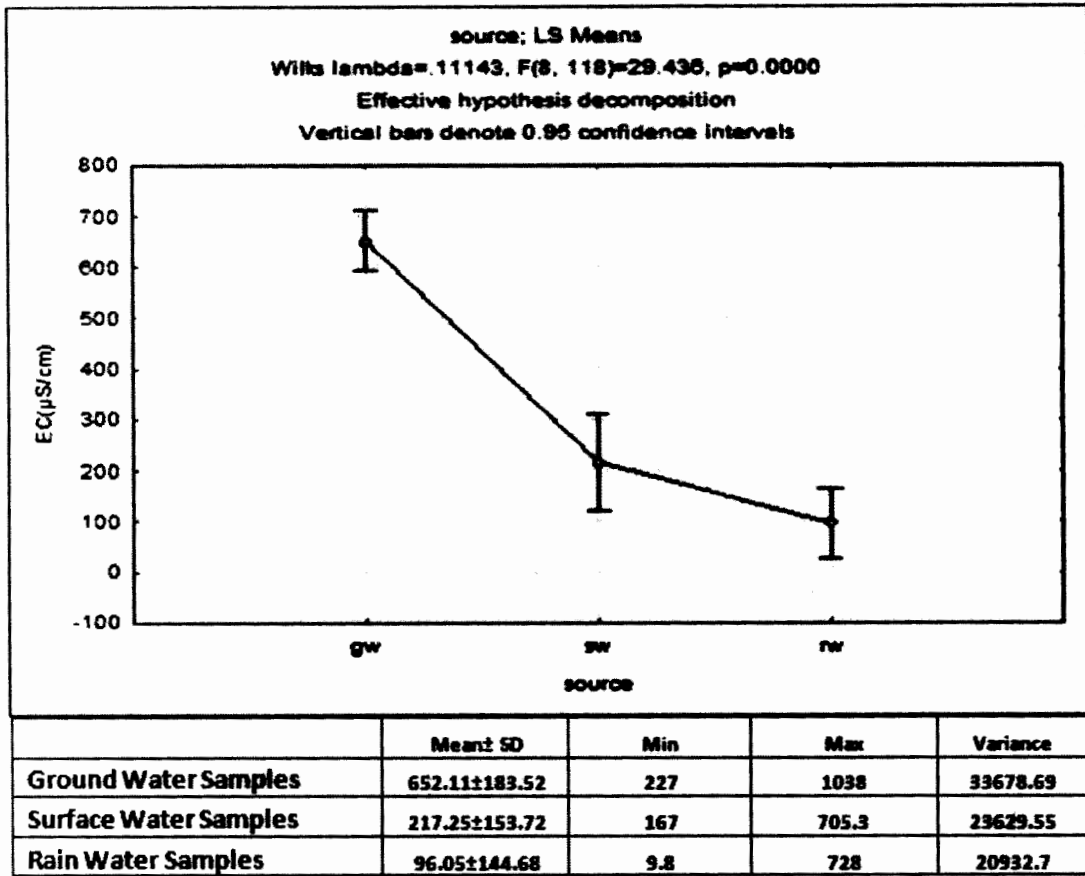|	Mean± SD	Min	Max	Variance
Ground Water Samples	652.11±183.52	227	1038	33678.69
Surface Water Samples	217.25±153.72	167	705.3	23629.55
Rain Water Samples	96.05±144.68	9.8	728	20932.7

Figure 3.2 EC of water samples

heavy traffic loaded area. The EC values of Islamabad rain-water was within the range of $15.0 - 157$ µS/cm.

A significant positive correlation existed ($r=0.9$) between nitrate concentration and EC is presented by positive linear correlation in figure3.3.
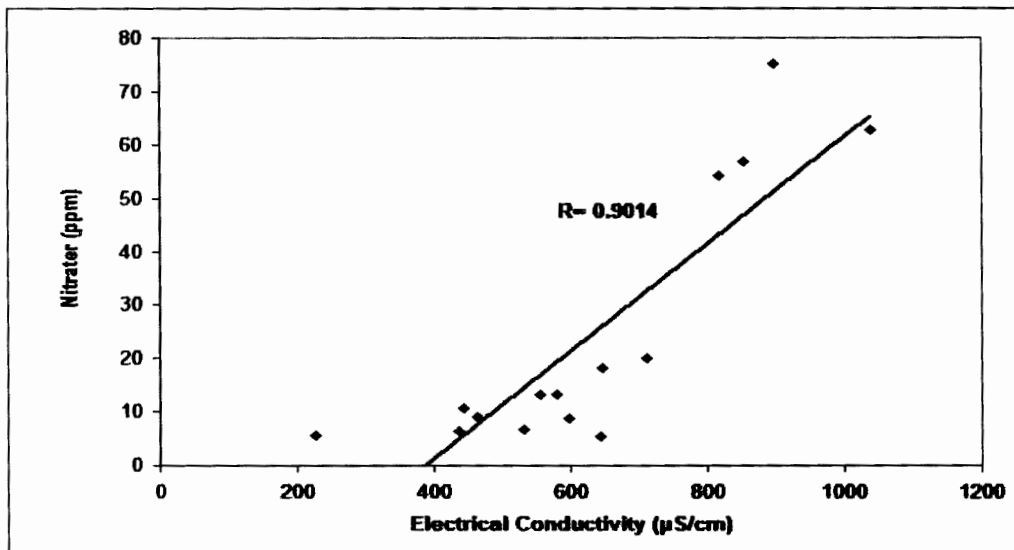

Figure 3.3 Correlation between Nitrate concentration and EC in groundwater

3.1.1.3 Total Dissolved Salts (TDS)

The TDS values of water samples follow the same pattern as EC values. Highest in ground-water than surface and rain depicted by the Figure 3.4. Lowest TDS values of Islamabad ground-waters were observed in sector G-9/4 and highest values in sector G-10/2 ground-water. Most of the Ground-water had EC range between 0-500 mg/L.

The TDS of the Nala-lai was significantly higher than Lake Water. WHO (1984) guideline for total dissolved salts (TDS) is 1000 mg/L, Low values of TDS in ground water accounts for possibility of contamination with dissolved hydrocarbons, which reduces specific conductance as hydrocarbon components present higher resistivity. On the other hand, it is also possible that there could be an increase of specific conductance of ground-water due to presence of polar organic compounds in the form of organic acids and bio-surfactants which are produced during degradation Cassidy *et al.,* (2001). High values of TDS suggest that hydrolysis of sodium (Na) or potassium (K)
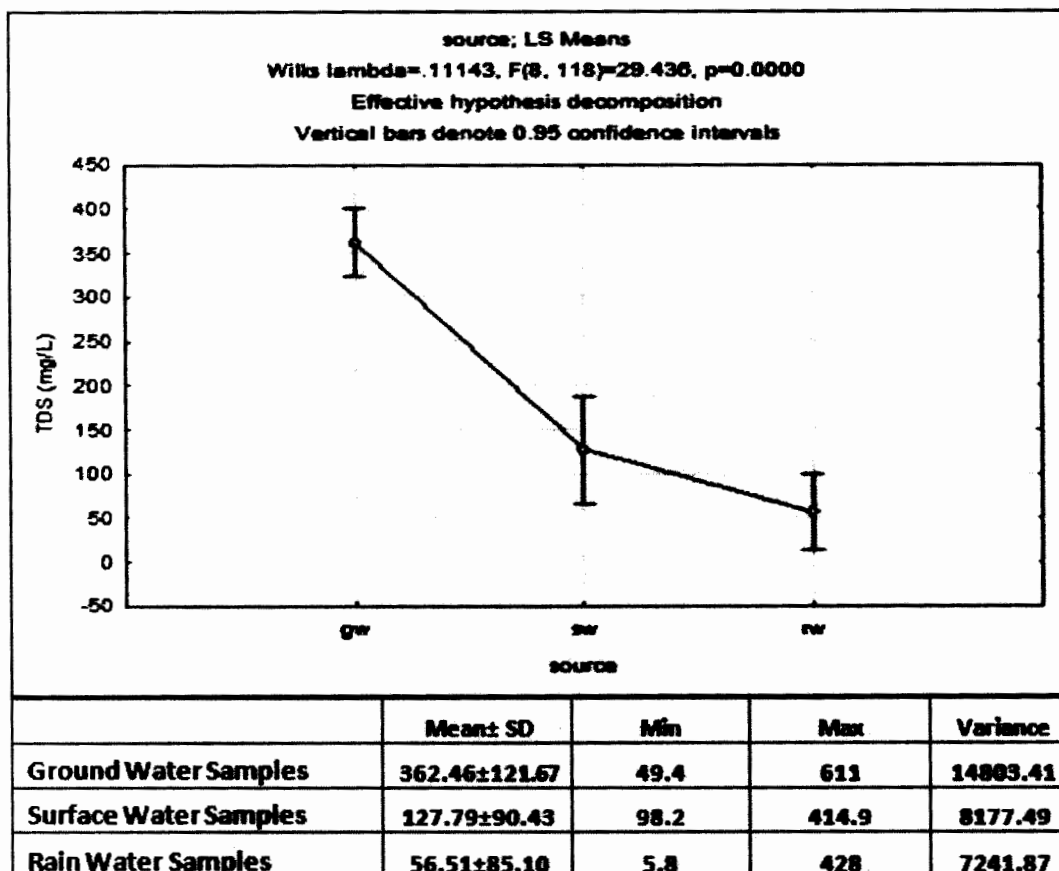

source; LS Means
Wilks lambda=.11143, $F_{(8, 118)}$=29.436, p=0.0000
Effective hypothesis decomposition
Vertical bars denote 0.95 confidence intervals

	Mean± SD	Min	Max	Variance
Ground Water Samples	362.46±121.67	49.4	611	14863.41
Surface Water Samples	127.79±90.43	98.2	414.9	8177.49
Rain Water Samples	56.51±85.10	5.8	428	7241.87

Figure 3.4 Total Dissolved Salts in water samples

silicates, is a countable factor in the chemistry of ground-water (Chae *et al.,* 2006).

Lowest TDS value was observed in rain-water collected from Kohinoor area, whereas highest value of TDS was found in Shamsabad area in Rawalpindi. The TDS values of Islamabad rain-water were in the range of 8.8 - 92.7 mg/L. Lowest TDS values were observed in sector H-10 residential area and highest in PINSTECH colony rain-water at Islamabad. TDS values of rain-water of surrounding area were in the range of 5.8-428.2 mg/L. Lowest TDS values were observed in Khanpur and highest in Attock area.TDS results had shown the same pattern as EC. As most of the rain water had TDS value above 50 mg/L, so the studied zone is polluted. High TDS values are due to suspended and dust particles in the atmosphere.

3.1.2 Chemical parameter

3.1.2.1 Validation of HPLC-Based Method for Nitrate Measurement

The analytical conditions have been optimized by varying the concentration, composition and pH of mobile phase reported by Butt *et al.*, 2001. Beside, UV detector response at various wave lengths, and flow rate have been optimized to get linear calibration curves. By injecting standards of different concentrations and measuring peak time indicated that the peak time was 3.2 minutes irrespective of analyte concentration (Figure 3.5). The peak separation time 3.2 minutes after injection was used for later analysis. Butt *et al.*, (2001) observed peak time of >5 minutes but the difference observed due to high concentration of mobile phase. Mobile phase concentration range of 1 to 5 mM of various ion-pairing reagents viz TBA-Cl and tetra-ethyl ammonium chloride (TEA-Cl) were tried for the separation of nitrate. This study achieved the best results when mobile phase comprising 80 % (v/v) 6.0 mM TEA-Cl (sodium dihydrogen phosphate (4.0 mM) as buffering reagent) and 20% pure acetonitrile was used. Mobile phase of pH 3.0 was obtained by 0.5 M H_3PO_4 (as per requirement), and it was passed through 5μm particle size C-18 reversed phase column having 150 mm and 4.5 mm inner diameter. The liquid passed at a flow rate of 0.5mL /min and nitrate detected at optimized wave length of 210 nm ultraviolet radiations.

Under optimum conditions, Nitrate could be determined without any matrix anions interference at even low concentrations in samples. In addition, the total analysis time was less than 5 minutes suggesting that the method had considerable potential for the rapid screening of environmental samples and food products for nitrate. The optimized condition for fast nitrate separation is shown in Figure 3.5. Nitrate exhibit

significant UV absorption at wavelengths of roughly 240 nm and below. Our choice of 205-210 nm as a detection wavelength limit simply represents a compromise between signal response for the analyte and back ground noise levels.

3.1.2.2 Analytical Performance Characteristics

Detector linearity was investigated by standard calibration curves constructed over the range 0.1–400 mg/L for nitrate with $R2$ >0.99 in water, rain and food samples. The methods applied to various environmental samples were reproducible. On the basis of results and its reproducibility it can be suggested that this methodology can prove useful in environmental monitoring of nitrate.

The proposed methodology was applied for the determination of nitrate in various kinds of water sample (surface, rain and groundwater).The peaks involving species other than nitrate in these chromatograms were not confirmed except water.
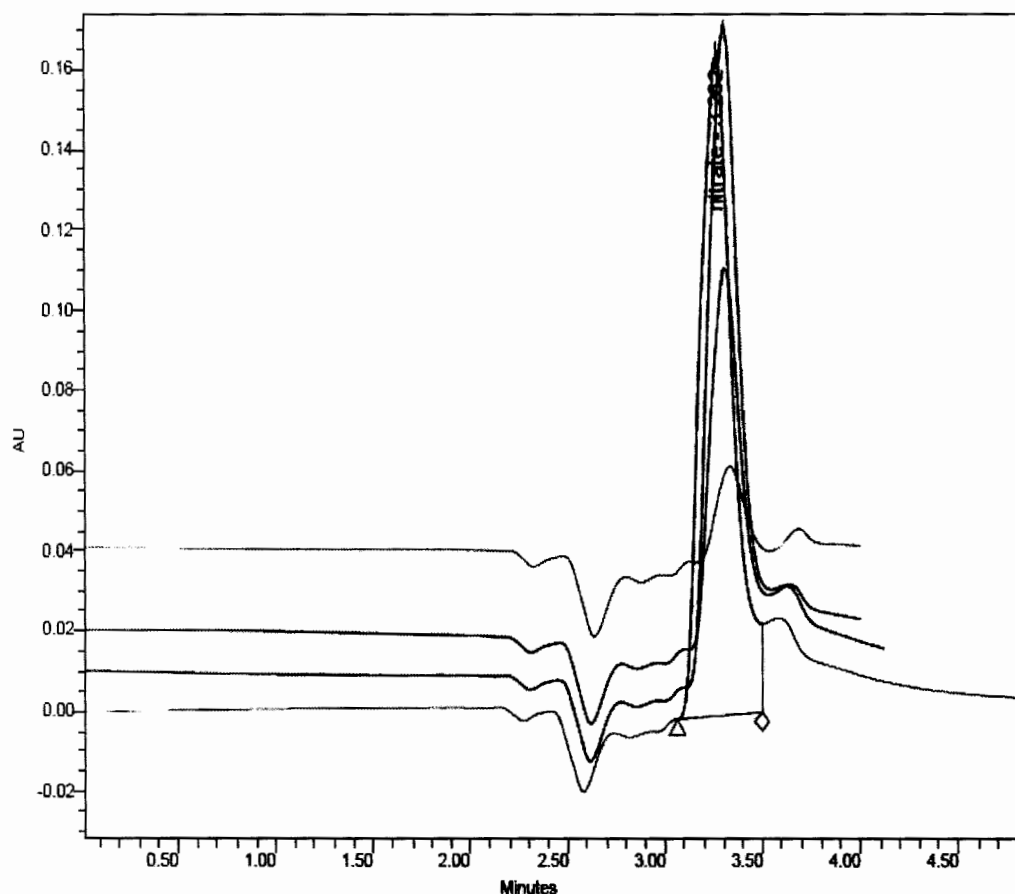

Figure 3.5 Nitrate standard peaks

The extraction and detection method used would not affect nitrate determination in meat and vegetables samples. pH was monitored and maintained to minimize

conversion of nitrite to nitrous acid or nitrous oxide. In post harvest storage of vegetables the action of indigenous bacteria or the presence of nitrate reductase enzymes especially when they are left at room temperature cause an increase in nitrate concentrations in vegetables(Ozdestan & Uren, 2010).

3.1.2.3 Nitrate assessment in water samples

This section comprises nitrate assessment in all natural water to determine nitrate concentration and sources of pollution. The results for nitrate concentration are presented in Figure 3.6 for ground-water, rain and surface-water from Rawalpindi, Islamabad and surrounding areas. The graph depicts that maximum nitrate concentration values had observed in ground water and rain water had lowest concentration. Most of Ground-water had nitrate concentration ranges between 0 to 40 mg/L. The Nitrate concentration of Islamabad ground-water was in the range of (3.9-83.02) mg/L. Lowest Nitrate concentration was observed in sector E-8, Barma chowk, Rawal town and sector H-9/2 area and highest in sector F-10/1 ground-water.

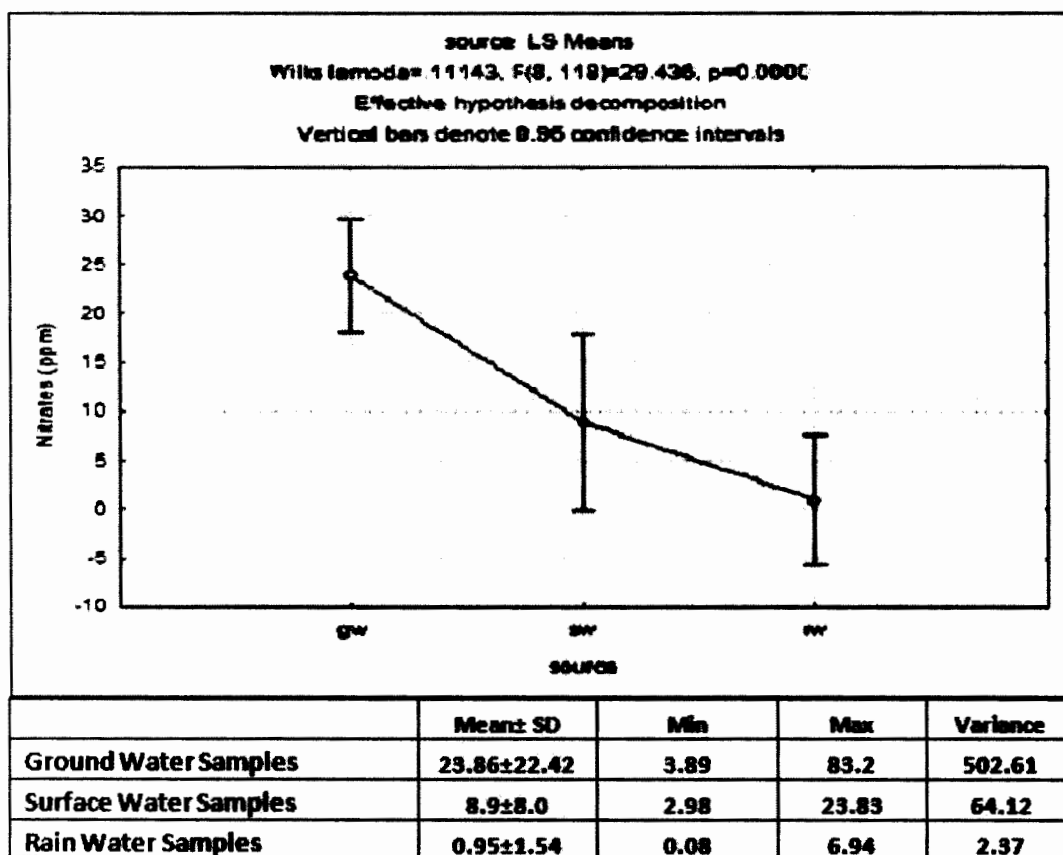|	Mean± SD	Min	Max	Variance
Ground Water Samples	23.86±22.42	3.89	83.2	502.61
Surface Water Samples	8.9±8.0	2.98	23.83	64.12
Rain Water Samples	0.95±1.54	0.08	6.94	2.37

Figure 3.6 Nitrate concentration in water samples

The Nitrate concentrations in most of the Rawalpindi ground-water were in the range of (8.6-75.21) mg/L. The mean and standard deviation of nitrate concentration in

Rawalpindi ground-water samples were 19.13±19.57 mg/L. Lowest Nitrate concentration were observed at Murree road and highest in Gawal Mandi ground-water. Four groundwater samples had nitrate concentration above permissible limits of drinking water set by WHO (45 mg/L) in Rawalpindi ground-water. All these tube wells were close to Nala-Lai and infiltration of waste water into aquifer resulting in increased nitrate levels. It is also notable that electrical conductivity (EC) values of these tube wells were higher than rest of tube wells (>800).Water samples from Rawalpindi city had high concentration of nitrate as compared to Islamabad groundwater. In present study, the mean and standard deviation of nitrate concentration in Islamabad ground-water samples were 32.64±25.72 mg/L. Nitrate concentration in Islamabad ground-water samples were generally less than permissible limits set by WHO (45mg/L) and 90 % of samples had less than standard levels. Only 2 out of 20 samples (10%) had nitrate Concentration above set standard levels. The increased population and intensive agricultural activities can be the reason for nitrate contamination (Liebscher *et al.,* 1992). Low nitrate concentrations (5-7 mg/L) were recorded in water samples of tube well located in vicinity of Rawal dam and Soan River (G 18, G19, G 20 and G16). It appears that these tube wells are being recharged from Rawal Lake and Soan River water (WHO, 2004).

Nitrate concentration in ground-water of this study was quite similar to nitrate concentration found in ground-water around the globe. In a study carried out in Bahar city in Hamadan province in Iran during 2006, nitrate concentration in 30% of groundwater samples out of 135 wells were above 44 mg/L. The maximum concentration found was as high as 75 mg/L (Jalali & Kollahchi, 2005). Farshad & Imandel in 2002 observed that nitrate and nitrites concentration in water samples (located in the outskirts of Tehran) were 51.96 mg/L and 5.9 mg/L, respectively and they were above WHO limits and the national guidelines. A survey carried out in an agricultural area of Canada (New Brunswick), nitrate concentration in approximately 20% of the 300 wells sampled exceeded WHO limits (Ecobichon *et al.,* 1985). In another survey of ground-water in Canada, 60% of wells were observed with nitrate concentration exceeding 45mg/L. The maximum concentration was recorded 182 mg/L. The population load and increased agricultural practices can be a reason for nitrate contamination (Liebscher *et al.,* 1992). However, nitrate concentrations in Canadian municipal water supplies are generally less than 5 mg/L and only a few samples had a nitrate concentration greater than 45 mg/L ([Environment New

Brunswick, 1983). Among various sources of nitrate contamination one is fertilizer applications. It is well established fact that that most of the nitrate lost from crop fields moves through subsurface tile flow. Nitrate can accumulate in soils over years and spring rain fall may flush it into tiles and streams. Nitrate in water is derived from three primary sources, rain fall, nitrogen amendments (fertilizers, manures etc.) and decomposition of soil organic matter.

Analytical data on nitrate concentration in ground-water was compared with WHO guidelines value for nitrate that is, 45mg/L (annexure II) in order to evaluate the status of nitrate contamination (WHO, 1996a ; WHO, 1996b). It was revealed that 40 % samples have high concentration of Nitrate in Rawalpindi area while only 10% samples were higher nitrate concentration than permissible limits in Islamabad ground-water. High concentration of nitrates in ground-water samples may be due to various factors. A heavy dose of nitrogen oxide is being released into the environment by industries, fertilizers, livestock, manures and atmospheric sources are among the top contributors to nitrate contamination of underground-water supplies. Analytical findings of the study give rise to the prediction that nitrate concentration is more commonly found in the ground-water of agricultural regions and sources like waste drains (Nala-Lai), stagnant ponds, sewage etc. may be the multiple reasons of nitrate contamination in Rawalpindi/ Islamabad ground-water. Ground-water from hand pumps, wells which is drawn from relatively shallow aquifer and shallow ground-water is more susceptible to nitrate contamination particularly in areas with more porous and well drained soils. Concentration of nitrate in the well water depends on the type of soil and bedrock present, and on the depth and construction of the well. It may occur in both shallow and deep well supplies however; generally shallow wells less than 120 feet deep are more susceptible to nitrate contamination, where soil is porous and the underlying material is gravelly. Since, it is very soluble and completely mobile in dissolved form so it can readily move with water through the soil and heavy rainfall or over irrigation moves the nitrate into ground-water systems that may be used for drinking purpose. In general, nitrate concentration is highest in ground-water near the land surface where nitrogen sources are present (Hallbety *et al.,* 1993). Ground-water that occurs in fractured rocks in mountainous area typically flows in stream. Thus, nitrates that were initially lost through leaching to ground-water can contribute to the pollution of surface-water such as streams, rivers and lakes. Similarly, areas with shallow water table or sinkholes are more vulnerable to

nitrate contamination, because, they do not evaporate, Nitrates are likely to remain in water until consumed by plants or other organisms.

Signs and symptoms of medical complexities of excessive nitrate concentration at extensive scale had not been observed in the examined region however, the problems of hyperthyroidism (goiter) or the insulin dependent diabetes may possibly be prevalent in the affected regions due to excessive nitrate in take through ground-water and it is left to be investigated. In addition to chemical processes, various multiple mechanisms which govern the movement and growth of the nitrate in the prevailing hydro-geological environment include microbial denitrification, volatilization of ammonia and uptake of nitrogen by plants (Tahir & Rasheed, 2008). From the research point of view it is recommended that an insight into physical, chemical and biogeochemical processes is useful in developing a long term projection for the groundwater quality of the study areas and in evolving appropriate surveillance strategies to check nitrate in the aquifers. Numerous cases of nitrate intoxication has been observed by WHO as a result of ingestion of water contaminated with high concentration of nitrate, almost 98% of which were associated with nitrate concentrations in the range of 44 - 88 mg/L (WHO, 1985).

In Nala-Lai nitrate concentration was higher than the WHO standards while nitrate concentration in lake waters was quite below the permissible limits set by WHO for drinking water and was almost negligible in some samples. High concentration of nitrate in Nala-Lai might be attributed to domestic input. The connection between domestic waste/ agricultural activity and stream nitrate loadings cannot be denied. Elevated concentrations of nitrate have often been noted in streams draining high levels of domestic production, nitrogen fertilizer application, and surface drainage. Run off from uncontrolled livestock activities can also be a causal factor. In the case of livestock, improvement in waste handling can minimize runoff, but the situation regarding cropland is comparatively complex. Nitrate concentration in rain-water of Rawalpindi area were in the range of 0.15-3.45 mg/L with average of 0.86 mg/L. Lowest Nitrate concentration was recorded in rain-water collected from Kohinoor area, whereas highest nitrate concentration was found in Pirwadhai. The Nitrate concentration of Islamabad rain-water was in the range of 0.08 - 2.44 mg/L with average of 0.53 mg/L. Lowest Nitrate concentration were observed in sector H-10 Islamic university and highest in Karachi company rain-water. Rain-water of

surrounding area was in range 0.13 – 6.94 mg/L with average of 1.74 mg/L. Lowest Nitrate concentration was observed in Khanpur and highest in Attock area.

These concentrations suggest that a range of sources contribute to rain chemistry, such as industries, terrestrial, agricultural, fossil fuel burning and marine. There was a relatively small difference in the mean ionic nitrate concentration at Rawalpindi and Islamabad. However, the differences in rain of surrounding areas suggest variation in rain chemistry. This is because rain events are influenced by dominating source at the time of the event. In some previous studies, (Galloway *et al.,* 1998; Galpin & Turner, 1999; Alastuey *et al.,* 2001) analyzed rainwater samples for studying the chemical composition of rainwater, and understanding of the source types that contribute to rainwater chemistry(L.oye-Pilot *et al.,* 1986 ; Ayres *et al.,* 1995 ; Marquardt *et al.,* 2001) studied the effect of power station emissions on rain quality and the influence of Sahara dust on rain acidity to illustrate contributions from anthropogenic and natural sources to rain chemistry. Galpin & Turner (1999) found South African rainwater chemistry to be influenced by a combination of industrial, terrestrial, biomass and marine sources.
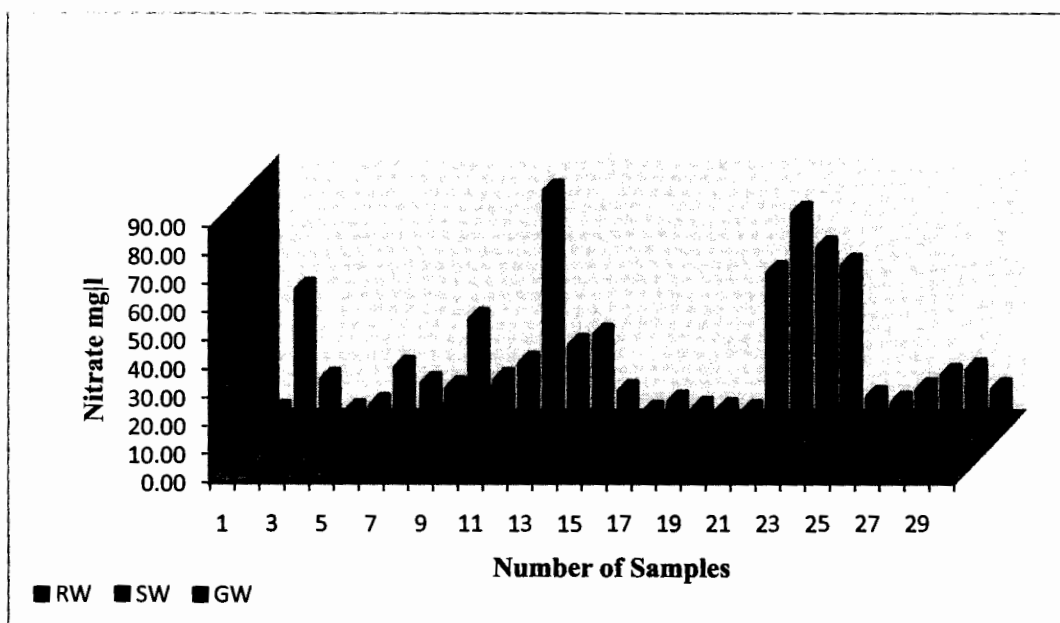

Figure 3.7   Concentration of Nitrate in water samples

Nitrates arise mainly from NOx gases. Natural and anthropogenic N compounds are emitted to the atmosphere as oxides of N (NO_XS), ammonia, and organic-N (Finlayson-Pitts & Pitts, 1997). Anthropogenic activities are the main source of inorganic-N (e.g., NO_XS) to the atmosphere. For example, at Pirwadhai area in Rawalpindi and Karachi Company in Islamabad where the dominant sources of the

Nitrate compounds were from fossil fuel combustion in motor vehicles and electrical power generators. Due to anthropogenic sources there is an elevated concentration of nitrate in rain-water of these areas. In surrounding area rain-water nitrate concentration was higher in Attock and Wah which are relatively bigger cities with lot of traffic, oil fields and industrial area (Hattar), so have high concentration comparatively. The relationship between atmospheric pollution and amounts of nitrate in rain-waters is evidenced by the fact that the highest concentrations are measured in areas with huge traffic load have, so the average nitrate concentration of Rawalpindi city was higher as compared to Islamabad. Figure 3.7 depicts concentration of nitrate in all water samples.

3.1.2.4 Concentration of Nitrate in Food Products

This section contains assessment of nitrate concentration in food stuff (vegetables and meat) to determine its concentration, source type and their concentration range in permissible limits.

3.1.2.4.1 Vegetables

A total of 12 vegetable samples were tested for nitrate concentration. These included spinach and cabbage collected from different areas grown under different environmental conditions. Samples were collected from agricultural forms of Attock , Hassanabdal, Wah Cantt and Rawalpindi/Islamabad. Nitrate concentration in spinach was determined in leaves as well as in stem due to difference of nitrate accumulation in different plant parts (Fytianos & Zarogiannis, 1999).

There was a large variation in mean concentration of nitrate in spinach from different localities. In Spinach leaves, nitrate concentration was in the range of 10.95-375.3 mg/kg and average is 103.6 mg/kg while in stem nitrate concentration was in the range of 106.5-276.8 mg/kg and average is 221.63. Maximum nitrate concentration in spinach leaves was observed in Attock (375.3 mg/kg) which was collected during monsoon period and low concentration was recorded in Rawalpindi area (10.8 mg/kg). In contrast nitrate concentration in stem was maximum in spinach from Islamabad (276.8mg / kg) and lowest in Wah Cantt (106.5 mg/kg). In general, except for two locations nitrate concentration was higher in stem than leaves of spinach.

Cabbage samples from 5 locations were examined for nitrate concentration. Nitrate concentration in cabbage was recorded as low as 253.85 mg/kg to as high as 319.85 mg/kg and average was 279.42 mg/kg. However nitrate concentration was not significantly different except Hasanabdal area. Many studies around the world have been conducted to measure the nitrate concentration in vegetables. Figure 3.8 depicts nitrate concentration in water and food samples. Food samples had higher nitrate concentration than water.
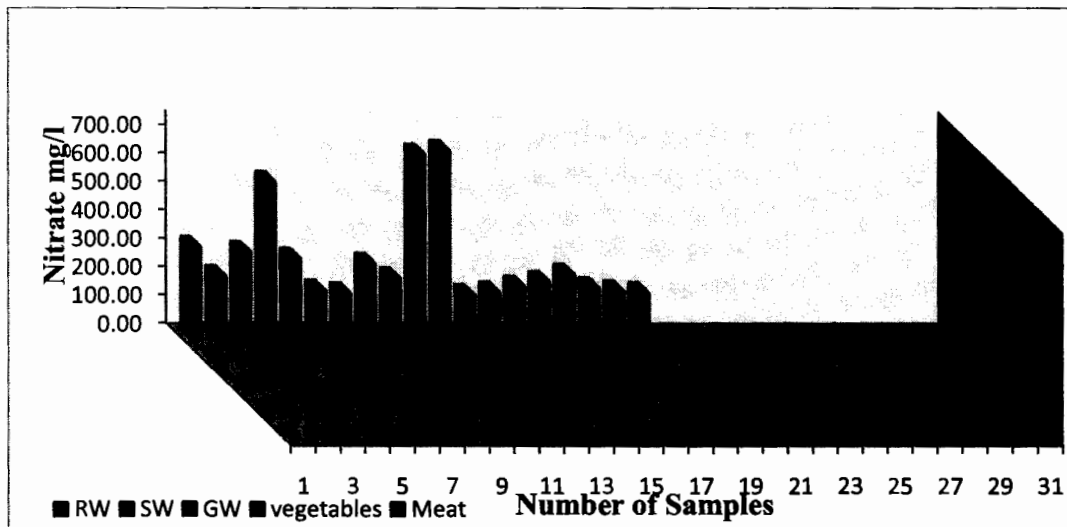

Figure 3.8 Concentration of Nitrate in water and food Samples

Nitrate concentration estimated in vegetables from Attock while other do not exceed the pollution level NO_3-N>325 mg/kg (Wang *et al.,* 2002). More recent surveys have shown no correlation between nitrate concentration and the incidence of met-haemoglobenemia until nitrate concentration exceeds 443 mg/kg. Many factors govern the nitrate concentration in crops, one of these is prevailing agricultural practices for instance it was observed that the nitrate concentration in vegetables which are cultivated in organic soils was higher than that grown under normal system. Environmental and microclimate conditions are important factor that governs the nitrogen accumulation in plant tissues. It has been observed that nitrate concentration in vegetables is affected by the high sunlight intensity. Nitrate concentration in plant tissues is increased by increasing the nitrate reductase activity which converts the nitrogen in plant to nitrate in high intensity light. Growing conditions such as stress, temperature, (drought), hot or dry, winds may affect the nitrate in plants (WHO, 1995). It has also been found that vegetables grown in winter, the time of year (with low temperature and less sunlight) has higher nitrate concentration (European

Commission, 1998). Vegetables grown in green houses (heated environment) have higher nitrate concentration than those grown in open fields, because of the lower light intensity, and higher nitrogen mineralization (McKnight *et al.,* 1999; Swallow, 2004). Different types of crops have different nitrate concentrations (Chung *et al.,* 2003). Leafy vegetables such as spinach, lettuce, and cabbage, and root vegetables such as carrots, beet, and broccoli, tend to accumulate large amount of nitrate though at a much lower level than do the leafy vegetables (Trembly *et al.,* 2001). The herbicide application may also affect the nitrate accumulation in plants. Other factors that may affect the nitrate concentration in vegetables are shading rate which increased the nitrate concentration (Pevicharova *et al.,* 1995), harvesting time (Wu & Wang, 1995), late harvested vegetables have the lowest nitrate concentration, the harvest date had no significant effect on either nitrate or nitrite concentration of the greenhouse grown vegetables. All these factors as well as other factors related to local environmental conditions might be the reason behind the variation in the results we obtained with that of other results.

The spinach collected during monsoon season from Attock and Cabbage from Hasan abdal show the highest nitrate concentration among all the other vegetables tested. All cabbages were small in size ,which has also an effect on nitrate concentration , growing plant accumulate more nitrate and use it for shoots and root development, as plant matures leaves use nitrate for photosynthesis and thus nitrate concentration reduces as plant matures. This shows high nitrate accumulation (Jarvan, 1993; sheehy *et al.,* 2004). In previous studies Cabbage had always shown lower nitrate concentration as (Chug *et al.,* 2003; Swallow, 2004), compared to spinach; our result is not in agreement with previous studies. The larger dark green colored leaves have large amount of chlorophyll, so they do not store nitrate and use it in protein synthesis, as it has been observed in current investigation that spinach has low nitrate concentration than cabbage.

In this study spinach leaves had low nitrate concentration as compared to stem. No previous study has been found to compare this result here it may be suggested that all vegetable samples had been collected during monsoon seasons and so due to lack of light intensity, nitrate in spinach tends to accumulate more in stem than leaves, as stem have vascular bundle, which can store more nitrate than leaves, when they are no longer in use for photosynthesis. Highest nitrate concentration had observed in

spinach leaves collected from Attock area, there may be two reasons, the crop could be irrigated from waste water (sample collected near industrial area) having high nitrate concentration that has accumulated in leaves or low light intensity may be the reason . However, in cucumber, the highest concentrations of nitrate were found in the neck, and skin tissues in some studies.

Nutrient medium might be another factor that could affect the nitrate concentration in spinach. The concentration of nitrate in all regions of the spinach was higher when nitrate constituted 75% or more of the total nitrogen in the nutrient medium, but was reduced by increasing the concentrations of the ammonium (NH_4).As mentioned earlier, comparison of nitrate concentration in stem and leaves had shown a lower nitrate concentration in leaves, and this might be due to various fertilization doses. As spinach is usually planted a complementary crop, therefore most of the farmers do not apply recommended dose of fertilizer. Nitrate concentrations in leafy vegetables are higher than the tuber vegetables. Green leafy and root vegetables tend to accumulate large amount of nitrate (Chug *et al.,* 2003; Swallow, 2004).

3.1.2.4.2 Meat

A total of 13 meat samples were examined for nitrate. These included meat in raw form as well as cured meat products, collected from different areas, markets and stores. In comparison, nitrate concentrations were found higher in meat as compare to vegetable (Figure 3.9).

Area	Spinach				
Attock	V-1(Leaves)	17.755	V-1cabbage	292.9	**Hotdogs**	331.2
	V-1(Stem)					
Attock	V-4(Leaves)	375.3			**Caned hot dogs**	313.2
	V-4 (Stem)					
Hasan abdal	V-2 (Leaves)	19.9	V-2cabbage	319.85	**Beef kabab (menu)**	84.1
	V-2(Stem)					
Wah cantt	V-7(Leaves)	221.15	V-7cabbage	253.85	**Nuggets**	166.7
	V-7(Stem)					
Khanpur	V-3 (Leaves)	18.95			**Chicken (desi)**	220.15
	V-3(Stem)					
Rawalpindi	V-5(Leaves)	10.95	V-5cabbage	270.85	**Mutton**	656.6

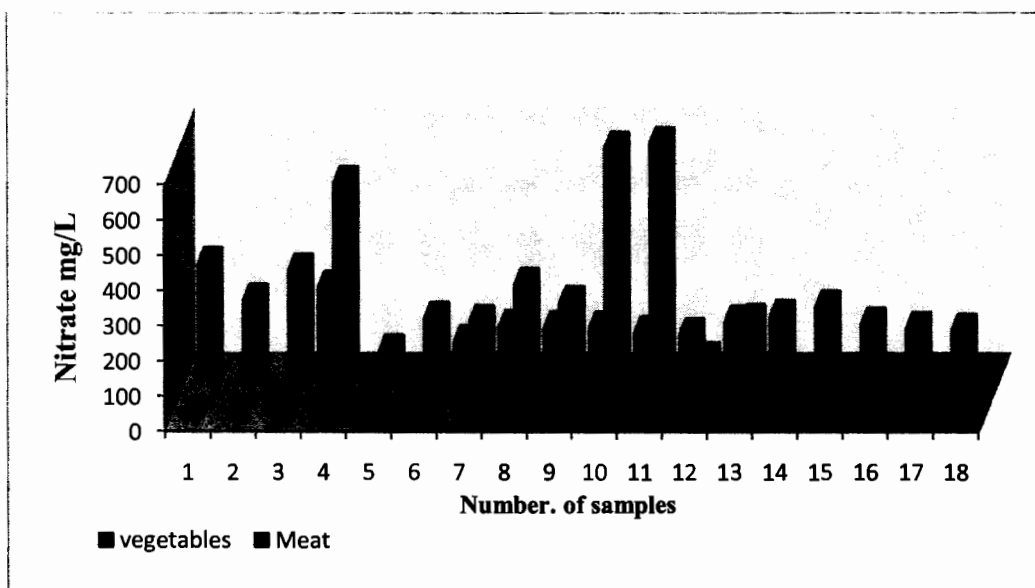|					
	V-5(Stem)	241.55			669
Islamabad	V-6(Leaves)	61.85	V-6cabbage	259.65	
	V-6(Stem)	276.8			

Figure 3.9 Concentration of Nitrate in Vegetable and Meat Samples

There was a high variation in mean concentration of nitrate in raw form and cured meat products. Nitrate concentration in raw meat ranges (61.5-669 mg/kg) and average was 341.54 mg/kg. Highest nitrate concentration was observed in fish collected from Rawal dam. Lowest was observed in farm-chicken of Rawalpindi. While in cured meat products the nitrate concentration ranges (84.1-558 mg/kg), average was 265.33 mg/kg. Highest concentration was observed in chicken bologna and lowest in beef kabab

Different countries have set their maximum limits for the addition of nitrate and/or nitrite salts as preservatives in cured meat. Under the Australian Food Standard Code 1.3.1 schedule 1,125 mg/L of nitrate in a form of potassium or sodium salt is permitted in dried, cured, and slow dried cured meat; whereas in commercially sterile and canned cured meat, the maximum nitrite (potassium or sodium salts) permitted is 50 mg/kg. For slow dried cured meat, the maximum allowed nitrate (potassium or sodium salts) is 500 mg/kg [FSANZ, 2007–2008). Nitrate concentration in various cured meat products was higher than maximum allowable limit set by Food Standards Australia and New Zealand (FSANZ) (1) at mg/kg. However, there the upper set limit

for fresh meat is 150 mg/kg (FSANZ). Maximum permitted level for residual nitrate concentration in final product in most of countries is 200 mg/kg (Hyytia *et al.,* 1997). Nitrate concentration was determined in the range of 84.1–558.5 mg/kg in cured meat products, and 61.65-669 mg/kg in raw meat. Concentration of nitrate remained higher than the set limits. Thus violation of maximum permitted limit.

CHAPTER # 4

CONCLUSION

&

RECOMMENDATIONS

4 CONCLUSION & RECOMMENDATIONS

4.1 Conclusions

The newly developed RP-IP- HPLC method was proved to be an excellent method for nitrate determination in environmental waters and food products as it is less time consuming. The maximum nitrate elution was achieved by using mobile phase comprising 80% tetra-ethyl ammonium chloride (6.0 mM) and 20% pure acetonitrile (4.0 mM sodium dihydrogen phosphate was used as buffering reagent). pH of mobile phase was adjusted at 3.0 (0.5 M phosphoric acid as per requirement is used). Nitrate peak was eluted at 3.2 minutes using optimized wave length of 210 nm in isocratic mode.

Among environmental matrices, ground water showed highest nitrate concentration than rain and surface water. Nitrate concentration in Islamabad rain-water was in the range of (0.08-2.44) mg/L. The lowest nitrate concentration was observed in sector H-10, whereas the highest nitrate concentrations observed in Karachi company rain-water. Most of the rain-water samples had nitrate concentration in range of 0-0.5mg/L. Nitrate concentration in Rawalpindi rain-water was in the range of (0.15-3.45) mg/L. Highest Nitrate concentration was observed in Pirwadhai area. The results indicate that high nitrate concentration was observed in heavy traffic loaded areas. The Nitrate concentrations in Rawalpindi ground-water were in the range of 5.41--75.21 mg/L. The 90% of Islamabad ground waters had nitrate concentration less than permissible limits set by the WHO 45mg/L. In Spinach, the leaves had nitrate concentrations in the range of 10.95 to 375.3 mg/kg, while in stem nitrate concentration was in the range of 106.5-276.8 mg/kg. Nitrate concentration in cabbage was estimated in the range of 253.85- 319.8 mg/kg, whereas in spinach nitrate concentration was higher in stem as compared to leaves due to low intensity of light. The concentration of nitrate in cabbage was high as compared to spinach. Highest Nitrate concentration was observed in Cabbage collected from Attock while others do not exceed the pollution level (NO3-N>325 mg/kg). Nitrate concentration in raw meat was (669-61.5 mg/kg) and the highest nitrate concentration observed in fish sample, collected from the Rawal dam. In all others surface-water nitrate concentration was found within permissible limits, set by the WHO except for Nala

lei, which had high nitrate concentration due to domestic and industrial inputs from nearby areas.

4.2 Recommendations for Future Study

For further research and studies following recommendations are made:

1. The several known standard methods for the determination of nitrate in food products and environmental waters are not suitable, because they require expensive instruments and chemicals. The optimized RP-IP-HPLC method is recommended for nitrate and other anion determination in environmental matrices and food products.

2. The literature on the occurrence of nitrate in food products especially in cured meat and baby foods is not available in Pakistan, Consequently, a study providing reliable data on the potential risk of excess nitrate exposure in Pakistan in infants is recommended.

3. Strict Legislations should be implemented to monitor the appropriate levels of nitrate in food commodities and in drinking water.

4. The water wells should be dug at distances from waste water ; moreover the depth of wells should be increased to avoid infiltration from the surface.

5. As being sole sources of water reservoirs for twin cities, Rawal Lake, Khanpur dam and Simli dam should be monitored through Environmental Protection (EPA) Agencies to check its chemical and biological contamination.

CHAPTER # 5

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ANNEXURE I

ANNEXURE II

Table 1 Maximum Nitrate permissible

| Environmental Samples	Maximum Level (mg NO₃/litre)	Law enforcing authorities
Ground Water	45 mg/L	WHO
Surface Water	45 mg/L	WHO
Rain Water	45mg/L	WHO
Food Samples	Maximum Level (mg NO₃/mg)	
Vegetable	325 mg/kg	FSNAZ (Food Standards
Meat	360 mg/kg (cured)	Australia and New Zealand)
	150mg/kg (raw)	

High Performance Liquid Chromatography (HPLC) (Water Breeze System)
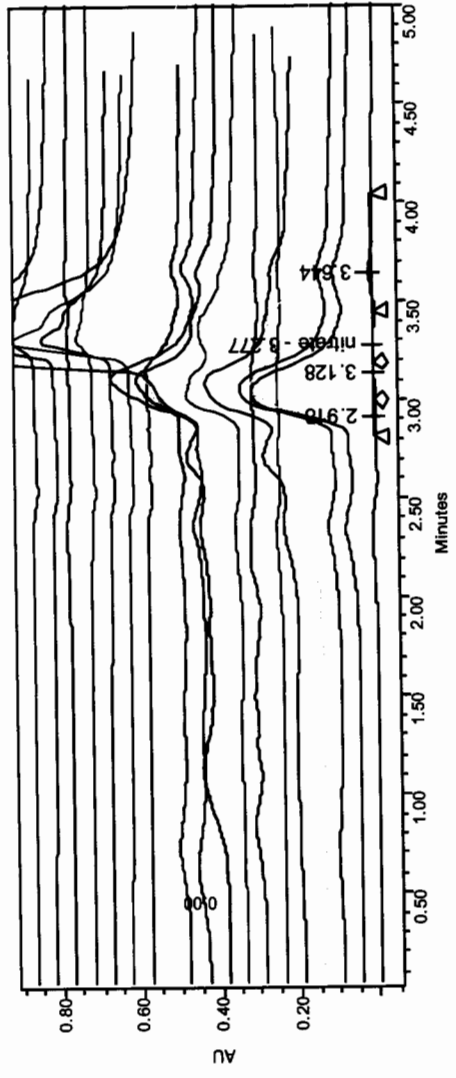
60
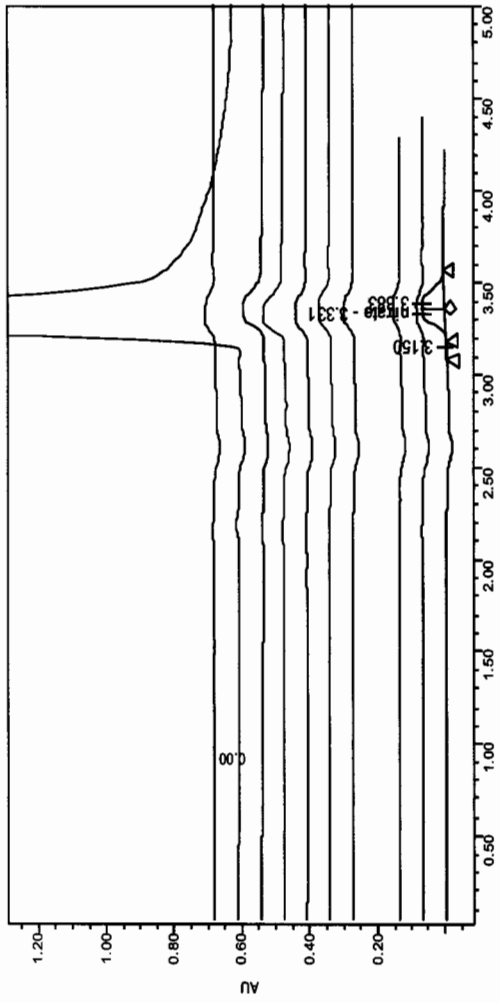
ANNEXURE III

Nitrate Peaks for Surface Water Samples
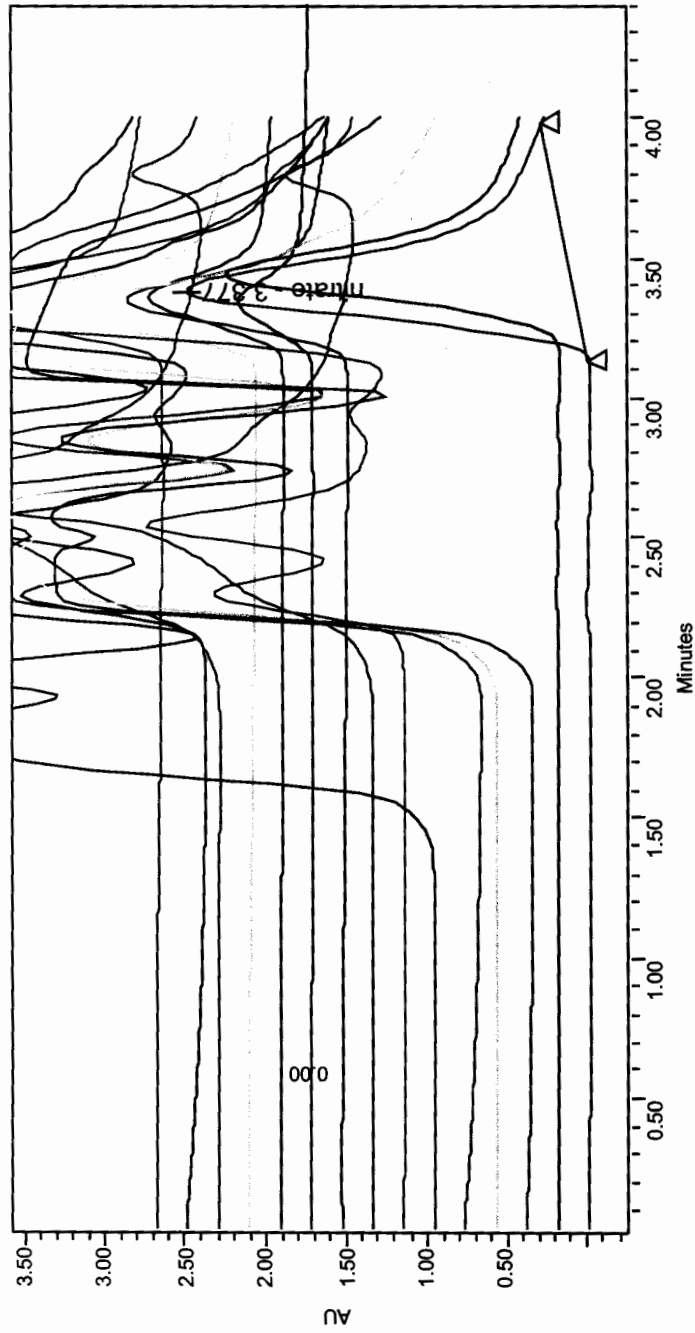
ANNEXURE IV
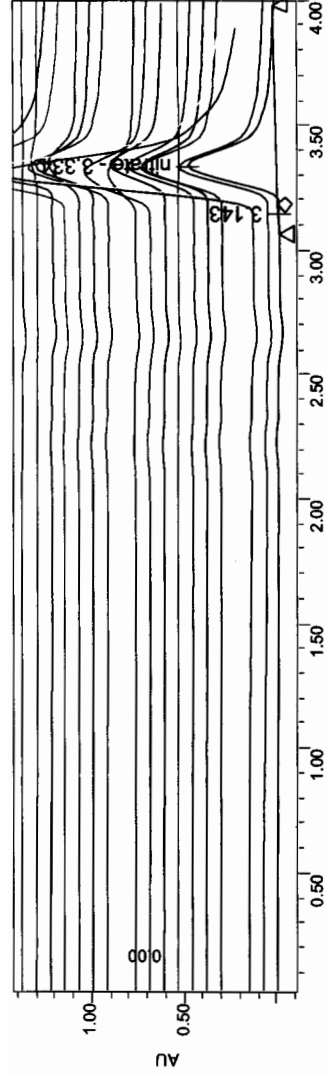
MinutesNitrate Peaks for Ground Water Samples

ANNEXURE V

Nitrate Peaks for Spinach (Leaves) Samples

ANNEXURE VI

Nitrate Peaks for Meat Samples