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Estimation of selected Nitrogen Compounds, Nickel and Phosphates in foodstuffs by continuous flow systems

by

Makhapa Abia Makhafola

Submitted in partial fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR

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Supervisor: Prof. J.F. van Staden

Department of Chemistry

University of Pretoria

Degree: Philosophiae Doctor

SYNOPSIS

Flow injection analysis offers distinct advantages in reproducibility, flexibility, sample throughput and cost performance. Since it was introduced in 1975, there are more than 10 000 publications to date.

The present study focuses on the development of process analysers for the determination of nitrogen compounds (namely, nitrate, nitrite and protein), nickel, and phosphate in various combination in samples originating from various fields, such as foodstuffs, water, and fertilisers. Nitrite is potentially unstable; it decomposes with time under acidic medium with an increase in

decomposition rate as the concentration of acid increases. Raman spectroscopy was employed as a means of determining the rate of decomposition of nitrite in solutions at various pH values.

A simple method for the determination of nitrite in foodstuffs by flow injection analysis (FIA) is described. The foodstuffs containing nitrite are digested in a microwave oven and then treated with 1 mol/ℓ NH_4Cl solution at pH 9. The simultaneous determination of nitrate and nitrite in foodstuffs and water was also studied. Cadmium (coarse powder) was used to reduce nitrate to nitrite. The effect of pH, length of reductor column, and various types of cadmium reductor on the yield of nitrite are investigated.

The flow injection method was developed for the spectrophotometric determination of nickel in cured meat. Dimethylglyoxime (DMG) in acetate buffer at pH 6.4 nickel forms a red complex which is measured at 475 nm. The effects of chemical and physical parameters in flow injection analysis were studied. A new bienzymatic amperometric sensor is proposed for the assay of proteins in milk. The sensor is based on two enzymes, namely, carboxypeptidases A and L-amino acid oxidase. The use of flow injection analysis and Raman spectroscopy for the determination of phosphate in foodstuffs and fertilisers, is also compared in this study.

Bepaling van geselekteerde stikstofverbindings, nikkel en fosfate in voedingstowwe deur kontinue vloeisisteme.

deur

Makhapa Abia Makhafola

Studieleier: Prof. J.F. van Staden

Department Chemie

Universiteit van Pretoria

Graad: Philosophiae Doctor

SAMEVATTING

Vloei-inspuitanalise bied besliste voordele met betrekking tot herhaalbaarheid, buigsaamheid, monsteromset en koste-werkverrigting. Sedert die aanvanklike bekendstelling in 1975 het reeds meer as 10 000 publikasies op die gebied verskyn.

Hierdie studie is gerig op die ontwikkeling van prosesanaliseerders vir die bepaling van stikstofverbindings (naamlik nitraat, nitriet en proteïen), nikkel, en fosfaat in verskeie kombinasies, in monsters afkomstig van verskeie terreine, soos voedingstowwe, water en

kunsmisstowwe.

Nitriet is potensieel onstabiel; dit ontbind geleidelik in suurmedium met 'n toename in ontbindingstempo soos die suurkonsentrasie verhoog. Ramanspektroskopie is gebruik om die ontbindingstempo van nitriet in oplossings by verskillende pH-waardes te bepaal.

'n Eenvoudige metode word beskryf vir die bepaling van nitriet in voedingstowwe deur middel van vloei-inspuitanalise (*Flow Injection Analysis* - FIA). Die nitrietbevattende monsters word in 'n mikrogolfoond verteer en dan behandel met 'n 1 mol/ℓ NH_4Cl oplossing by pH 9. Die gelyktydige bepaling van nitraat en nitriet in voedingstowwe en water is ook bestudeer. Kadmium (growwe poeier) is gebruik om nitraat na nitriet te reduseer. Die effek van pH, reduktor-kolomlengte, en verskeie tipes kadmiumreduktor op die nitrietopbrengs is bestudeer.

Die vloei-inspuitmetode is ontwikkel vir die spektrofotometriese bepaling van nikkel in gepekelde (geprosesseerde) vleis. Nikkel vorm 'n rooi kompleks met dimetielglioksiem (DMG) in asetaat-asynsuurbuffer by pH 6,4, wat by 475 nm gemeet kan word. Die effekte van chemiese en fisiese parameters in vloei-inspuitanalise is ook bestudeer. 'n Nuwe biënsimatiese amperometriese sensor word voorgestel vir die bepaling van melkproteïene. Die sensor berus op twee ensieme, naamlik karboksipeptidase A en L-aminosuuroksidase. Die gebruik van vloei-inspuitanalise en Ramanspektroskopie vir die bepaling van fosfaat in voedingstowwe en kunsmisstowwe word ook in hierdie ondersoek vergelyk.

DEDICATIONS

I would like to dedicate this project to my Dear Wife, Noella, for her incredible endurance of flow injection-induced loneliness, and to my children Motheo and Kagišo, who limited their fair complaints about lost weekends and holidays to a reasonable minimum. This is also to my Mother, Gertrude, who is no longer with us but her word of encouragement will always be with me. Robala ka kgotso Mama.

I LOVE YOU GUYS

THE MISSING PART

ACKNOWLEDGEMENTS

Thanks to God in the name of Jesus for the gift of life and career. I would like to thank my promoter Professor Koos van Staden, for guiding me through difficult times. This project would not be completed without your guidance. To my Father (Solomon), brothers (James and Lleka) and sisters (Lobisa and Selina) thank you, may God bless you. To Dr R.I. Stefan, thanks for your contribution.

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CHAPTER 1

FLOW INJECTION ANALYSIS-AN INTRODUCTION

1.1. Introduction

Flow injection analysis (FIA) originated from the concept of air segmented flow analysers (SFA) which were initially given major prominence in the early 1960s with the introduction of commercially available air segmented analysis systems [1-2]. Air segmented flow analysers were widely used in clinical laboratories for automatic routine determination of a variety of species in blood and urine samples for medical diagnostic purposes. By the mid-seventies Růžička and Hansen [3] in Denmark and Stewart and co-workers [4] in the USA blew a fresh breeze through chemical analysis known as flow injection analysis. Since then the FIA technique become known as a fast, flexible and adaptable way of doing automatic wet chemical analysis.

Růžička and Hansen [5] define FIA as "an information-gathering from a concentration gradient formed from an injected well defined zone of a fluid, dispersed into a continuous unsegmented stream of a carrier". Flow injection has been applied to colorimetric methods using well established chemistries also found in both air segmented and discrete analysis systems. Such analysis can be simple involving no more than the addition of the sample to the moving reagent/carrier stream. FIA is applicable to other detection systems, for example, ion selective electrode or as a means of sample introduction to atomic absorption spectrometry.

FIA consists of four major components (Figure 1.1.), namely,

(1) A propelling system, which transport the carrier stream to the detector.

(2) An injection system, introducing the liquid sample into the carrier stream.

(3) A reaction zone, to introduce reactants and achieve the appropriate mixing in the moving stream.

(4) Detector, continuously monitor the flowing stream.

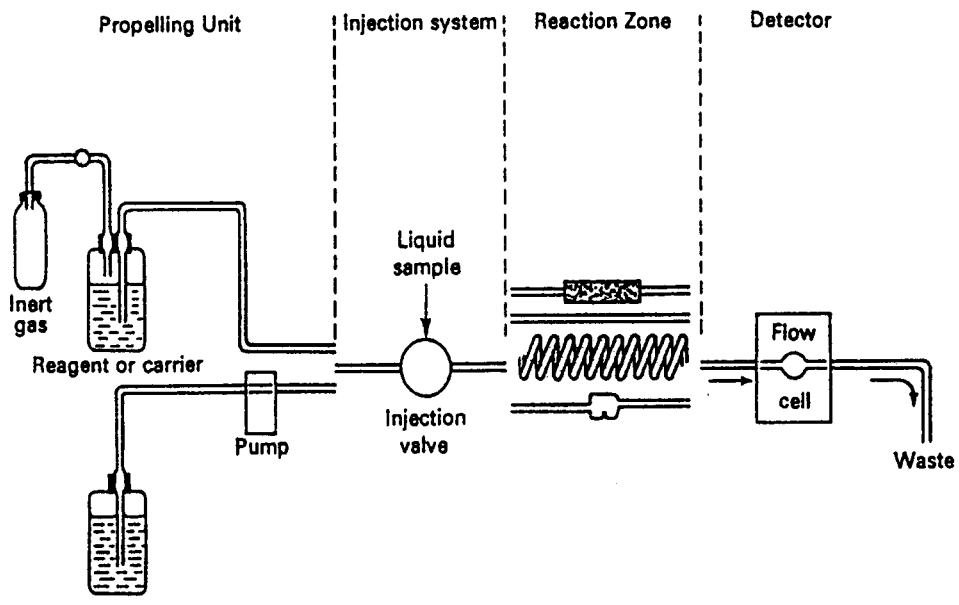

Figure 1.1.: Schematic diagram of a single line FIA [6]

In FIA a reagent (colorimetric) is moved by a peristaltic pump (propelling system) directly into an injection port which permits injection of samples into flowing stream. The sample and reagent then pass through a reaction zone (reaction coil) where they mix and produce a signal at the detector. Excess dispersional and cross contamination are avoided in properly designed systems without air bubbles and where mixing of samples and reagent could be easily realised [7].

FIA differs from earlier air segmented systems in that the sample is injected directly into a moving stream without the addition of air. The sample-reagent zone mixes and reacts as it moves downstream towards the detector, the degree of dispersion is controlled by a variety of factors, their impact being specific to the analytical system in use. It is the control of this dispersion which is at the heart of the technique and coupled with short, highly reproducible retention times separates FIA from other continual flow analysers and provides the potential for sampling rates up to 200 per hour.

The concept of FIA is base on the following principles, (a) sample injection, (b) controllable sample dispersion, (c) reproducible timing, (d) variable flow rates, (e) high sample throughput and (f) absence of any stabilization time. This approach yields a highly reproducible readout even when the mixing is incomplete, the chemistry does not reach equilibrium and the signal transient.

1.2. Comparison between FIA and SFA

FIA is a concept of continuous flow analyser of discrete samples. The advantages of flow injection are: speed of analysis (according to Varcarcel and Castro [8] up to 54% of FIA methods in the literature up to 1987 had a sample frequency between 50-120 samples per hour); manifolds used to construct FIA systems are relatively inexpensive and are easily assembled and/or exchanged (contributes to its simplicity); lower reagent consumption (up to 20-100 times lower than the manual techniques); versatile (adapt to a variety of applications in analytical chemistry); free from air bubbles, low carry over; FIA can be used to obtain excellent and fast results without using glassware (which are replaced by tubes, pumps and valves) and without exposing the analyst to toxic reagents. Another outstanding feature of FIA is low flow rate used which means

low reagent consumption, flow rates are also reproducible. FIA apparatus is suitable for miniaturization.

Considering all these advantages there are limitations to FIA. FIA methods are considered to be less sensitive than their manual and SFA counterparts. Less sensitivity is brought about by the fact that equilibrium is not attained before measurements are made, as well as the physical dispersion or dilution of the sample in the carrier. The only way of overcoming this limitation is to incorporate a pre-concentration or separation manifold in a FIA system.

The main difference between SFA and FIA is that the latter makes use of air bubbles. SFA are based on the use of air segmented stream, the purpose of the segmented being to preserve the identity of their individual samples. The air-bubbles are used to prevent cross contamination.

The disadvantages of SFA are: apparatus are bulky; stream pulsate rather than to flow regularly because of the compressibility of air; streams have to be de-bubbled before they reach the flow cell before being repumped; size of air bubbles has to be controlled; pressure drop and flow velocities vary in the presence of air for different tubing material; wetting reagent is required; air bubbles in plastic tubes act as electrical insulators which result in a build-up of static electricity as a results it disturbs potentiometric sensors; the movement of a carrier stream cannot be exactly controlled or instantly stopped or restarted. SFA systems are too technically complex to miniaturize. Flow rates are less reproducible than with FIA.

FIA may be considered a hybrid of SFA and high performance liquid chromatography (HPLC). FIA is cheaper than the HPLC. Table 1.1 is a summarised comparison of the three techniques.

Table 1.1.: Comparison of operating characteristics for FIA, SFA and HPLC

Parameter	FIA	SFA	HPLC
Start up time	20 s	about 20 min	about 20 min
Sample introduction	injection	aspiration	injection
Sample volume	small ($\mu\ell$)	large (mℓ)	small ($\mu\ell$)
Analytical stream	unsegmented	segmented	unsegmented
Manifold	simple	relatively complicated to allow for introduction and removal of air	complicated due to packed column
Manifold diameter	< 1 mm I.D.	2 mm I.D.	< 1 mm I.D.
Lag phase	neglible	significant	neglible
Pump speed	variable	fixed	variable
Pumping pressures	low	low	high
Column	possible	possible	yes
Steady state	not attained	usually required	--
readout time	seconds	minutes	minutes
Wash cycle	not required	essential	not required
Reagent stream	yes	yes	possible
Reagent consumption	low	higher	--
Sampling rate	up to 400 per hour	about 60 per hour	lower than 60 per hour
Reproducibility (CV)	0.5-2%	0.5-2%	0.5-2%
Reaction time	0-60 seconds	> 5 minutes	--
Data deduction	integration or peak height	peak height	integration

When comparing parameters of FIA and SFA in Table 1.1. we can conclude that FIA is a better technique than SFA.

1.3. The aim of the study

FIA has been successfully applied in all major fields of analytical chemistry, environmental, food and clinical analysis. Laboratories which had previously used SFA have introduced the concept of FIA to complement SFA not to replace it. FIA offers distinct advantages in reproducibility, flexibility, sample throughput and cost performance. Since its inception in 1975 [3], there are more than 8000 publication to date.

The aim of the study includes the development of process analysers for the determination of nitrogen compounds (viz, nitrate, nitrite and protein) nickel and phosphate in various combinations from samples originating from various fields mainly foodstuff. Chapters 5-10 describe the experimental work done on the determination of compounds mentioned. For each determination the following parameters were critically evaluated and optimised:

* Accuracy
* Precision
* Linear range
* Detection limit
* Sample interaction
* Interferences
* Sampling rate

The chemistry of nitrite is such that under even mildly acidic conditions a significant proportion of the anion will be in equilibrium with its conjugate acid, (HNO_2) nitrous acid. This compound

is highly reactive and will undergo a series of reactions with many nucleophilic species which are likely to be present in the sample with the net result that the analyte is rapidly destroyed. Therefore it is necessary to study the stability of nitrite ion under acidic condition related to sample condition. Raman spectroscopy method is proposed for the kinetic study of the decomposition of nitrite to nitrate in acid samples [9].

Nitrite determinations are very frequent in environmental analysis. Nitrite, in some instances in combination with nitrate, is added to cure meats such as bacon, ham, sausages, etc. to prevent food poisoning from *Clostridium botulinum* and also to confer the characteristics colour and organoleptic properties to the product [10]. Nitrite play a role as an important precursor in the formation of nitrosamines which have been shown to be potent carcinogens. Thus, methods for the nitrite determinations if foods and other biological systems are becoming important. A FIA method is proposed for the determination of nitrite in foodstuffs [11].

Nitrate and nitrite play an important role in the nitrogen cycle which involves the complex interaction of different ecosystems of the biosphere. Both nitrate and nitrite are present are present in food and water, and it is from these sources that humans are exposed to these ions. There is a need, therefore, for a quick, accurate and precise method for nitrate and nitrite determination. The FIA method is proposed for the simultaneous determination of nitrate and nitrite in food and water [12].

Orthophosphate are useful in foodstuffs because they form soluble complexes with many cations something that plays an important role for increasing the bioavailability of those cations. They are useful in food systems because they stabilise colloidal suspensions. Food phosphates are

among the group of food additives known in the US as "generally recognised as safe (GRAS)" [13]. The reliable FIA method is proposed for the determination of phosphates in food. Also proposed is the quantitative determination of phosphate in fertilisers and cured meat using Raman spectroscopy. Comparison between FIA and Raman spectroscopy for quantitative phosphate determination are discussed.

Analysis results of trace elements in nutrients are produced on a large scale to estimate the dietary intake of nutritive and toxic elements and to decide on the acceptance or rejection of foodstuffs. Obviously data for such purposes must be accurate; if not, the consequences could be serious for public health and for economy. Nickel is one of the trace elements found in foodstuffs, it is not highly toxic, but its compounds have produced toxic effects. Ordinary human diet is 0.3-0.5 mg nickel per day [14], therefore it is necessary to monitor the level of nickel in foodstuffs. The FIA method is proposed for the microanalysis of nickel in foodstuffs.

Proteins determination are of great interest in diverse fields, and many methods have been developed particularly with a view to routine and automated procedures [15-16]. In the field of food technology, the Kjedahl [17] method was commonly used as a major method. There is a demand to explore new methods of monitoring proteins in foods, which is simple, reliable, rapid, and inexpensive which is applicable to minute volumes of samples. There are several considerations in the design of flow-through electrochemical detectors (potentiometric and amperometric) that can be interfaced with FIA. Owing to its simplicity, amperometric is one of the most often used electroanalytical methods. The direct amperometric and the automated FIA-amperometric procedures for protein assay in milk is proposed.

In this project the best FIA systems for the determination of nitrogen compounds, phosphate and nickel in food and water are automated and the prospects of application as process analysers are investigated. The advantages of the proposed FIA methods are speed, precision, and relatively low cost, plus its ability to be adapted for use in detecting a wide range of compounds.

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"Every time a man puts a new idea across he finds ten men thought of it before he did-but they only thought of it"

By Honoré Balzac

CHAPTER 2

FOOD SCIENCES

2.1. Introduction

The simple definition of food science is the study of experimenting with foods. Ott [1] define food as "any substance that is eaten or otherwise taken into the body to sustain physiological and psychological life, provide energy, or promote nutrition". Food as a substance consists of known chemicals such as water, protein, carbohydrates, lipids, minerals and vitamins, pigments and flavouring compounds. It also consists of unknown chemicals that impart the sensory characteristics of colour, flavour, texture, nutritive characteristics and other effects. Now we can modified the definition of food science based on the information given as "the study of basic constituents of foods (such as carbohydrates, lipids, proteins, water) and the chemical microbial, and physical actions and reactions that cause nutritional, sensory, and other changes before, during, and after processing".

Fox and Cameron [2] defined food sciences as "a coherent and systematic body of knowledge and understanding of the nature, composition and behaviour under various conditions, of food materials". Food science must be applied effectively and this is the province of food technology. The difference between food science and food technology is that the former is involved in determining whether the foods meet the dietary needs of individuals or countries and how to store and preserve food with minimum nutritional loss. The latter is involved in that food must

be grown, stored, processed, preserved and transported on a large scale.

Nutrition is the study of various nutrients in relation to their effect upon the human body. There are six types of nutrients which are present in the diet of a healthy human body, viz, fats, carbohydrates, proteins, water, mineral elements and vitamins. The body also requires a continuous supply of oxygen. If the human body lack minimum amount of any nutrient is going to result in a state of malnutrition. A general deficiency of all nutrients produces under-nutrition, and extreme cases result in starvation. It is very important to maintain the balance of nutrients in your body. The two main basic functions of nutrients is to maintain the body structure and provide energy required to perform external and internal activities. Figure 2.1. is the relation between nutrients, their functions in the body and important foods that supply them.

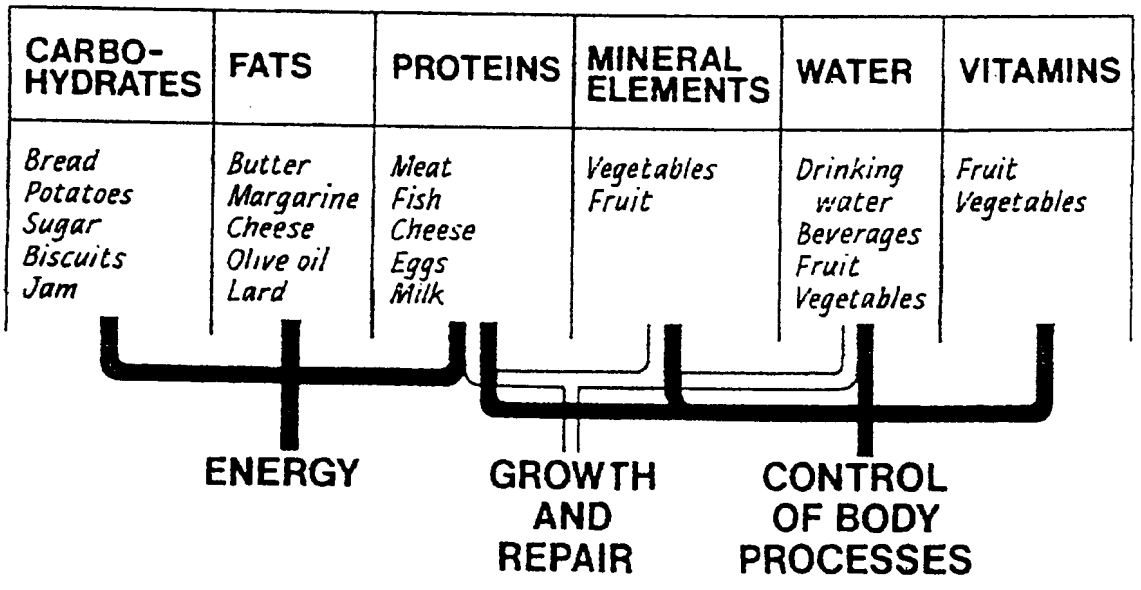

Figure 2.1.: The nutrients [2]

2.2. Scientific method

The early 17th century was the renaissance of modern science. Scientists wanted to know more about nature. Their approach to learning resulted into the scientific method. It is very important to understand scientific method because it has provided us with the knowledge of food science, nutrition, and other sciences. It helps in solving controversies over complicated problems. Scientific method is basically an "inductive method, that is using logical reasoning from a particular facts or individual cases to reach a general conclusion" [1].

The preliminary information needed for the scientific method include speculation or assumption, gathering of information (literature survey), thinking about the speculated problem based on the information gathered, and speculate how to solve the problem and what might be the problem. The scientific method include the following sequence of steps that need to be followed:

(1) Develop an assumption (hypothesis).

(2) Experimental plan (how to approach the problem).

(3) Evaluation of data (verification of data).

(4) Recycling procedure.

Once the food production is finished, the quality of food needs to be assessed whether the foodstuff meet the requirements of a consumer, i.e., the consumer will accept these products and pay for them. There are two types of food quality assessment, namely, sensory assessment of food quality (SAFQ) and objective assessment of food quality (OAFQ).

2.2.1. Sensory assessment of food quality (SAFQ)

SAFQ is defined by the Sensory Division of the Institute of Food Technologists [3] as a "scientific discipline used to evoke, measure, analyse, and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, taste, touch, and hearing. SAFQ is important to most food experiments because it set the standard on how food looks, feels, smells, and tastes. Many types of sensory tests are used in food research. The test selected will depend on the objectives of the investigation. The sensory methods are supplemented with chemical and physical methods of food quality.

There are three types of tests that are used for sensory method, viz, different test (discriminative), quality test (descriptive) and acceptance (preference test). The difference tests are of the two types, i.e., duo-trio test and triangle test. The duo-trio test is applicable to fruits and vegetables whereas the triangle test is applicable to beverages.

2.2.2. Objective assessment of food quality (OAFQ)

OAFQ are methods that does not rely on the observation of human sensors (individual). They are investigated by using an instrument or a standard procedure. The objective methods include the following:

(1) Chemical methods, this include the determination of nutritive value of foods.

(2) Physicochemical methods, this include pH measurement.

(3) Microscopic examination, to check the properties, that is, structure or physical

arrangement of food components.

(4) Physical properties, such as temperature, amount of liquid, shelf life etc..

2.3. Bioavailability of nutrients

Foodstuffs are complicated insoluble substances that must be converted into simpler, soluble, more active substances before they can be used by the body. That is, the foodstuff must be in a bioavailable state so that the body can be able to utilise it. Bioavailability has been defined as the proportion of the nutrient that is digested, absorbed and metabolised through normal pathways [4]. Digestion is both physical and chemical. The physical process involves the breakdown of large foods portions into smaller ones while the chemical process involves the breakdown of large molecules into smaller ones. The chemical process involved in digestion are brought about by enzymes. Nutrients such as water, many vitamins, other mineral salts, and simple sugars does not need digesting.

Nutrients cannot be utilised by the body until they have passed into the blood stream, a process which is known as absorption. The nutrients are distributed to all the cells of the body by the bloodstream where they can sustain the complex process of metabolism. The three terms absorption, digestion, and metabolism are all included in the term bioavailability.

The research project focuses on the following compounds, nitrogen compounds (nitrate, nitrite, proteins), nickel and phosphate, which are available in both food and water. We will only discuss their bioavailability and also their toxicological effects.

2.4. Nitrogen compounds

Elementary nitrogen is related to the complex proteins required by human beings. This relation is summed-up in the nitrogen cycle (Figure 2.2.). Elementary nitrogen occurs in almost limitless quantities in the atmosphere. Combined nitrogen is widely distributed in the soil in the form of salts and also in the form of organic compounds. Combined nitrogen forms an essential part of the body structure, which requires a continuous supply of nitrogen in a suitable form. It is not possible for the body to synthesise its nitrogen into a suitable form. This means that the body must be supplied with nitrogen which has already been converted into suitable form.

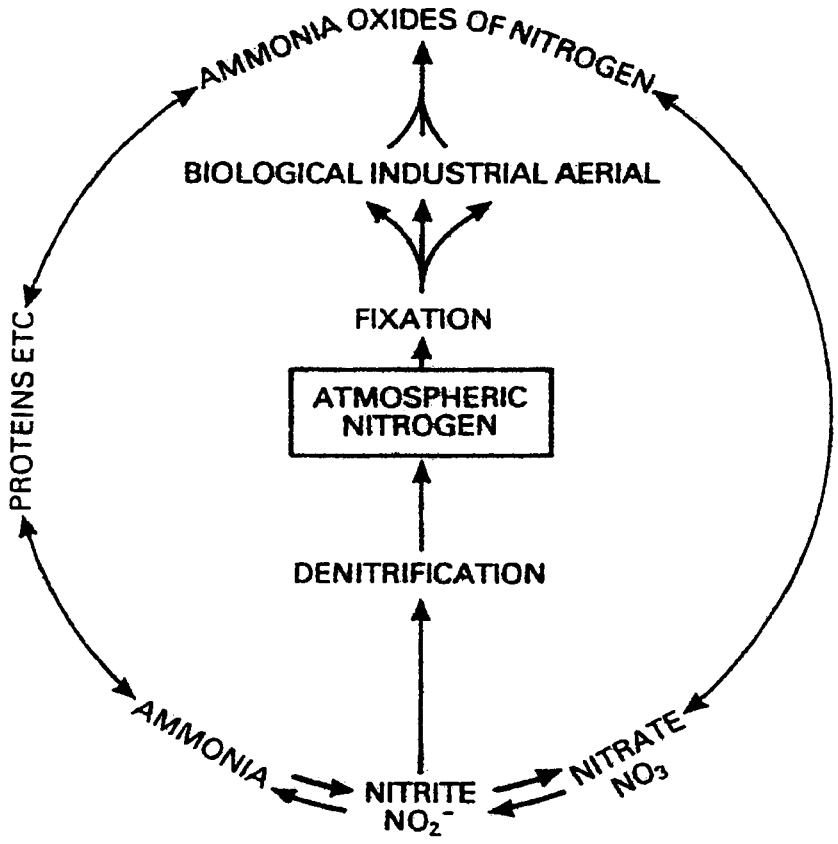

Figure 2.2.: The Nitrogen Cycle [5].

2.4.1. Proteins

Proteins are complex substances known to man. This fact can be illustrated by comparing the molecular weights and formulae of proteins with those of other substances. Proteins are a very important group of nutrients. They are found in the cytoplasm of all living substances, in both animals and plants.

Proteins are organic substances which contain the elements carbon, hydrogen and oxygen. They resemble both fats and carbohydrates. Some proteins contain sulphur and phosphorus. Protein molecules are extremely large and consist of long chains of chemically bonded amino acids. About twenty different amino acids occur in the proteins found in foods [6]. Amino acids are called amphoteric because they contain both acidic (-COOH) and basic (-NH_2, amino group) groups. A typical protein molecule contain about 500 amino acids which are joined together by peptide links. A protein molecule consists of a single polypeptide chain or a number of polypeptide chains joined by cross-linkages.

Proteins vary in structure (Figure 2.3.) but they can be classified into two main groups, (namely, globular proteins and fibrous proteins) according to the shape of the molecules. Molecules of globular proteins are rounded in shape but are not necessarily spherical. They form colloidal solutions and are affected by acids, alkalis and heat. Fibrous proteins are almost straight (inelastic or elastic proteins). They are relatively insoluble and not affected by acids, alkalis, and moderate heat.

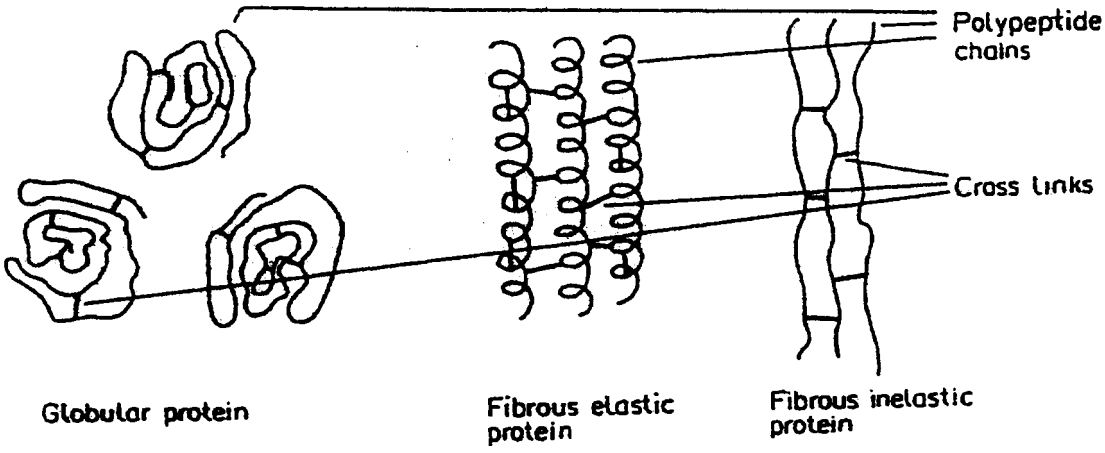

Figure 2.3.: Types of proteins [6].

2.4.1.1. Proteins in foods

When food proteins are eaten, the proteins are hydrolysed during digestion to produce amino acids. After absorption the amino acids are transported by the blood cells. In the blood cells the amino acids recombine and new proteins are formed.

The quality of food protein may be judged by its protein content, the number of essential of essential amino acids it contains and the degree to which its protein is digested and absorbed by the body. The Biological Value (BV) [6] of a protein is used as a measure of protein which is converted into body protein:

$$BV = RN/AN \times 100$$

where RN is retained nitrogen and AN is absorbed nitrogen. The BV does not take into consideration the digestibility of the food protein. Not all the protein taken into the body is

absorbed from the intestine.

The Net Protein Utilisation (NPU) is the term used to measure protein quality which takes digestibility into consideration. NPU is the percentage of dietary protein which is converted into body protein. It is given by the following equation:

$$NPU = BV \times Digestibility$$

where Digestibility = (nutrient digested and absorbed) / (nutrient eaten) × 100. The BV and NPU of variety proteins are given in Table 2.1.. The digestibility of carbohydrates is about 95% and that of proteins and fats is about 90%. Vegetables proteins have a lower BV than animal proteins.

Table 2.1.: BV and NPU of proteins from a variety of sources [6].

Protein source	BV %	NPU %
Egg	97	96
Meat	82	78
Fish	79	77
Milk	77	71
Soya bean	73	61
Rice	67	63
Peas	64	47
Peanuts	55	43
Corn	50	45
Wheat	49	48

2.4.1.2. The effects and functions of proteins in the body

Proteins are indispensable components of living matter, particularly as structural components and as enzymes responsible for the metabolic events of the organism. Some proteins are involved in the contractile process, others in metabolic regulation, while others serve as antibodies, the defence mechanism against disease. Most of the protein supplied by food is used for growth and repair, the excess protein is used for energy (1 g of protein provides 17 kJ of energy).

Main symptoms of protein deficiency are wasting of the muscles, retarded growth and distended abdomen caused by oedema (fluid in the tissues). Deficiency of protein also causes anaemia because protein is necessary for the formation of red blood cells.

2.4.2. Nitrate and nitrite

Nitrate and nitrite are found throughout the environment because they are the fundamental components of the nitrogen cycle (Figure 2.2.). The major source of human nitrate exposure is from the oral ingestion of the performed ion [7]. The main contributors are from vegetables and potable drinking water, with a lesser contribution from cured meats. The permitted levels of nitrate and nitrite added to cured meats have been decreased in recent years [8].

The degradation of nitrate (denitrification) to nitrogen and/or oxides of nitrogen takes place in the soil and water . Waste material from plants and animals returns combined nitrogen to the soils in which part is recycled and part returned to the atmosphere to complete the nitrogen cycle. Bacteria of the Rhizobium species present in the root nodules of legumes such as beans

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contribute strongly to the nitrogen cycle in reducing elemental nitrogen to ammonia [5]. Ammonia is then converted (nitrification) to nitrate which proceeds via the intermediate formation of nitrite through a two stage process involving different microorganisms. According to Christy *et al.* [9] more than 90% of the nitrogen absorbed by plants is in the form of nitrate.

2.4.2.1. Nitrate and nitrite in foods

The occurrence of nitrate in the majority of foodstuffs and water suppliers is well established [10]. The concentration vary enormously between different foodstuffs, for example, milk contain about 1 mg/kg of nitrate and whereas in certain vegetables the amount exceed 1000 mg/kg [11]. Concentrations of the nitrate ion can differ markedly from samples of the same origin. Pickston *et al.* [12] have reported values of nitrate ion for beetroot ranging between 630-6800 mg/kg.

Nitrite occurs rarely in food and water. Its occurrence is due to the microbial reduction of nitrate to nitrite. Nitrite in combination with nitrate are added to cured meat to prevent food poisoning from *Clostridium botulinum* and also to confer the characteristics colour and organoleptic properties to the product. The amount of nitrate and nitrite added are controlled by legislation of specific countries. Nitrate and nitrite are not allowed to be added to baby foods.

Nitrate is very stable chemically throughout the relevant pH of foods. It can be reduced to nitrite in contact with metals such as occurs during the cooking of food in aluminium utensils [13]. Nitrite is unstable particularly at acidic pH values at which it can disproportionate to yield nitrate and nitrogen oxide [14]. Ascorbic acid (vitamin used as an acid in the manufacture of cured meats) reacts readily with nitrite, a major product in the absence of oxygen being nitrogen oxide.

Great care must be exercised in determining nitrite in the presence of scavengers such as ascorbic acid with which it can react at acidic pH values required for its measurement as a diazonium salt.

2.4.2.2. Effects of nitrate and nitrite

Until the mid 1940's [15] there was little interest in the amount of nitrate and nitrite in the human diet. There is little evidence of acute toxicity associated with nitrate whilst nitrite, although known to cause dilation of blood vessels and production of methaeoglobin, was disregarded as a general hazard. The health aspects can be divided into acute toxicity and the effects of chronic dosage. The chronic toxicity of nitrate is due to its reduction to nitrite in the body.

2.4.2.2.1. Acute effects

The major source of toxicity of nitrite is the formation of methaemoglobin (Met-Hb) from oxyhaemoglobin. Ingestion of sodium nitrite in large amounts can be fatal, the lethal dose being 2-9 g as sodium nitrite [16]; the figure of 4 g sodium nitrite is widely accepted. The major effects of nitrate appear to be mediated indirectly through its end ogenous metabolites, nitrite and N-nitroso compounds, most of which are known to have profound toxicological effects.

2.4.2.2.2. Chronic effects

There are no reports of chronic toxic effects of nitrate that are not related to the effect of the product of bacterial reduction of nitrite. The major health hazards cited in the literature are the infant methaemoglobinaemia and N-nitroso related carcinogenesis.

2.5. Phosphates

Phosphorus occurs in natural waters and wastewater as phosphates. Phosphate is classified as a mineral element (which is phosphorus) in foods. These are classified as orthophosphate, condensed phosphates (pyro-, meta-, and other polyphosphates) and organically bound phosphates. Organic phosphate are formed primarily by biological processes. Phosphates also occurs in bottom sediments and in biological sludges, both as precipitated inorganic forms and incorporated into organic compounds. They are contributed to sewage by body wastes and food residues.

Phosphates constitute a very large group of compounds which van Wazer and Callis [17] defined as "those chemical substances in which anion consists of phosphate tetrahedral which may be linked together by sharing corners". The phosphate series begins with the single phosphate group which may exist as the triply charged orthophosphate, which may exist as the triply charged orthophosphate anion (PO_4^{3-}), or may participate in molecules where one, two or more of the four oxygens are covalently bonded to other atoms. The group is a tetrahedron formed by four oxygen atoms surrounding a phosphorus atom.

Phosphorus constitutes only approximately 1% of the total weight of human body mostly in the form of hydroxy apatite in bones and teeth [18]. Phosphorus compounds intervene in metabolic cycles to an extent that makes them essential to life. Orthophosphate form soluble complexes with many cations in increasing the bioavailability of those ions. Phosphorus is involved in energy transfer mechanisms whereby chemical bond energy is transformed into other chemical bond or into other forms of energy, including kinetic energy in muscle movement [19].

2.5.1. Phosphate in foods

Phosphorus is found in foods of animal or vegetable origin, associated with lipids in the form of mono- or diesters referred to as phospholipid. Important phosphorus compounds in vegetable tissues are the phytic acid (inositol hexaphosphate) and its calcium and magnesium salts. These substances are involved in vegetable structural support. Considering the omnipresence of phosphorus in food constituents of nearly every origin, it is natural that this element be constantly found in the human diet in concentration that varies with the type of food and also within a food group.

Phosphate is often associated with proteins in biological systems. The most important phospho-proteins found in foods are those associated with eggs (phosvitin) and milk (casein). The uses of food phosphates in the diary industry include protecting condensed and sterilised milk against age gelation, increase the solubility of dehydrated milk and whey powders, production of novel dairy based desserts, and improving texture, melting, and spreadability of processed cheese [18].

Phosphates are used in fats and oils. The most important function of phosphate in oil extraction is to increase protein solubility in the aqueous phase and to reduce protein lipid interactions that hinder extraction and decrease yields. They are also used in food flavouring, special egg composition, sugar, beverages, food colouring, and food coating.

Food phosphates are among the group of food additives known in the U.S. as "generally recognized as safe" (GRAS) [18]. This classification implies that the use of a particular food

additive is safe within the limits defined by specific regulations concerning the maximum concentration of food product in which the additive may be used. Food phosphates have gained recognition as effective antimicrobial agent used in food products which include dairy products [20-21], meats (fresh and cured) [22-23], bakery products (include cereals and grains) [24], vegetables and fruits [25-26].

2.5.2. Nutritional and toxicological aspects of phosphates

Because of the prevalence of the element phosphorus and of phosphorus compounds in many food products, concerns about the potential toxicity of added food phosphates has been a major regulatory argument against a more widespread allowance for use of these additives in food [27].

Recommended daily intake of phosphorus for adult humans has been set at < 30 mg/kg body weight per day unconditionally and at 30-70 mg/kg body weight per day conditionally by the World Health Organization (WHO) [28]. Table 2.2. present the US recommended daily allowance (RDA) for calcium and phosphorus depending on sex and age group. Recommended daily intake of phosphorus is commonly associated with that of calcium, the ratio are 1:1 or >1:1 of calcium to phosphorus are usually specified as optimal for human nutrition. Feldheim [30] has indicated that such ratios may be appropriate for babies and infants but not justifiable for adults.

Table 2.2.: U.S. RDA for calcium and phosphorus [29].

Group	Age (years)	Calcium (mg)	Phosphorus (mg)
Infants	< 0.5	360	240
	0.5-1.0	540	360
Children	1-10	800	800
Males	11-18	1200	1200
	19-51	800	800
Females	11-18	1200	1200
	19-51	800	800
Pregnant	—	400	400

The available literature on human physiological effect of added phosphates is relatively scarce. Bell *et al.* [31] studied the physiological response of human adults to foods treated with phosphates (1 g of P per day). Their studies resulted in subjects suffering from mild diarrhea during the first week but this disappears by the second week. Other well known uses of polyphosphates that might have health related effects are in the prevention of discolouration caused by iron and manganese. Polyphosphates and ferric phosphate salts have been shown to enhance iron absorption. Phosphates bind to protein and form stable complexes in ways that might alter protein digestibility and nutritive value.

Contamination of water with phosphates from fertilisers has been studied for its potential effects on aquatic environment [32]. Although eutrophication of even larger bodies of water may occur in the presence of high phosphate and nitrogen contamination, this effect seems to be absent when high phosphate levels only are introduced in water. Long term exposure to low phosphate levels has not affected plankton or animals, although short term exposure to high phosphate

levels have caused severe environmental damage .

2.6. Nickel

Metallic nickel has been known for about 200 years, but interest in its biochemical and toxicological activities is fairly recent [33]. Nickel occur at 0.08% of earth crust, it also occur at trace amount of sea water, petroleum and coal. It occurs at about 0.1-6 mg/ℓ fresh weight in plants and animals. Soils contain 40 mg/ℓ nickel on average [34]. The most stable form of nickel is Ni^{2+}.

Nickel is toxic for most plants and fungi. Growth of woody plants is limited /or prevented by high nickel concentrations in soils [35]. Land plant tissue contains about four times more nickel than does animal tissue. Some marine animals like oysters and lobsters are richer in nickel.

Acidic foods take up nickel from cooking and storage vessels. Nickel is poorly absorbed, as a result, nickel ingested from foods is relatively non-toxic. Certain nickel salts, such as the cyanide, chlorides, carbonates, hydroxides, nitrates and sulfates are used in nickel plating. Various nickel salts are utilized as pigments, for example, nickel dimethylglyoxime, a red coloured complex is used in sun-fast paints, lacquers and cosmetics [33]. Nickel phosphate is used as a yellow pigment for water colours and oils. Nickel sulfate serves as a mordant in dyeing and printing fabrics.

2.6.1. Nickel in foods

The essential minerals and trace elements in foods from animal origin are mainly present as

organic complexes, very often as metalloproteins having functional properties. In vegetable foods the metals are mostly bound to structural or storage components such as fibre and phytate. Various enzymes may be the "carrier" for minerals and trace elements.

Nickel will be bound to albumin or other proteins in animal foods, in vegetable foods is bound to phytate or phenolic group such as tonnins in tea or coffee. Trace amounts of nickel in water (< 200 $\mu g.\ell^{-1}$), air ($1 \times 10^{-10} \mu g.\ell^{-1}$), and meat (±0.2 mg/kg) are not considered as toxic [33]. Ordinary human diet is 0.3-0.5 mg nickel per day. Total nickel intake varies with the types of food that are consumed [36]. Vegetables are higher in nickel content than animal muscles. Typical Western diets of mixed foods were found to supply 300-500 μg of nickel daily [33].

Table 2.3.: Nickel contents in animal tissue [33].

Tissue	Calves ($\mu g/g$)	Rats ($\mu g/g$)
Heart	1.11	3.0
Kidney	2.08	1.7
Liver	0.76	1.3
Lung	2.94	2.4
Spleen	0.00	6.2

According to Underwood [37] only 1-10% of the dietary nickel ingested is absorbed. Animal studies by O' Dell *et al.* [38] seem to indicate that the animal body either absorbs nor retains nickel readily. Of the ingested nickel 90%, i.e., excreted via the faeces and 10% via urine. Under ordinary circumstances, nickel is found in all tissues and body fluids in relative low concentrations. Table 2.3. contains a comparison of nickel levels in these animal tissues. Levels

of nickel ranging from 0.02-1.46 μg/g have been reported for human liver and lungs respectively.

Nickel requirements are as yet unknown. Fairly recently studies have indicated that nickel is an essential element in an animal nutrition. Its physiological role has not been delineated as yet. Significant concentrations of nickel have been shown to be present in RNA and DNA [35, 39]. Nickel is now considered to be among the newer essential metals.

2.6.2. Toxicology of nickel

Exposure to nickel carbonyl is the most hazardous of all industrial exposure to nickel [40]. This highly volatile compound, formed during a process employed for the production of nickel. It is toxic even in low concentrations. Initial symptoms of exposure include nausea, dizziness, headaches and chest pain, disappear within a few days. Except for certain industrial exposures, nickel is rather toxic for most plants and fungi.

Nickel was recognized fairly early as a potential carcinogen. The carcinogenic actions of nickel and its salts have been observed in numerous animal experiments over the years [41-42]. Workers exposed to inhalation of aerosols of soluble nickel salts, that is, those in nickel plating operation and in nickel refineries are probably at greatest risk. Nickel dermatitis caused by wearing nickel plated watches, other jewellery, spectacle frames, etc., it is well known as a dermatological syndrome that is readily cured by removing the source of exposure. A "split ear" syndrome caused by nickel plated earings has been described [43]. Stainless steel kitchen appliances and nickel containing detergents have been implicated as causative agents in contact dermatitis [33].

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"Science is built with facts, as a house is with stones. But a collection of facts is no more a science than a heap of stones is a house."

By: Jules Henri Poincaré

CHAPTER 3

RAMAN SPECTROSCOPY

3.1. Introduction

The early 1920s brought a rapid development in the interest of light scattering of electromagnetic radiation by charged particles. Several physicists [1-4] predicted that radiation scattered from molecules contains not only photons with the incident-photon frequency but also some with a changed frequency. The physicists made the tests with samples ranging from liquids, solids and gases. Raman [5] was the first to obtained positive results of experiments with liquid benzene. He was awarded the 1931 Noble Prize in physics for his discovery and the phenomenon now carries his name. The first experiments were carried out using sunlight and filters and relied on visual observation of colour changes in scattered light. The experiments used 600 mℓ of sample and required a 24 hour exposure to obtain measurable spectra [6].

The first instrument used for recording Raman spectra is schematically presented in Figure 3.1. The instrument consists of the following: mercury lamp (for radiation), filter, consisting of sodium nitrite solution, sample (monochromatic light is used to illuminate the sample), glass prism (disperse the scattered radiation), photographic plate (which is used to record the spectra). The resulting spectrum consisted of a very strong Rayleigh scattering (corresponding to the incident radiation wavelength) and very weak Raman scattering bands. Raman scattering is most easily seen as the change in frequency for a small percentage of the intensity in a monochromatic

beam as the result of interacting with some material.

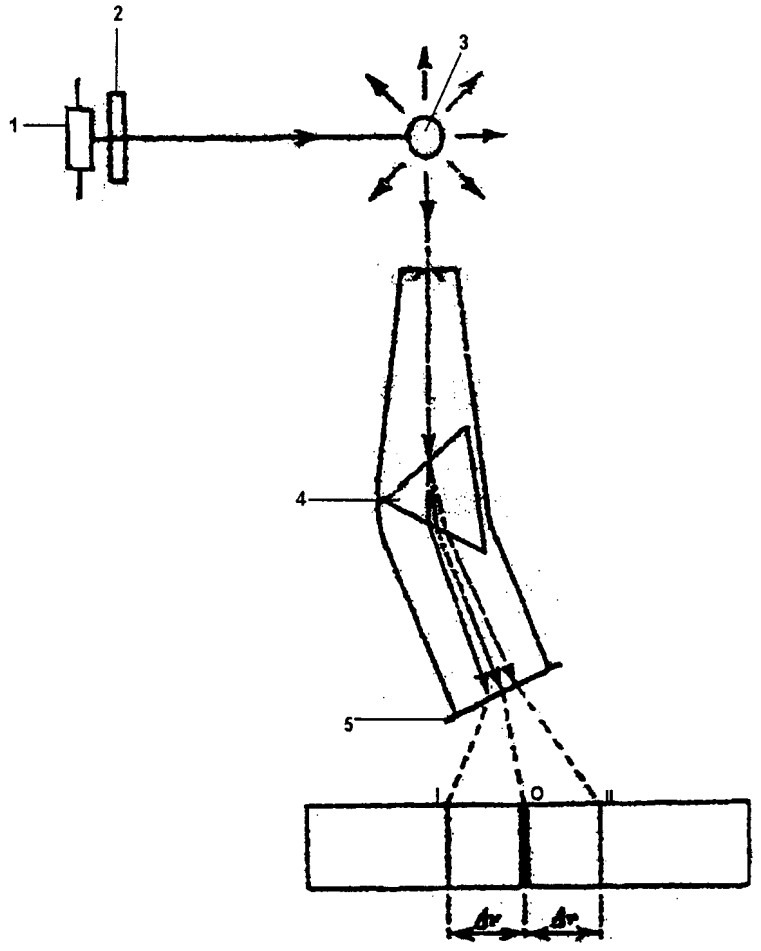

Figure 3.1.: First Raman spectrographs [7], 1 = mercury arc lamp, 2 = solution filter (saturated nitrite solution), 3 = sample, 4 = prism, 5 = photographic plate. 0 = Rayleigh scattering line, I = Raman Stokes scattering band, and II = Raman anti-Stokes scattering band.

Raman spectroscopy has grown fast in both qualitative and quantitative analysis since the discovery of lasers in the early sixties [10]. The Raman renaissance took place with the development of commercial continuous wave (CW) visible lasers [9]. Laser spectrometers allow the recording of spectra for small sample volumes, coloured samples, solids, liquids, gases, samples over a wide range of temperatures and pressures, dilute solutions, crystalline powders

and in variety of other non-standard conditions.

Raman spectroscopy has contributed to the development of rotational-vibrational spectra. Theory of Raman scattering shows the same type of quantized vibrational changes that are associated with infrared (IR) absorption [8]. The advantage of Raman spectroscopy is that it provides vibrational spectra that are rich in highly reproducible detailed features, providing the possibility of highly selective determinations. Raman spectroscopy is superior to IR spectroscopy for investigating inorganic systems because aqueous solutions can be employed, the low frequency spectral region is easily obtained, glass or quartz cells can be employed instead of using sodium chloride windows which are atmospherically unstable.

3.2. Theory of Raman spectroscopy

The Raman effect can be described as the inelastic scattering of light by matter. Raman scattering is seen as a change in frequency for a small percentage of the intensity in a monochromatic beam as a result of interacting with some material. Raman spectra are acquired by irradiating the sample with laser source of monochromatic radiation. During irradiation, the scattered radiation is measured at an angle of 90°C with a suitable spectrometer.

3.2.1. Excitation

Spectral excitation is carried out by radiation in Raman spectroscopy. When a photon of light interacts with a molecule it can be scattered in one of the following three ways, viz,:

(1) Stokes scattering, photon undergoing inelastic loss of energy,

(2) anti-Stoke scattering, photon undergoing inelastic gain of energy,

(3) and Rayleigh scattering, photon undergoing inelastic and thus retain its energy.

Figure 3.2. is a representation of a Raman spectrum that was obtained by irradiating a sample of carbon tetrachloride, it shows all the three emitted radiation. Raman peaks are found on both sides of the Rayleigh peak, the pattern of shifts on each side is identical. The Stokes lines are bigger than the anti-Stokes line, because of this reason generally only the Stoke lines are used. Fluorescence causes interference with the Stokes shift but not with the anti-Stokes. Anti-Stokes signals may be used for fluorescence samples.

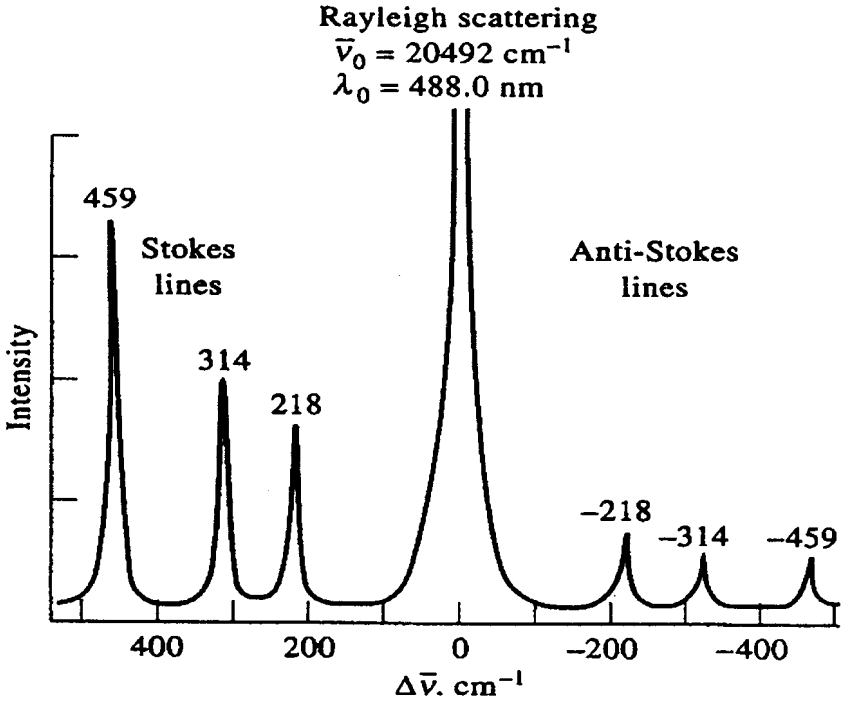

Figure 3.2.: Raman spectrum of carbon tetrachloride [8].

3.2.2. Wave model of Raman and Rayleigh scattering

The classical interpretation of the Raman effect regarding the scattering of light assumes that the molecule is experiencing a dipole vibration. The dipole moment is caused by electromagnetic radiation. Electromagnetic radiation generates an electric field (E) which can be expressed as:

$$E = E_0 \cos(2\pi\nu_{ex}t) \qquad (1)$$

where E is an electric field, E_0 is the maximum amplitude (equilibrium field strength), ν_{ex} is the frequency of radiation, and t is time. The electric field induces a dipole moment (μ) which will emit or scatter radiation (Rayleigh scattering) and is given by the following equation:

$$\mu = \alpha E = \alpha E_0 \cos(2\pi\nu_{ex}t) \qquad (2)$$

where the proportionality constant α is known as the polarizability of an analyte bond. The polarizability constant is the measure of the deformability of the bond in the electric field.

In order to be Raman active, the polarizability bond (α) must vary as a function of the distance between nuclei according to the following equation:

$$\alpha = \alpha_0 + (r - r_{eq})(\delta\alpha/\delta r) \qquad (3)$$

where is α_0 is the polarizability of the bond at the equilibrium internuclear distance r_{eq} and r is the internuclear separation at any instant. The change in internuclear separation varies with the

frequency of the vibration v_v as given by:

$$r - r_{eq} = r_\mu \cos(2\pi v_v t) \qquad (4)$$

where r_μ is the maximum internuclear separation relative to the equilibrium position. Substituting equation (3) into (4) gives:

$$\alpha = \alpha_0 + (\delta\alpha/\delta r)\, r_m \cos(2\pi\, v_v t) \qquad (5)$$

We can then obtain an expression for the induced dipole moment μ by substituting equation (5) into (2) then it becomes:

$$\mu = \alpha_0 E_0 \cos(2\pi v_{ex} t) + (\delta\alpha/\delta r)\, E_0 r_m \cos(2\pi\, v_v t) \cos(2\pi v_{ex} t) \qquad (6)$$

Using the trigonometric identity for the product of the two cosines, this can be rewritten as:

$$\mu = \alpha_0 E_0 \cos(2\pi v_{ex} t) + (\delta\alpha/\delta r)\, E_0 r_m/2 \times [\cos 2\pi (v_{ex} + v_v)\, t + \cos 2\pi (v_{ex} - v_v)\, t] \qquad (7)$$

There are three terms in the above equation (7) which represents the three major phenomena observed in Raman spectroscopy. The first term describes the Rayleigh scattering (v_{ex}); the second term ($v_{ex} + v_v$), is the anti-Stokes; and the third term is the Stokes Raman scattering ($v_{ex} - v_v$). For the Raman scattering to occur $\delta\alpha/\delta r$ must be larger than zero.

3.2.3. Intensity of normal Raman peaks

Raman intensities are directly proportional to the concentration of reactive species. Placzek [11] originally derived the polarizability theory of expressing the observed intensities of the Raman Stokes and anti-Stokes scattering. The theory is applicable to non-rotating molecules. Placzek theory is applicable to normal Raman scattering and does not include the resonance Raman scattering. The Placzek theory provides the following equation:

$$I = K(\nu_{ex} + \nu)^4/\nu \times NI_0/1 - e^{-h\nu/kT} \times [45(\alpha^s)^2 + 13(\alpha^a)^2] \qquad (8)$$

where K is a constant, N is the number of molecules, I_0 is the incident intensity, α^s is the symmetric part or isotropic part, and α^a is the asymmetric or antistrophic part. The constants 45 and 13 are described by Long [12] and are the result of the experimental geometry. As a result this yields the ratio (the ratio depends on the absolute temperature (T) of the sample) of Stokes to anti-Stokes intensity,

$$I_{Stokes}/I_{anti\text{-}Stokes} = (\nu_{ex} + \nu)^4/(\nu_{ex} + \nu)^4 \, e^{-h\nu/kT} \qquad (9)$$

3.2.4. Raman depolarization ratios

Raman measurements provide information on the intensity and frequency of molecules. Another additional information which is obtainable from Raman measurements is that of structural determination, which is called the depolarization ratio. Polarization is a property of a beam of radiation whereas depolarization describes a molecular property [8]. Depolarization (p) ratio can

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be defined as:

$$p = I_{\perp} / I_{\parallel} \qquad (10)$$

where $I_{\parallel}$ is the intensity parallel to the incident plane and $I_{\perp}$ is the intensity perpendicular to the polarized radiation. The depolarization ratio may be obtained experimentally by inserting a polaroid sheet between the sample and the monochromator (Figure 3.3.). The p is dependent upon the symmetry of the vibrations responsible for Raman scattering. The depolarization ratio can be useful in identifying complex organic molecules [13] and also in the study of low-symmetry molecules [14]. The main disadvantage is that of interlaboratory reproducibility.

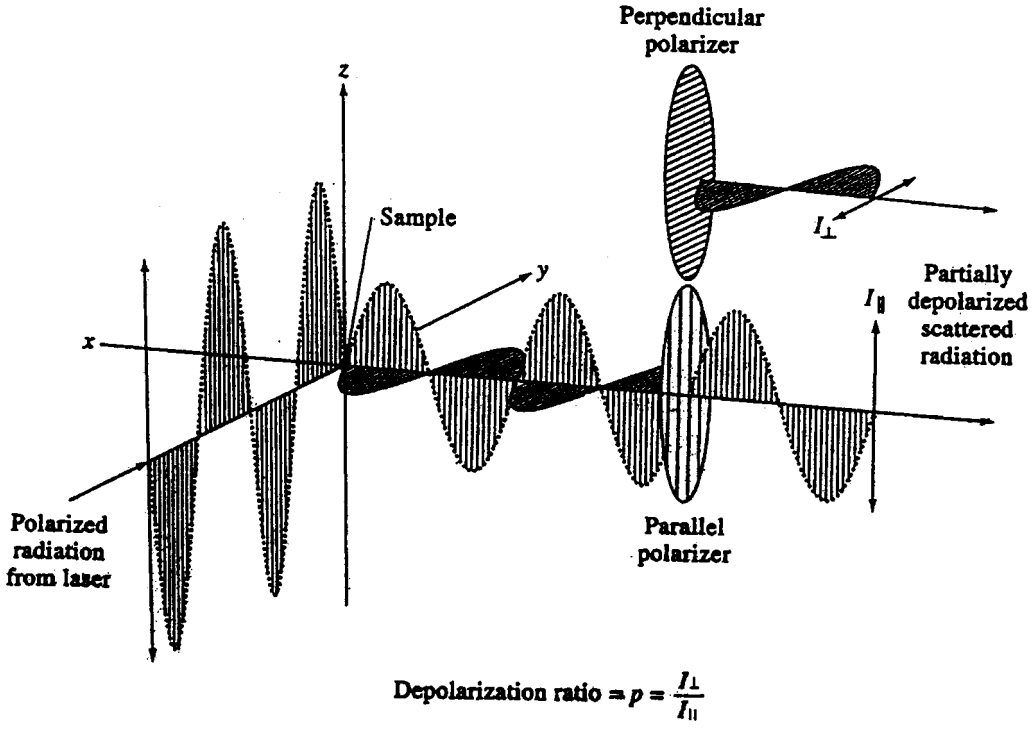

Figure 3.3: Depolarization resulting from Raman scattering [8].

3.3. Instrumentation

Raman spectroscopy consists of three basic components: a laser source (usually a continuous wave gas laser), sample handling system (illumination system), and a suitable spectrometer. Figure 3.4 is a typical schematic diagram of Raman spectrophotometers.

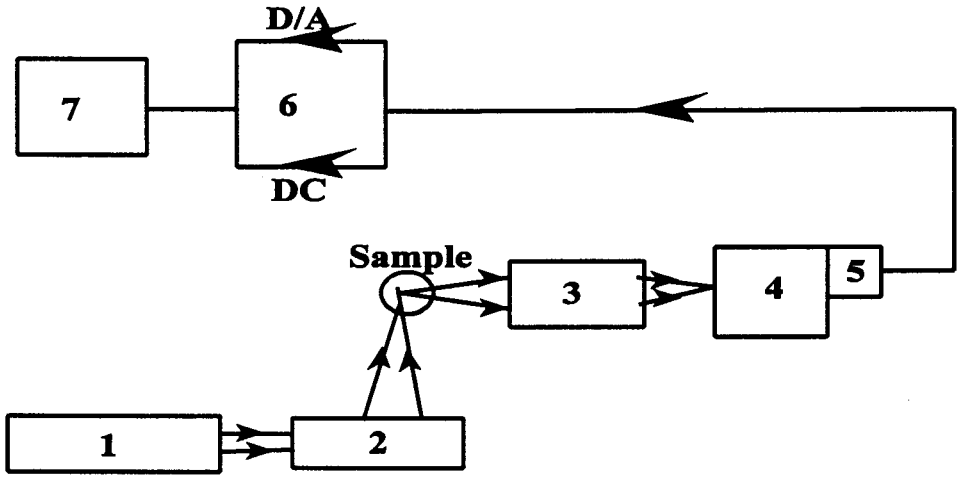

Figure 3.4.: Schematic diagram of Raman spectrophotometer [20], 1 = Laser; 2 = Collimating optics; 3 = Collecting optics; 4 = Monochromator; 5 = Photo-multiplier; 6 = Photon counting detector (DC = direct current amplification and D/A = digital to analog converter); 7 = Recorder.

3.3.1. Sources

The sources for Raman spectroscopy are divided into pulsed and continuous wave (CW) lasers. The most popular source is the CW laser (Figure 3.5.), the pulsed lasers are applicable in ultraviolet resonance Raman spectroscopy and time-resolved Raman measurements. The CW

lasers give a continuous supply of photons and are by far the most popular used sources. The most common lasers that are used for Raman spectroscopy are presented in Table 3.1.

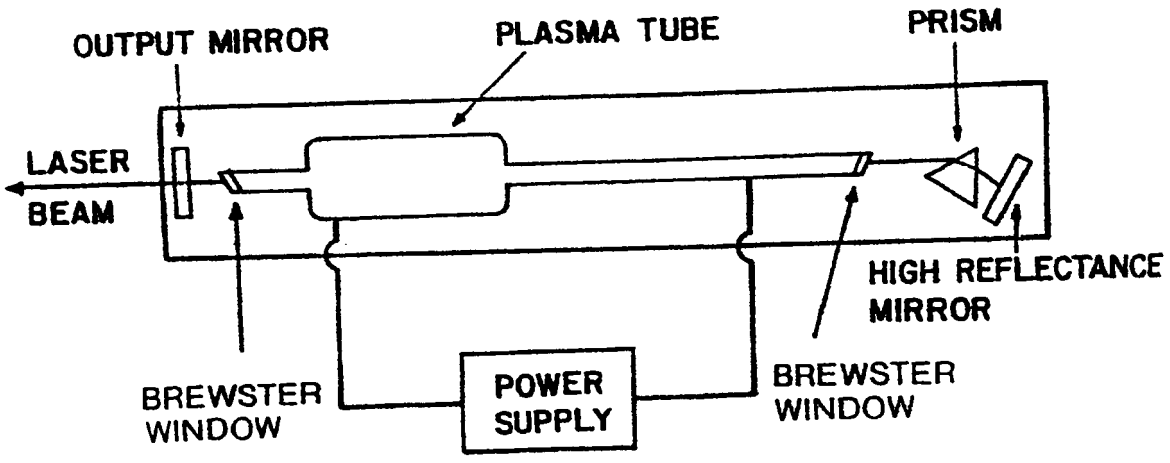

Figure 3.5.: Schematic diagram of a gas laser [15].

Table 3.1.: Common Laser Sources for Raman Spectroscopy

Type of source	Wavelength in nm
Argon ion	488.0 or 514.5
Krypton ion	530.9 or 647.1
Helium/Neon	632.8
Diode laser	782 or 830
Nd/YAG	1064

The first three sources are the most popular gas lasers employed in Raman spectrophotometers. The last two sources, which emit near infrared (N-IR) radiation, are used as excitation sources. There are two main advantages of N-IR sources, (a) is that they can be operated at much higher

power and (b) the fluorescence is much less.

3.3.2. Sample illuminating system

The two major advantages of Raman spectroscopy as compare to IR is the ease of sample handling and analyses of aqueous samples. The latter is important for biological, inorganic and water polluted samples. Glass quartz can be used for windows, lenses, and other optical components instead of sodium chloride plates. Gases, liquids and solids samples can be analysed very fast (within a fraction of a second). Figure 3.6. shows two systems for handling liquid samples.

Solid samples are ground first to a fine powder before analyses. Samples are packed in ampoules or capillaries, depending on the amount of solid sample available, and they are analysed exactly the same as the liquid samples. Polymers can be analysed directly without prior sample preparation.

Colourless samples can be measured by irradiation with a laser beam whose wavelength is in the normal Raman scattering. If the sample is coloured efficient absorption of the laser radiation may occur which will result in heating or decomposing the sample. The problem can be minimised by defocussing the laser beam at the sample or another alternative is to insert a cylindrical lens between the laser and the sample.

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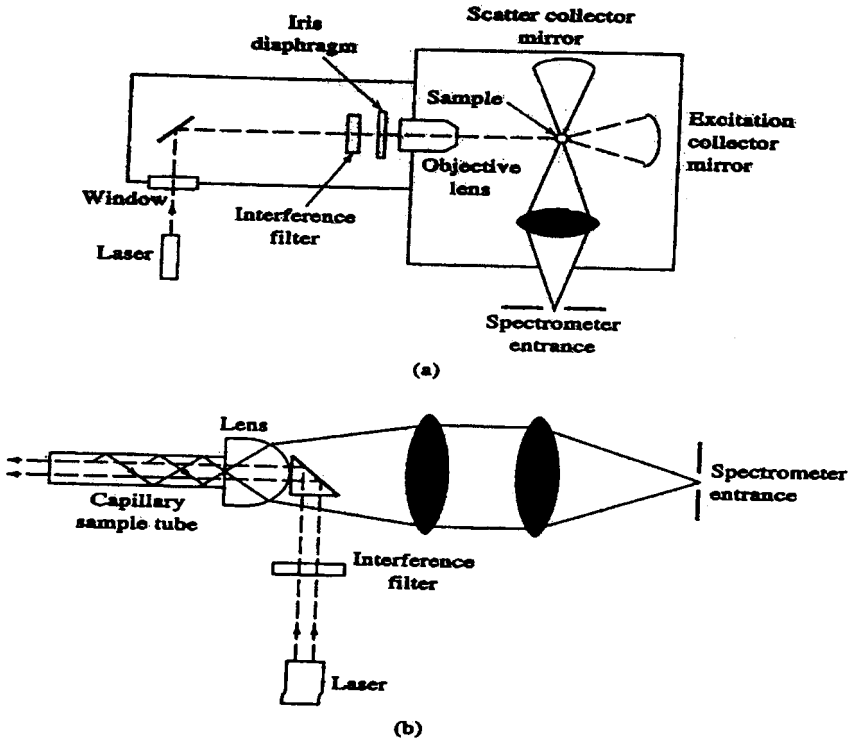

Figure 3.6.: Two sample handling systems [8].

3.4. Raman spectrometers (monochromators)

The original Raman spectrometers and UV dispersive instruments were similar in design. They also used similar components. All of the Raman scattering work is carried out using a dispersive monochromator. A monochromator disperse collected light across its exit slits to a detector (usually a photomultiplier tube). Single, double, triple and single plus double monochromator systems are used.

The spectrometer uses narrow slits and result in a very narrow band width, whereas spectrographs uses much wider slits and a grating which produces much less dispersion and results in a broad band of light. The mainly used spectrometer for Raman studies is the double monochromator (Figure 3.7.) which uses two gratings. This spectrometer is used in conjunction with a

photomultiplier tube (PMT). The detection system used are divided into single-channel (PMT) and multi-channel (array detector) systems. The single channel detection system involves the counting of photons by PMT, whereas the multichannel detection system includes an array detector which has an intensifier coupled to it. Campion and Woodruff [17] have reported on the use of array detectors. The current choice of Raman spectrometers is between the double and triple monochromators. Double or triple monochromator is required for a low frequency Raman spectroscopy which is less than 200 cm^{-1}. Recently Raman spectrometers are either Fourier transform (FT) instruments or multi-channel instruments based upon charge-coupled devices. The FT-Raman spectroscopy can eliminate the problem of fluorescence interference because the measurement takes place in the N-IR.

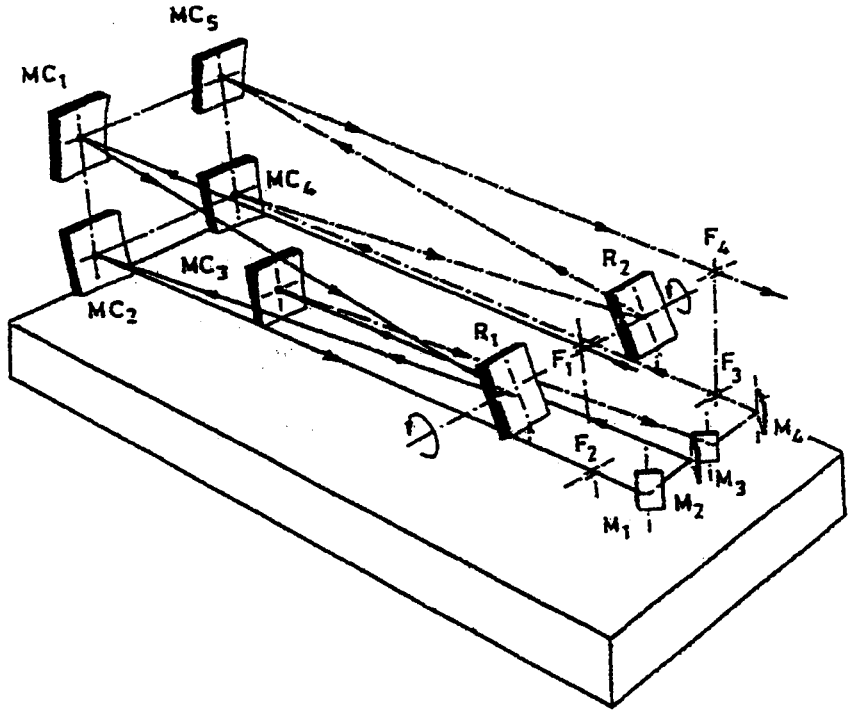

Figure 3.7.: Schematic diagram of a double monochromator [7]. F_1 **= entrance slit;** F_4 **= exit slit; MC 1-5 = are concave mirrors; Mn = plane mirrors; and Rn = plane gratings.**

3.5. Applications of Raman spectroscopy

Raman spectra allow the identification of molecules and the study of their structure, the characterization of chemical reactions, kinetic studies (thermodynamic functions), and qualitative and quantitative analysis of biological, organic and inorganic molecules. The technique is mainly used for structural and qualitative analysis. Quantitative analysis by Raman spectroscopy has not yet been exploited widely. This lack of use has been due largely to the high cost of the instrument relative to that of absorption instrumentation.

Limitations of Raman spectroscopy shares with other emission techniques the handicap of being a single-beam technique and this lead to uncorrected variations in source, sample, and optics. Another limitation is the "Raman scattering" phenomenon which is weak as compared to a process such as absorption of radiation. The major limitation is that of strong fluorescence from many samples. Fluorescence is approximately 10^6-10^8 times stronger than Raman scattering [14]. This limitation can be alleviated by chemical or physical manipulation of the sample. The chemical or physical manipulation can be achieved by addition of quenchers, clean-up of the sample, or burn-out of the fluorescence by preliminary exposure to high-intensity radiation. The fluorescence limitation has received considerable attention [18-21].

3.5.1. Qualitative analyses

The major application of Raman spectroscopy is that of qualitative and structural analysis. Samples ranging from biological, diagnostics (medicinally related), environmental, geological, industrial, etc., can be analysed using Raman technique.

For the qualitative analysis of organic species the Raman spectra are similar to IR spectra in that they are of the vibrational-rotational type. They both provide useful information on the functional group identification and fingerprinting of analytes. More information can be obtained from Raman spectra of organic compounds like olefines than from IR spectra [8].

One of the advantages of Raman spectroscopy over that of IR, is the study of inorganic species in solution. The vibrational energies which provides frequencies and intensities (in the range 100-700 cm^{-1}, region where IR spectrum is very difficult to detect) help in identifying the inorganic species composition, structure, stability, and effect of temperature and pressure [8]. Organic compounds contain similar structural elements whereas the inorganic compounds are formed from a wider range of elements. The other factor is the difference in bonding found in organic compounds which is of covalent nature and that of inorganic compounds which is of ionic nature. It is easier to identify organic compounds by IR spectroscopy due to their characteristic bands. Sharp bands which are observed in Raman spectra make interpretation of compounds much easier. This is the reason why Raman spectroscopy is also useful for the identification and determination of structures of simple inorganic molecules with covalent bonding, for example, centrosymmetric and diatomic molecules.

Raman spectroscopy has been applied widely for the study of structure and function of biological systems. The reason for its uses in biological samples include small sample requirement, the minimal sensitivity toward interference by water, fingerprint-like spectra which can be obtained for species as small as low-molecular-weight metabolites and as large as living organisms, availability of UV and N-IR excitation sources (which minimise fluorescence interferences), spectral detail, and the conformational and environmental sensitivity. The biological samples that

were reported include proteins [22-25], nucleic acids [26-28], and lipids [29].

3.5.2. Quantitative analyses

The principal field of application of laser Raman spectroscopy has been in the determination of structure and identification of samples. Raman spectroscopy has not yet been exploited widely for quantitative analysis. Due to precise focussing of laser beams, it is possible to perform quantitative analyses of very small samples. The quantitative analysis used in Raman spectroscopy is similar to those used in all other procedures of instrumental analysis. These procedures involve comparison of spectra for the unknown sample and a standard. From the scattering process the measured intensity (I_R) of the scattered radiation is directly proportional to the concentration, C, of the analyte. The equation of the straight line is:

$$I_R = J.C \qquad (11)$$

where J is the slope of the graph called scattering coefficient. Knowledge of J for a particular species allows one to calculate the concentration C from measured I_R.

The quantitative analysis of Raman spectroscopy has been exploited as far back as 1971 [30]. The basics of quantitative analysis is described, including the use of internal and external standard to compensate for instrumental and sample variations. Raman spectroscopy was also used for routine analysis in determination of pollutants in natural water samples [31-32]. During the last two decades the quantitative analysis of Raman spectroscopy has grown very fast. Applications have included the determination of phenols, sulfur oxyanions, pharmaceuticals, antitumour,

aromatic amines, azo dyes, and ethanol fermentation broth [33].

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"Basic research is when I'm doing what I don't know I'm doing."

By: Werner von Braun

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CHAPTER 4

FIA-BASICS

4.1. Introduction

As discussed in Chapter 1, the concept of FIA is founded on the following principles; sample injection; controllable sample dispersion (which distinguish it from other techniques); reproducible timing; variable flow rates; baseline resolution between each sample; high sample throughput; and absence of any stabilization time. FIA method utilizes the rapid injection of an aqueous sample either manually (through a septum device) or automatically by some automatic injection system into a continuously moving unsegmented carrier stream of water or reagent solution. The injected sample forms a slug or zone that are chemically processed in a manifold system and transported into a flow through detection system to generate an analog signal (i.e., peak height or peak area and the return time, T) which is directed to a recorder.

This chapter will focus on theoretical backround of FIA and the essential components in FIA system.

4.2. Theoretical Backround

When the sample is first injected into the carrier stream, a well defined sample-plug is formed in the stream. As the sample-plug is swept downstream through the reaction manifold system of

narrow bore tubing the plug disperses freely into and, thus mixes with the carrier stream under laminar flow conditions to form a gradient. The magnitude of this dispersion is dependent on the operating parameters applied to the system. The operating parameters include, sample volume, carrier stream flow rate, reagents stream flow rate, pressure in the system, type of tubing, tube length, tube bore size, coil diameter and viscosity differences.

The efficiency of FIA lies in the ability to manipulate the dispersion of the sample-plug so that the degree thereof suits the requirements of an analytical procedure. Varying the values of operating parameter (mentioned above) confers a significant degree of control over the dispersion characteristics and facilitates optimization of a flow injection systems for many diverse applications.

4.2.1. Dispersion

Ruzicka and Hansen [1] defined dispersion as "the dilution of a sample volume undergoes when injected into a flowing stream". In FIA the extent of the dispersion is usually expressed in terms of peak height (or area). The ratio of the injected concentration(C_0), of a given component to that corresponding to any point on the dispersed peak concentration (C) at the detector is known as:

$$D = \frac{C_0}{C} = \frac{H_0}{H} \qquad (1)$$

D is influenced by the three interrelated and controllable variables, namely, sample volume, tube length, and pumping rate. This is demonstrated in Figure 4.1. (a), sample volume is varied whereas other two variables are kept constant. In Figure 4.1. (b) the tube length is varied whereas

the other two variables are kept constant. Figure 4.1. (a) shows the need for highly reproducible injection volumes when dispersion of 2 and greater are used, and it is also important to control other variables in order to obtain good precision.

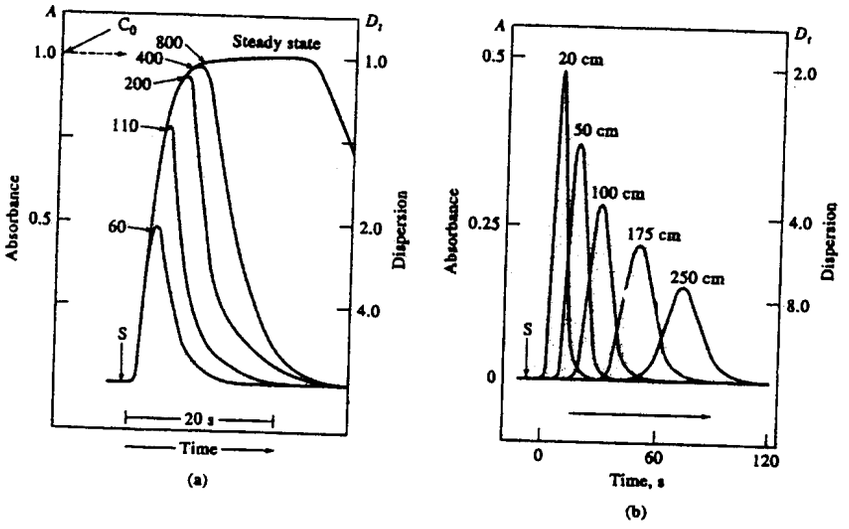

Figure 4.1.: Effect of sample volume and length of tubing on dispersion [2]. (a) Tube length: 20 cm; flow rate: 1.5 mℓ/min; indicted volumes in $\mu\ell$. (b) Sample volume: 60 $\mu\ell$; flow rate: 1.5 mℓ/min.

Dispersion is time dependent, which means an injected sample will have mixed to different extents at different points along the flow-line. There are two mechanisms which contribute to the dispersion of the injected sample, namely, convective transport (turbulent flow or laminar flow) and diffusional transport (axial or radial). Both of these concepts are present in Figure 4.2.. The convective transport occurs under laminar flow conduits, which yields a parabolic velocity profile with sample molecules at the walls. Diffusional transport gives rise to axial and radial diffusion. There is the third type of transport which originate from density gradients, but its contribution to the final dispersion is rather small since density differences between the fluid and the sample zone are generally negligible.

The value of dispersion (D) is greater than unity and reflects the dilution factor of the analyte under investigation. Depending on the degree of dispersion obtained, FIA systems may be classified as high (D > 10), medium (D = 3-10) or limited dispersion (D = 1-3). The simplest way of measuring D is to inject a well defined volume of dye solution into a colourless carrier stream and continuously monitor the absorbance. The height of the recorded peak (H) is measured and compare with the distance between the baseline and the signal recorded when the flow cell is filled with undiluted dye (H_0) [3].

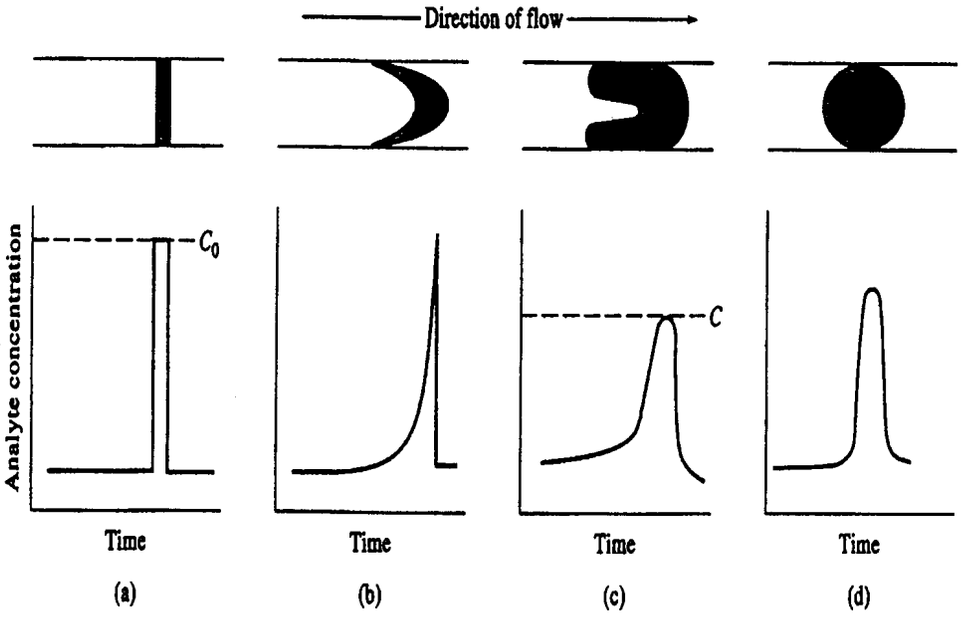

Figure 4.2.: Representation of concentration contours at stages in the dispersion process: (a) no dispersion, (b) dispersion by convection, (c) combination of convection and diffusion, and (d) dispersion by diffusion. [2]

4.2.1.1. Limited dispersion

Limited dispersion is of course used in estimations where little or no dilution of the sample is

required, e.g., in the determination of pH, use of ion-selective electrodes, and introduction of sample into flame photometers or atomic absorption spectrophotometers. High sampling rates of up to 700 per hour is possible due to the little dispersion of the sample.

4.2.1.2. Medium dispersion

According to Fang [4], systems with a dispersion coefficient between 2-10 are classified as medium dispersion systems. Medium dispersion manifolds have been used for most of the analytical procedures, in which the sample zone is mixed with the carrier stream (and perhaps with several reagents in sequence) to produce a product that can be sensed by a flow-through detector. Most of the colorimetric procedures fall under this category of dispersion. Not only must sufficient mixing take place between the sample zone and the reagent but also sufficient time must elapse to allow adequate formation of products before the detector is reached. Most colorimetric procedures are based on fast reactions and are ideally suited to this approach. For slower reactions the residence time is increased by decreasing the flow velocity and by increasing the coil length, which would increase dispersion and band broadening. Very slow reactions are probably best accommodated by using air-segmented Auto Analyzer apparatus.

4.2.1.3. Large (High) dispersion

Systems with high dispersion, are normally used where samples are highly concentrated for the detector. In such cases components such as mixing chambers may be required to achieve the high degree of dilution [4]. Large dispersion can be used either to obtain a suitable sample dilution or to stretch a concentration gradient over a longer period of time. More highly dispersed patterns,

best produced by inserting a mixing chamber between the injection part and the detector. They are useful when performing flow-injection titrations.

4.2.2. Theoretical models for dispersion

A number of theoretical models have been proposed to describe or predict a general expression C = f (t) accounting for the signal profile and relating its characteristic (travel, residence and baseline to baseline times, and peak height or area) to the experimental parameter of FIA system that includes flow rate, reactor length, tube diameter etc..

This section covers several models that have been developed for laminar flow conditions to define the theoretical principles of FIA and device mathematical expression of the form C = f (t) which accounts for the physical behaviour of an injected plug.

4.2.2.1. Taylor's model

The application of Taylor's model has been discussed by several authors [5-9]. With all the controversies surrounding this model, it is accepted that this model only holds for low flow rates and very long reactors. As a results this help to compensate for radial concentration changes and favour the prevalence of diffusion phenomena. Taylor's model is applicable to a Gaussian distribution defined by C = *f* (t) in a form which depends on the chosen parameters, namely,

$$C = f(t) = \frac{m}{r^2} \cdot \frac{1}{4\pi Dt} e^{-\left[\frac{(x - L)^2}{4Dt}\right]} \quad (2)$$

and

$$C = \frac{C_0 V_i}{q\sigma \cdot \sqrt{2\pi}} e^{-\left[\frac{(t-t_r)^2}{2\sigma^2}\right]} \qquad (3)$$

where; C is the concentration of the sample at a given time,

C_0 is the initial concentration of the injected sample,

V_i is the volume of the injected sample,

m is the injected solute mass ($m = C_0 V_i$),

t is the time after injection,

σ is the parameter corresponding to the standard deviation (Gaussian distribution),

x is the axial distance from the injection point.

This model can be applicable when the flow rates are low, reactors are long, and the injected volume is negligible compared to the volume of the reactor ($V_i << V_r$). The model is not applicable under normal FIA conditions [5, 9], but the Arris-Taylor modification is applicable to the normal FIA system [10].

4.2.2.2. Tanks-in-series model

This model which is closely related to the concept of age distributions function. It was first discussed in connection with FIA by Ruzicka and Hansen [3] and later by Betteridge [8]. This model is similar to liquid chromatography in that it uses theoretical plates. The mathematical expression derived from this model is:

$$C=\frac{1}{(\overline{t_r})_N}\left\{\frac{t}{(\overline{t_r})_N}\right\}^{(N-1)}\cdot\frac{1}{(N-1)}e^{\left[-\frac{t}{(\overline{t_r})_N}\right]} \qquad (4)$$

where $(\overline{\ }_r)_N$ is the mean residence time of an element of fluid in a given tank. The method fails for small N values. This model is a better improvement than the Taylor's model [6], but it is not ideal for short reactors. It offers an accurate dispersion for single bead reactors according to Reijn et. al [11].

4.2.2.3. Mixing chamber model

This model was developed by Pardue and Fields [12] as an improvement of the tanks-in-series model [13]. The deduction of the method was based on the following four conditions:

1. Chemical reaction should be fast, because reaction rates are not classified as variables.
2. The sample should reach the mixing chamber without undergoing dispersion across the two interfaces.
3. The analyte and the reagent should mix immediately thoroughly inside the chamber.
4. Dispersion should not occur between the chamber and the detector.

This model consists of three stages, viz,:

1. **First stage:** At the time (t_0) when the sample start to enter the chamber, it mixes and reacts immediately with the reagent, because of its displacement and reaction. The reagent concentration of $C^0_{by} = C^0_b$ is reduced due to displacement. The decrease in the

reagent concentration due to chemical reaction and dilution in the chamber is given by:

$$-\frac{dC_{bg}}{dt} = \frac{qC^{0}_{as}}{nV_g} + \frac{qC_{bg}}{V_g} \qquad (5)$$

and

$$C_{bg} = \left(\frac{C^{0}_{as}}{n} + C^{0}_{bg}\right) e^{\left(-\frac{qt}{V_g}\right)} - \frac{C^{0}_{as}}{n} \qquad (6)$$

which is valid between t_0 and t_1, where C_{bg} is the reagent concentration, C^0_{as} is the initial sample concentration, t is time in seconds, and V_g is the volume of the chamber.

2. **Second stage:** $t_1 < t_2 < t_3$, the rest of the samples enters the chamber. At this stage the concentration of the reagent is zero ($C_{by} \approx 0$), therefore the sample concentration (C_{ag}) start to increase with time according to the following equation:

$$-\frac{dC_{ag}}{dt} = \frac{q}{V_g} C^{0}_{as} - \frac{qC_{ag}}{V_g} \qquad (7)$$

and

$$C_{ag} = C^{0}_{as}\left[1 - e^{-qV(t - t_1)}\right] \qquad (8)$$

it is valid between t_1 and t_2, where C_{ag} is the sample concentration in the chamber, q is the flow rate (mℓ/min), V_g is the volume of the chamber, and C^0_{as} is the initial sample concentration.

3. **Third stage:** This is the stage where sample starts leaving the chamber unreacted. The reagent enters the chamber and then react with the remaining sample. Due to sample leaving, the concentration of sample decreases in the chamber and is given by equation (7) and the following equation:

$$C_{ag} = C_{ag,\max} \cdot e^{-\frac{q}{V_g}(t - t_2)} - nC_b^0 \left[1 - e^{-\frac{q}{V_g}(t - t_2)} \right] \quad (9)$$

it is valid between t_2 and t_3 (end point); where C^0_b is the concentration of the undiluted reagent in the flowing stream, C_{ag} is the sample concentration in the chamber, q is the flow rate (mℓ/min), and V_g is the volume of the chamber.

The concentration of the sample in the mixing chamber increases to reach a maximum value at t_2 after which the concentration decreases again. The rate of decrease of the sample concentration in the chamber depends on C^0_{as} relative to C^0_b, as expressed in the above mathematical equation. The same expression is valid when the carrier stream is contains no reagent and the injected sample is only diluted in the chamber ($C^0_b = C_{bg} = 0$).

4.2.2.4. General model

This model take into consideration both the convective and diffusional transport. It describes the overall physical phenomena as:

$$\frac{\partial C}{dt} = D_m \left(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial r^2} + \frac{1}{r} \cdot \frac{\partial C}{\partial r} \right) - u_{\max} \left(1 - \frac{r^2}{a^2} \right) \frac{\partial C}{\partial x} \quad (10)$$

where C is the concentration at the point (x, r, t), D_m is the molecular diffusion coefficient of a sample. The left hand side corresponds to diffusional transport. The first term within the brackets accounting for axial diffusion (dependence of C on ℓ) and the other four for radial diffusion (dependence of C on r).

4.2.3. Practical definition of the dispersion

The dispersion at the detector is given by the position and shape of the analyte signal. Therefore, practically dispersion is defined from the parameters characterizing the transient signal. An FIA peak is characterized qualitatively by:

1. Its position as defined by the travel time, t_a.
2. Its bandwidth, characterized by the baseline-to-baseline time, Δt.
3. The coordinates of the band maximum (T, C_{max}).

4.2.3.1. Růžička's dispersion coefficient

Dispersion was proposed by Růžička et. al [14] in 1977 and called it the dilution factor, and is also known as "practical dispersion" or simply "dispersion". Dispersion can be expressed as the dilution of the sample from the point of injection (C_0) to detector (C) in a FIA manifold [14] .

It was the only parameter used to characterize passage of the sample through the system. It can also be expressed as a ratio between peak heights obtained for undiluted signal (h_0) and for recorded peak signal (h_{max}).

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$$D_{max} = \frac{0}{h_{max}} \qquad (11)$$

There is a direct relationship between the property used for detection, the magnitude of the transduced signal recorded and the concentration of the sample or its reaction product. The dispersion coefficient can be determined from the ratio of the height of the signal found in the absence of dispersion to that of the FIA signal, thus:-

$$D_{max} = \frac{k_0 h_0}{k_1 h_{max}} = \frac{h_0}{h_{max}} \qquad (12)$$

where D_{max} is the dispersion at maximum signal (D>1); k_0 and k_1 are proportionality constants; h_0 is the magnitude of the response recorded when the sample solution is used as a carrier; and h_{max} is maximum response recorded of sample injected into a carrier. When $k_0 = k_1$ the relationship between response and concentration is linear. From the equation it can be seen that the larger D, the less intense the signal (sensitivity) and the broader the curves recorded.

4.2.3.2. Vanderslice's expressions

Vanderslice et. al [10] reported a numerical solution for the general diffusion-convection equation by providing two simple expressions which relate the travel time and the baseline-to-baseline time to the geometric and hydrodynamic characteristics of the FIA system. This equation has two numerical solutions,

$$t_0 = \frac{109RD^{1.025}}{f} \cdot \frac{h}{q} \qquad (13)$$

and

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$$\Delta t = \frac{35.4\ R^2\ f}{D^{0.36}} \cdot \left(\frac{l}{q} \right) \qquad (14)$$

where t_0 is the travel time in seconds, Δt is the baseline-to-baseline time in seconds, q is flow rate (mℓ/min), f is Vanderslice's accommodation factor, R is the tube radius (mm), D is the molecular diffusion coefficient (cm^2/sec), h and l are various tube lengths (cm).

The above equations provide time related information on the FIA signal, but has no correspondence to aspect such as coordinates of the maximum (T, C_{max} or D). The accommodation factor (f) is very important in both equations in that it is an adjustable parameter to the experimental observation [6].

4.2.4. Influence of various factors on the dispersion

The overall dispersion in FIA is considered as the sum of the dispersions originating from the four main parts of the system [15].

$$D = D_{injection} + D_{transport} + D_{detector} + D_{connectors} \qquad (15)$$

Factors that influence dispersion are sample volume, hydrodynamic factor (like flow rate), geometric factors (reactor shapes from open, coiled and packed), knotted reactors, normal packed tubes (single bead string reactor), dilution in a closed-loop system, and partitioning of the injected volume into two streams beyond a splitting point.

4.2.4.1. Sample volume

Figure 4.3. shows the influence of various injected sample volume on the dispersion coefficient. The figure reveals the following:

1. The travel time does not depend on the injected sample volume.
2. The residence time, and hence t', increases with the injected volume ($T_1<T_2<T_3<T_4$).
3. The baseline-to-baseline time also increases with the injected volume ($\Delta t_1<\Delta t_2<\Delta t_5<\Delta t_4$).
4. The dispersion coefficient decreases with increasing sample volume.

According to Růžička, D and V_i are inversely proportional to each other, that is,

$$D = \frac{k}{V_i} \qquad (17)$$

where k is proportional constant and V_i is the injected sample volume.

A large sample volume has no influence on the travel time, but increases the residence and baseline-to-baseline times. The peak forms a plated shoulder at very high sample volumes. In extreme cases where the sample volume is very large a portion (central) of the sample plug remains undiluted.

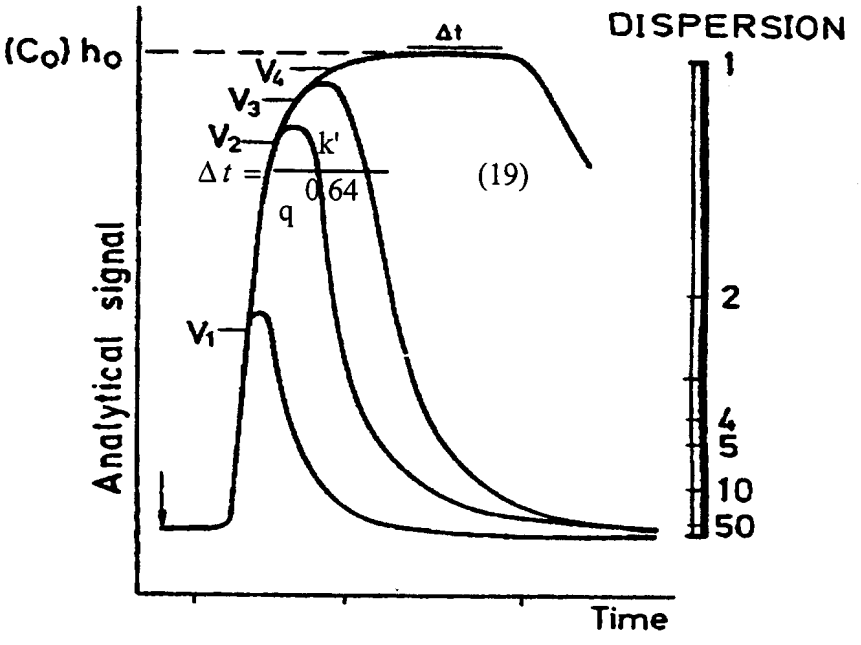

Figure 4.3.: Superimposed curves of the injected increasing sample volumes ($V_1<V_2<V_3<V_4$).

4.2.4.2. Hydrodynamic factors

Vanderslice's expression provide a relationship between flow rate (q), travel (t_a) and baseline-to-baseline (Δt) time as:

$$t_a = \frac{k}{\frac{0.125}{q}} \qquad (17)$$

and

$$t_a = \frac{k'}{\frac{0.64}{q}} \qquad (18)$$

Figure 4.4. shows that when the flow rate increases the dispersion decreases. This is confirmed

by the equation above. An increase in flow rate results in a decrease in the values of D, t_a, the residence time and the peak width.

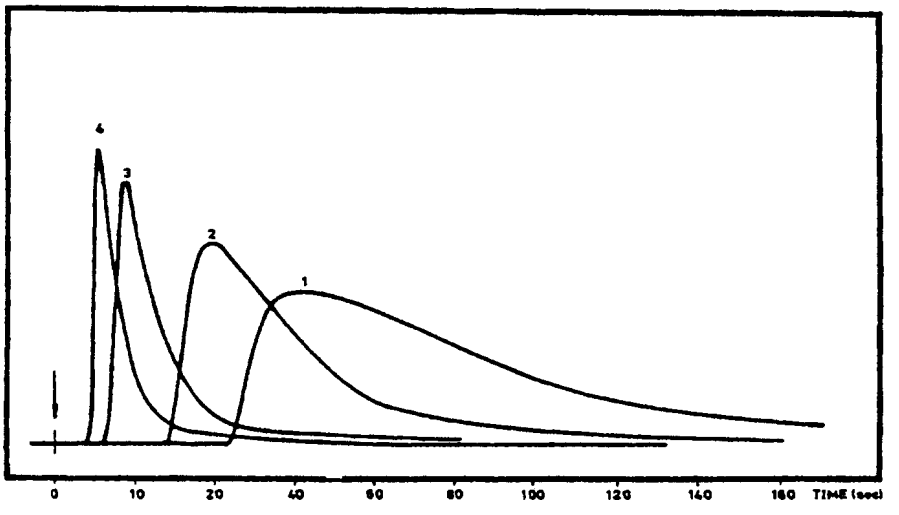

Figure 4.4.: Superimposed signals obtained when different flow rates are used in FIA ($q_1<q_2<q_3<q_4$). L = 100 cm; d = 0.5 mm; q_1 = 0.57 mℓ/min; q_2 = 0.95 mℓ/min; q_3 = 2.00 mℓ/min; q_4 = 3.00 mℓ/min.

4.2.4.3. Geometric factors

This deals with the influence of the reactor shape (i.e., open, coiled, packed) and its dimensions on the dispersion.

1. **Straight tubes:** They represent the simplest forms of reactors for FIA. The two most important parameters that play a critical role in these forms of reactors are length and diameter. If both the length and the diameter are increased , dispersion coefficient also increases this is shown by the equations given by Vanderslice:

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$$t_a = kL^{1.025} \qquad (19)$$

and

$$\Delta t = k' L^{0.64} \qquad (20)$$

The two equations are consistent with Růžička's expression:-

$$D = kL^{\frac{1}{2}} \qquad (21)$$

Both Růžička and Vanderslice expressions are proven to be correct by results shown in figure 4.5..

The dispersion, travel time and peak width increase with increasing tube diameter.

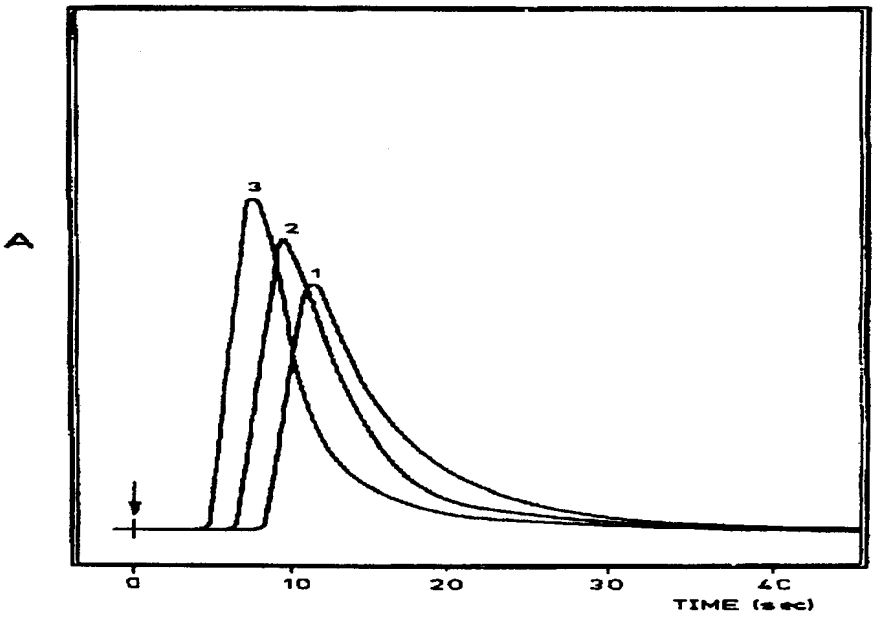

Figure 4.5.: Superimposed signals obtained when different reactor diameters are used.

L = 100 cm; q = 20 mℓ/min; d_1 = 0.70 mm; d_2 = 0.50 mm; d_3 = 0.35 mm.

2. **Coils:** Coiling is qualitatively defined by relating:

$$\lambda = \frac{\text{coil diameter}}{\textit{tube} \text{ diameter}} \qquad (22)$$

For λ (coiling) = 10, the dispersion is one-fourth of that for the uncoiled tube. The general trend of coiled tubes is that they decrease the dispersion experienced by a sample plug normally in a straight tube of similar length. Dispersion decreases with decreasing reactor coil diameter.

4.2.4.4. Knotted reactors

Dispersion decreases if the length of flexible tubing is knotted from end to end. Knotted reactors have the same effect as interified coils.

4.2.4.5. Normal packed tubes

Packed reactors are common in chromatography and chemical engineering. The ratio:

$$\tau = \frac{\text{tube diameter}}{\textit{particle} \text{ diameter}} \qquad (23)$$

is a useful parameter. When τ is between 5 and 50 axial dispersion is directly related to the particle size; the smaller the particle diameter the smaller the dispersion. The only limitation is when the packing is too light, high pressure arise which are not ideal for FIA. This result in the two characteristics of FIA, i.e., simplicity and low cost being deprived.

4.2.4.6. Single bead string reactors (SBSR)

The SBSR are made of ordinary Teflon tubes packed with tiny glass beads having a diameters of 60-80% of the inner diameter of the tube [16]. This type of reactors increase radial dispersion, which reduces dilution of the sample in the flow line, and therefore decreases dispersion. The advantages of SBSR are:- dispersion is 10 times less than that of open tubes; peak heights or dispersion coefficients are independent of the flow rate over the range 0.2-1.5 mℓ/min; sample throughout is high; and it allows different streams to merged easily.

4.2.5. Influence of chemical kinetic on dispersion

Dispersion is both a physical and chemical phenomena, therefore, it is influenced by chemical kinetics. Reaction rates are considered in FIA since measurement is made under non-equilibrium conditions. Contribution of chemical reaction in SFA is less significant since chemical equilibrium is attained by the time the sample reaches the detector.

A chemical reaction is modify by the value of the dispersion in two ways:

1. When some property of a reaction product is measured, the chemical contribution results in a decrease in the practical dispersion.

$$A_{sample} + B_{carrier} \xrightarrow{k_1} C_{measured} \qquad (24)$$

D becomes smaller when reaction rate constant, k_1, is higher.

2. When property of the reactants (A) which are consumed with time are measured, the chemical contribution increases the dispersion coefficient. The higher the reaction rate (k_1) the larger is D.

4.3. Basic FIA components

A typical FIA system (Figure 4.2.) consists of four essential components, namely, a propelling system, an injection system, transport (reactor) system, and a detection system. These different units are connected by Teflon connectors and Tygon tubing to form a flow-through-system.

4.3.1. Propelling system

The propelling system should be constant and perfectly reproducible in order to give a predictable residence time and a constant dispersion. The flow should be pulse-free, if not they should be well dumped by using a suitable attenuator. There are four general types of commercially available propelling system, namely; peristaltic pump, the displacement (piston) pump, gravity and gas pressure systems and constant head devices.

4.3.1.1. Peristaltic pumps

Peristaltic pump (Figure 4.6.) is the most popular type of propelling unit used in FIA. It is simple, cheap and easy to manage. It works on the base of rollers forcing liquid through an elastic tube. The number of channels (tubes) ranges between 1-16 (depending on the roller length).The commonest carrying up to five channels.

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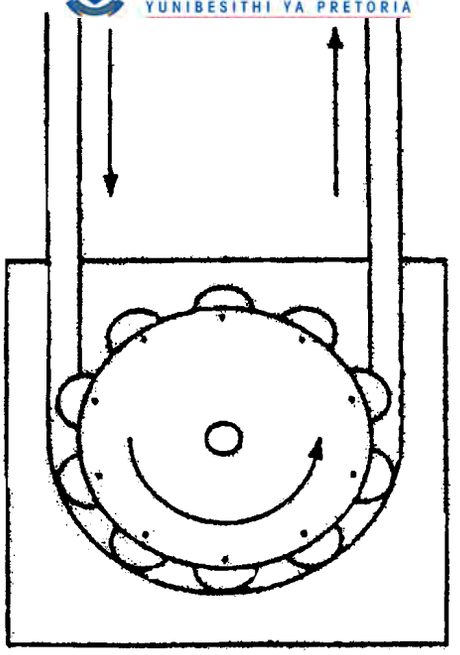

Figure 4.6.: Schematic diagram of a peristaltic pump (showing one channel).

Table 4.1.: Pump tubing materials.

Type of solution	Tubing material
Aqueous solution	PVC, Tygon
Dilute ethanol solution	PVC, Tygon
Dilute acids and bases	PVC, Tygon
Concentrated acids and bases	Fluoroplasts (Acidflex)
Alcohols	Modified PVC (Solvaflex)
Lower alcohols	Silicone rubber
Formaldehyde, acetaldehyde	PVC, Tygon
Acetone	Silicone rubber
Acetic acid and anhydride	Silicone rubber
Aliphatic hydrocarbons	Modified PVC (Solvaflex)
Aromatic hydrocarbons	Fluoroplasts (Acidflex)
Chloroform	Fluoroplasts (Acidflex)
Carbon tetrachloride	Modified PVC (Solvaflex)

The tubes are made of different materials. These materials are listed in Table 4.1.. This type of pump give pulsed streams, but this was overcome by rotating the pump head at a high rate and by having closely spaced rollers. One minor limitation, is that they generate static electricity, but it can be eliminated by short circuiting the inlet and outlet of each tube.

4.3.1.2. Displacement pumps

Displacement pumps have not been widely used for FIA, they are mainly used in HPLC. Their advantages include high volume delivery precision, high pressures and reproducibility timing. They are very expensive as compared to peristaltic pump. Displacement pumps produces a pulsed stream, especially single-piston pumps. Damper or restricters are required to obtain a pulse-free flow.

4.3.1.3. Pressure system pumps

Pressure systems are of the two types, viz, gravity and gas pressure pumps. Gravity based propelling system rely on the difference in height between the reagent and/or carrier reservoir and the FIA flow-line. A pulse free flow is obtained and the difference in heights determines the flow rate. The main disadvantage of the gravity based propelling systems is that you need to maintain a constant level of the liquids in the reservoir in order to achieve a constant flow; this may be accomplished by using reservoirs with a cross sectional areas.

Gas pressure systems use a pressure regulated inert gas to pressurise the reagent and carrier containers. The flow on each stream is controlled by a ball/screw restrictor valve. This is

demonstrated on Figure 4.7.. The resulting flow is pulse free and there is no generated electrical interferences.

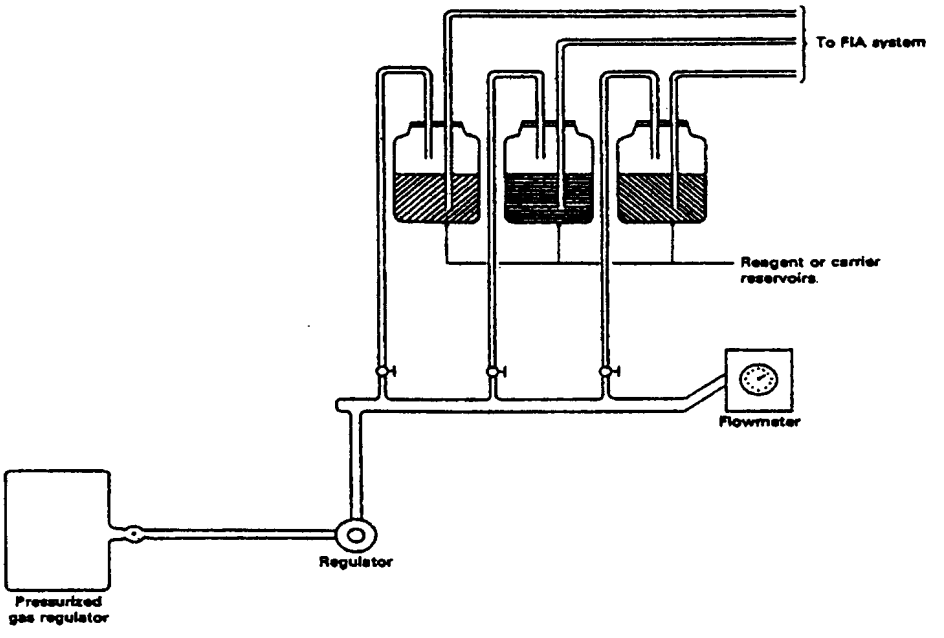

Figure 4.7.: Gas pressure system pump [18].

The main problems with these systems include regulating the flows on each stream and the possible bubble formation from different solubility of the gas in the various reagents. It consists of an inert gas supply fitted with a pressure control valve, a flowmeter (for checking flow supplied) and a distributor for delivering the gas to the different reagent or carrier solution reservoirs.

4.3.2. Sample injection systems

Sample injection systems should be able to provide highly reproducible insertion of an accurately

measured sample volume with minimum disturbance of the carrier flow. The requirement of precision is particularly important as the physical dispersion coefficient obtained can be critically dependent on the volume injected, with small changes in the volume producing large changes in peak height. There are two main types of sample injection systems that are used, namely, syringe and rotary valve.

4.3.2.1. Syringe

Syringe was the first sample injection system described by Růžička and Hansen [19] which relied on the use of a needless syringe (figure 4.8.).

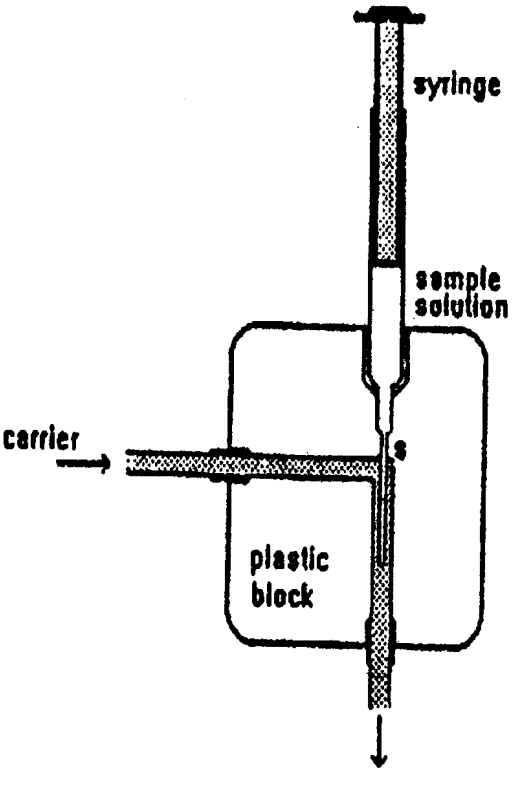

Figure 4.8.: Syringe-based injection system (s is septum).

This type of device is widely used in chromatography. The pressure exerted by the plunger displaced a septum, thereby momentarily creating a chamber accommodating the sample which was then forced through a channel into the carrier by return of the septum to its starting position (flap valve).

4.3.2.2. Rotary valve

The four-way rotary valve is the most common injection devices. Early FIA type of rotary valves were constructed so as to have an external sample loop which could be readily changed and a by-pass line so that the carriers stream continued to flow while the valve was in transition between the "fill" and "inject" positions. The valves that are frequently used in FIA are the 6- or 10-ports rotary valves as well as the 8-channel 16-port multi functional valve. The valve that was used for this project is the 10-port injector valve which is shown in Figure 4.9.

This valve has 5 channels engraved on the contact surface of the rotor and 10-ports on the stator or vice versa. Rotation of the valve between the two available position ("fill" and "inject") will cause each channel to connect at different set of ports, thus making manipulations such as sample loading and dilution possible. The advantages of rotary valves are reproducibility, wide sample range, rapidity, handy operation and capability for automization.

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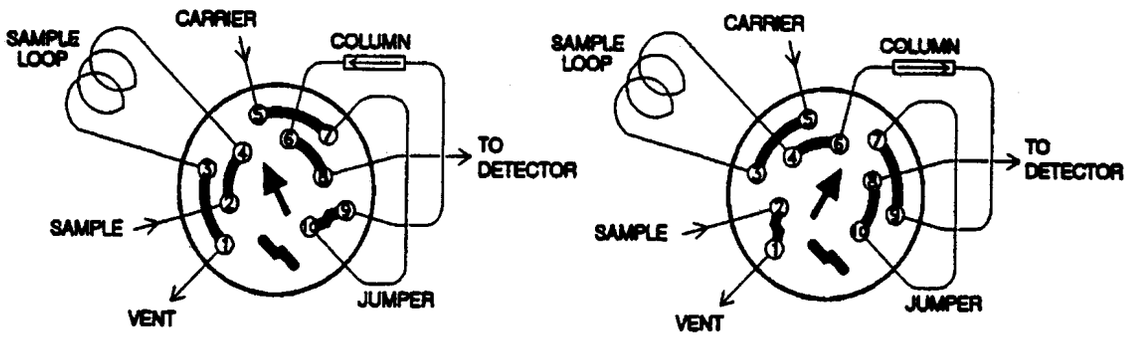

Figure 4.9.: Schematic diagram of a 10-port injector valve [20].

4.3.3. Transport and reactor systems (including connectors)

Components of the transport and reaction systems are intended both to carry the flowing stream along the manifold and to interconnect the various parts making up the working system. The reaction manifold system forms the heart of a FIA and in many ways the efficiency of a good FIA system starts here. The manifold system not only determines the accuracy, precision and reproducibility of the FIA system, but indirectly together with the pumping rate contributes to the sampling rate of a FIA system, but indirectly together with the pumping rate contributes to the sampling rate of a FIA method.

4.3.3.1. Tubes and connectors

The materials used for tubing are Tygon, Teflon, polyethylene and polypropylene. The internal diameter is between 0.1-2.0 mm, but for limited, medium and large dispersion is commonly 0.3, 0.5 and 0.7 mm respectively. Changes in diameter at connections result in irregularities markedly affecting the dispersion or the degree of dispersion will also depend on the inner diameter of the tube.

Different pieces of tubing are connected to one another as well as to the different components of the system by means of connectors. These connections should be void of dead volumes, leakage and introduction of air bubbles

4.3.3.2. Reactors

Varcacel and Castro [17] define reactors as units of the transport system acting on the residence time and the sample plug and endowing the latter with the required characteristics for measurement by the detection unit. FIA technique owe its versatility to a wide variety of reactors available. There are various types of reactors which are chosen relative to the particular needs of a specific analysis to be carried out.

4.3.3.2.1. Open tubes and coils

These are the simplest reactors consisting of a variable length of fixed-diameter tubing and located between the injection and sensing units. They are normally used for simple reactions. Poor precision will result from tubing that is allowed to change its configuration during the routine operation of the FIA system.

A variable length of open tubing which has been helically wound around a cylinder with a specific diameter is known as a coiled reactor. The coil length is a function of the residence time required for optimum carrier (reagent)-sample mixing, whereas its diameter influences sample dispersion.

4.3.3.2.2. Packed reactors

These reactors are made of a length of tubing filled with chemically inert or active material. Chemically active materials that are used include ion exchange resins, oxidants and reductants and enzymes. This type of reactor is not very often used in FIA because of complications that arise due to their physical nature.

4.3.3.2.3. Single bead string reactors (SBSR)

These reactors are packed with chemically inert polymer beads or glass with diameters 60-80% of that of the tubing. The tube is occupied by a single 'string' of beads. These type of reactors offer several advantages, viz, greater residence time, lower dispersion and smoother baseline.

4.3.3.2.4. Mixing chambers

A mixing chamber is used when a greater extent of mixing than is afforded by merging points or unstirred chambers or when complete sample-reagent homogenization is required. Advantage offered by mixing chamber is that the signal variation arising from changes in injection speed is reduced to such an extent that the injection can be done manually without an accessory intended to measure accuracy and reproducibility.

4.4. Detectors

The requirements of the detector in FIA are those normally associated with analytical

instrumentation in general, namely, fast response, low noise, high sensitivity, linear and dynamic range, reproducibility, good stability etc.. The detection system used in FIA comprise the most frequently used types of analytical detectors, namely, optical (spectroscopic and non-spectroscopic), electrical (amperometric, potentiometric, conductimetric, coulometric) and others such as thermo-chemical detectors.

4.4.1. Optical detectors

There are various types of optical detectors that are used in FIA, viz, spectrophotometers, spectrometers, chemiluminescence and fluorescence. Optical detectors are intensively used in FIA systems. They are as reliable as the electrochemical detectors. Optical detectors have also become popular in certain areas of FIA because they offer increase in sensitivity and have extended the FIA principle to more fields in chemical analysis.

4.4.1.1. Spectrophotometers

According to numerous publications to date, spectrophotometric detectors [21-22] are most widely used in FIA techniques. They are mainly used due to the simplicity of cell construction of the detection system.

A conventional batch spectrophotometer can easily be converted into a flow-through detector by simply substituting the conventional cuvette with a flow-through cell. Although a number of cells are available, in most cases Hellma U-shape type tubular glass flow cells with effective volumes ranging from 8-80 $\mu\ell$ are most popular. Depending on the spectral region covered, the cells are

furnished with either glass or quartz windows. A high precision and reliability with a high speed of analysis of analyte from matrix is obtained.

4.4.1.2. Spectrometers

Flame emission (FES), atomic absorption (AAS) and inductively coupled plasma emission spectrometers (ICP) have been used as detectors in FIA systems. The FIA system can be coupled directly to the nebuliser of these spectrometers. The advantages associated with the use of these spectrometers with FIA are, high sample throughput, multi-element analysis (ICP), lower volume ($\mu\ell$), minimum interference effect, and strong tolerance to high concentrations of dissolved solids in the sample. The main disadvantage associated with the spectrometers is the relatively large volume of the spray chamber (ca. 100 mℓ) as compared to the volume of sample used ($\mu\ell$ range).

4.4.1.3. Chemiluminescence and fluorescence

For some of the optical detectors like the chemiluminescence and fluorescence, the sensitivity is less than that of amperometric detectors. They are more suitable for trace analysis and their selectivity can be increased by the utilization of an enzymatic or immun.-reaction.

Fluorescence detectors cannot be used for detection of all types of substances. Advantages such as good sensitivity, low detection limit, miniaturization capability and relatively low cost are responsible for the extensive use of this technique. One major disadvantage is the scarcity of substances exhibiting fluorescent properties. They can be used successfully in a conduits of a FIA system for trace analysis [23-25].

Chemiluminescence detectors are more versatile than the fluorescence detectors. Burguera and Townshend [26] used the chemiluminescence detector for the determination of triethanolamine. An optimum FIA-chemiluminescence should meet the following requirements:-

1. The distance between the injection valve and the detection coil should be as short as possible.
2. The detection coil should be long enough to give a sufficiently intense signal.
3. The reaction conditions should be chosen so as to obtain the maximum intensity of chemiluminescence immediately after mixing rather than to maximise the overall yield.

This type of detector has been successfully being used in the conduits of an FIA system [27-33].

4.4.2. Electrochemical detectors

Electrochemical sensors are selective, sensitive and linear response over a wide concentration ranges [34]. Electrochemical detectors used in FIA are classified as those:

1. Based on the properties of the bulk of solution, eg., conductimetric [35] and capacitometric. These are non-destructive in character and non-selective. Their sensitivity is moderate and their linear dynamic range is limited. They have the advantages of being non-destructive and more or less universal. These are not used much in FIA.

2. Based on the properties of the solute, eg., amperometric [36-38], polarographic [39], coulometric [40-41] and potentiometric [42]. These are very sensitive and selective.

Their selectivity differs from that of optical detectors. Some of the electrochemical solute property detectors are (partly) destructive.

These detectors are electrochemical reactors in principle. Oxidation and reduction reactions take place at the electrode. An important feature is that the signal of this kind of detector is determined by the concentration or activity of the measured compound in the vicinity of the working electrode surface. The detector measures local rather than average concentrations in the flow channel.

Electrochemical detectors used in FIA can also be classified according to the geometry of the system. There are three different types , namely,

1. Annular detector, sensitive tube is inserted into the flow line.
2. Wire detector, metal electrode placed with its surface along the axis of flow
3. Cascade-type detector, the flow impinges frontally (ion-selective electrodes) or spherically (dropping mercury electrode).

4.5. Data acquisition

FIA, like all continuous flow analysers, monitors the response by the detection/measurement system to the changing concentrations of a determinant or its chemical derivative with time. The basic parameters that delimits on a FIA transient signal is the peak (either height, area or width). The peak height is the most commonly used technique particularly when data can only be obtained in the analogue form. The height or width of the peak is evaluated with respect to

baseline restoration time which determines sensitivity and sample throughput. The quality of peak is related to the extent of dispersion.

Data acquisition and computer aided flow analysis was obtained from the FLOWTEK package provided by MINTEK [43]. This specialised software can control a series of different FIA modes. The basic components of FIA are electronically controlled. The pump, rotary valve and the detector have to operate precisely synchronized. The response is displayed on the computer monitor as peak height, width, peak time and concentration. These data can be saved within the computer. A sequence of steps necessary for the analysis to be performed can be built up and stored for future use.

Data acquisition and signal amplification were achieved by using a PC-30-B interface board (Eagle Electronics, Cape Town, South Africa) and an assemble distribution board (MINTEK, Randburg, South Africa).

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"In the fields of observations, chance favours only the mind that is prepared."

By: Loius Pasteur

CHAPTER 5

KINETIC STUDY OF THE DECOMPOSITION OF NITRITE TO NITRATE IN ACID SAMPLES USING RAMAN SPECTROSCOPY

5.1. Introduction

This chapter describes the kinetic study of the decomposition of nitrite to nitrate in acid medium using Raman spectroscopy.

Nitrite, an intermediate stage in the nitrogen cycle, occurs in water as a result of the biological decomposition of proteinaceous materials. Nitrite and the oxides of nitrogen are recognized as important pollutants in water and air respectively. Nitrite in some instances in combination with nitrate, is added to cured meats such as bacon to prevent food poisoning from *Clostridium botulinum*[1] and also to confer the characteristics colour and organoleptic properties to the product. Infant methaemoglobinaemia [2] has been traced to high nitrite content. Some scientists now believe that the body may convert the nitrites to nitrosamines [3], which are known to cause cancer.

5.2. Chemistry of nitrites

The aqueous solution chemistry of nitrous acid and nitrites has been extensively studied. The nitrites vary widely in solubility, those of the alkalies and alkaline earth metals being slightly soluble. Moreover the latter are relatively unstable, some decomposing at room temperature. The

nitrites, like nitrous acid, function either as oxidising or reducing agents.

The chemistry of nitrite is such that under even mildly acidic conditions a significant proportion of the anion will be in equilibrium with its conjugate acid, HNO_2, nitrous acid [4]. This compound is highly reactive and will undergo a series of reactions with many nucleophilic species which are likely to be present in the sample, with the net result that the analyte is rapidly destroyed. This can be shown from the equation below:

$$NaNO_2 + HCl = NaCl + HNO_2$$

Nitrous acid is unstable forming an equilibrium mixture:

$$3\ HNO_2 \rightleftharpoons NO_3^- + 2\ NO\uparrow + H_3O^+$$

which in concentrated solution or on warming is largely displaced to the right. It is fairly weak acid with pKa 3.35 at 18 °C. The NO undergoes further reaction, so that the actual system is complex. One of these reactions is:

$$NO + OH \rightarrow NO^+ + OH^- \leftrightarrows NO.OH \rightleftharpoons HNO_2$$

The existence of NO^+ and NO^- help explain the kinetics nitrous acid as an oxidising agent. It reacts with ammonia, urea, sulfonates and some other nitrogen compounds to produce nitrogen.

Sodium nitrite ($NaNO_2$) is mildly toxic (tolerant limit ≈ 100 mg/kg body weight per day, i.e., 4-8

g/day for human). It has been much used for curing meat. It is also used industrially on a large scale for the synthesis of hydroxylamine, and in acid solution, for the diazotisation of primary aromatic amines:

$$ArNH_2 + HNO_2 \rightarrow HCl \rightarrow [ArNN]Cl + 2H_2O$$

The resulting diazo reagents undergo a wide variety of reactions including those of interest in the manufacture of azo-dyes and pharmaceuticals.

5.3. Chemistry of nitrates

Nitrates, the salts of nitric acid are readily soluble in water with the exception of the basic nitrates of mercury and bismuth. Nitrates are present naturally in soils, waters, all plant materials, and in meats. They are also found in small concentrations (1-40 $\mu g/m^3$) in air as a result of air pollution. Nitrates in soil and in surface and ground water result from the natural decomposition by microorganisms of organic nitrogenous material such as the protein in plants or animals. As with the salts of other oxoacids, thermal stability of nitrates varies markedly with the basicity of the metal and the products of decomposition are equally varied. The presence of organic matter or other reducible material also markedly affects the thermal stability of nitrates.

5.4. Choice of analytical methods

Many spectrophotometric methods [5-12] have been reported for the determination of micro amounts of nitrite, most of them being based on the modification of the Shinn [13] reaction

which is based on the well known Gries reaction. In this reaction the nitrite is diazotised with sulphanilamide and the product coupled with N-(1-naphthyl)ethylenediammonium dichloride to form a highly coloured azodye which is measured at 520 nm. A number of flow injection systems [14-18] have been developed using these modifications, a method invented by Růžička and Hansen [19].

Over recent years new techniques such as HPLC [20-22], derivatization gas chromatography [23-26] and chemilumescence [27-29] have been developed and applied with success to foodstuffs and water. In general the colorimetric technique gives results that agree well with other procedures.

Some routine laboratories [30] complained that problems were encountered when the FIA version of the Shinn reaction was applied to certain samples mainly those from an acidic origin. An investigation was launched to try and find a solution to the problem.

The FIA procedure was studied in detail to see if the origin of the problem was situated in the method. From the experimental work carried out it was found that there was nothing wrong with the colorimetric method, but that the problem was in the samples analysed. In contrast to the substantial literature on analytical methods for nitrite and nitrate, and their application to foodstuffs and water, there is a dearth of information on the bias introduced by incorrect storage of samples prior to analysis. Published surveys often detail analytical methods employed in full but provide very little information on the sample storage conditions used and the possible resulting bias. It was therefore decided to study the stability of the nitrite ion under acidic and non-acidic condition related to sample conditions.

Raman spectroscopy was used to determine the rate at which nitrite decomposes with time, and the type and nature of the end product formed. One of the earliest systematic analytical investigation of Raman spectroscopy, that of Irish and Chen [31] presents a survey of the analytical properties of a large number of organic and inorganic compounds. Included in this group was nitrate, one of the target compounds . Recently, Lombardi [32] and co-workers presented a paper on quantitative and qualitative analysis of inorganic compounds by Raman spectroscopy, and included in this group was both nitrate and nitrite, the target compounds. The work is based on application to acid and non-acidic samples that contain nitrite and nitrate.

5.5. Experimental

5.5.1. Reagents

All reagents were of analytical reagent grade and de-ionised water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used in all the experiments. Sodium nitrite and potassium nitrate were oven dried at 110 °C for 24 hours before solutions were prepared.

The following samples were prepared:

0.625 mol.ℓ^{-1} nitrite in de-ionised water

0.625 mol.ℓ^{-1} nitrate in de-ionised water

0.625 mol.ℓ^{-1} nitrite in 1 mol.ℓ^{-1} NH_4Cl adjusted to pH 3

0.625 mol.ℓ^{-1} nitrite in 1 mol.ℓ^{-1} NH_4Cl adjusted to pH 4.8

0.625 mol.ℓ^{-1} nitrite in 1 mol.ℓ^{-1} NH_4Cl adjusted to pH 6

0.625 mol.ℓ^{-1} nitrite in 0.01 mol.ℓ^{-1} HCl

0.625 mol.ℓ^{-1} nitrite in 1 mol.ℓ^{-1} HCl

5.6. Apparatus

5.6.1. Instrumental conditions

Raman spectra with a resolution of 3 cm^{-1} were recorded using the macro chamber of a Dilor XY multichannel spectrometer. The 514.5 nm line of a Coherent argon ion laser was used at 200 mW for excitation of the sample.

5.6.2. Samples geometry

The liquid samples were in glass tubes during recording of Raman spectra. In all cases except for recording of kinetic data the diameter of the tubes was 6 mm. For the kinetic measurements a diameter of 17 mm was used to allow gas (NO) to escape freely from the sample.

5.7. Limits of detection

The limit of detection for both nitrate and nitrite was 500 $mg.dm^{-3}$.

5.8. Data processing

Raman intensity is related to the concentration of the species which generates the line through the equation:

$$\mathbf{I_R = JC}$$

J = specific or molar intensity of the line.

C = concentration of the scattering molecules in $mol.dm^{-3}$

In kinetic measurements absolute values of intensities were used. These values are shown in Table 1. The first step in the kinetic analysis of reactions is to establish the stoichiometry of the reaction and identify any side reactions. From the data it was assumed that it is a first order reaction with respect to the reactant, so equation (1) was used to plot the graph of $\ln([I]/[I_0])$ against time, where I = Raman intensity of the vibrational mode of the species.

$$\ln([I]/[I_o]) = -kt \qquad .....(1)$$

TABLE 1: Absolute values of intensities for nitrite and nitrate

Intensity/NO_2^- at ≈1330 cm^{-1}	Intensity/NO_2^-at ≈815 cm^{-1}	Intensity/NO_3^-at ≈1050 cm^{-1}	Time in seconds
1165	806	1095	0
1074	737	1213	15
1050	700	1279	30
1045	697	1380	45
1045	697	1380	60
1036	689	1506	75
1030	681	1512	90
1019	672	1512	105
1013	668	1556	120
1001	665	1592	135
991	648	1599	150
965	647	1605	165
957	646	1606	180
956	633	1640	195
933	631	1648	210
924	612	1704	225
920	607	1706	240

5.9. Results and discussion

All the samples were analysed immediately after preparation. Figure 5.1 shows the Raman active

peak of nitrate in deionised water peak at around 1050 cm^{-1}. This is the $\nu_2(A_1)$ N-O stretching vibration in NO_3^- [32]. Figure 5.2 is the Raman spectrum of nitrite in deionised water with peaks at 1330 cm^{-1} (the $\nu_2(NO_2)$ vibration [32] and 815 cm^{-1} (the δ(ONO) deformational mode [32]). The ν_2 water bending mode at around 1640 cm^{-1} is also observed here. Both nitrate and nitrite in deionised water were similarly analysed a few days later to determine whether any changes had occurred in the samples. No changes could be observed.

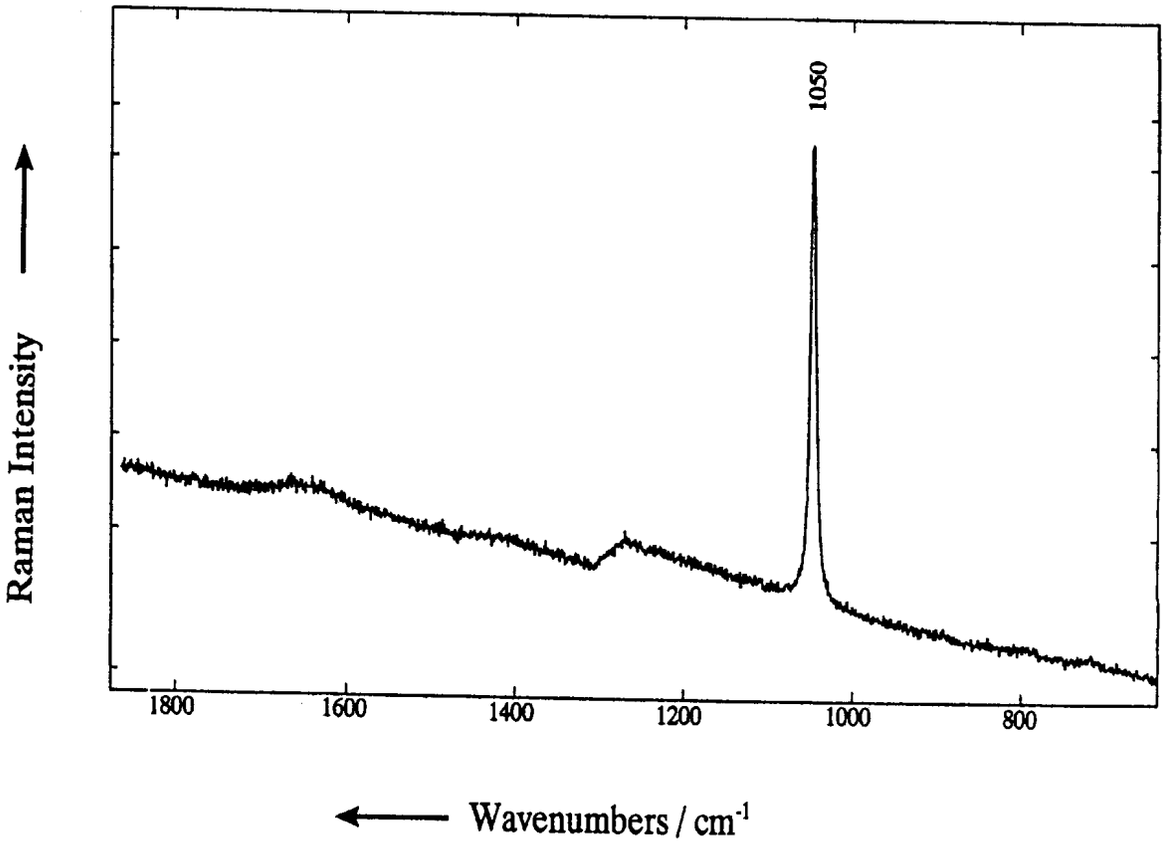

Figure 5.1.: The Raman spectrum of 0.0625 mol.ℓ^{-1} nitrate in deionised water, showing the N-O stretching vibration around 1050 cm^{-1}.

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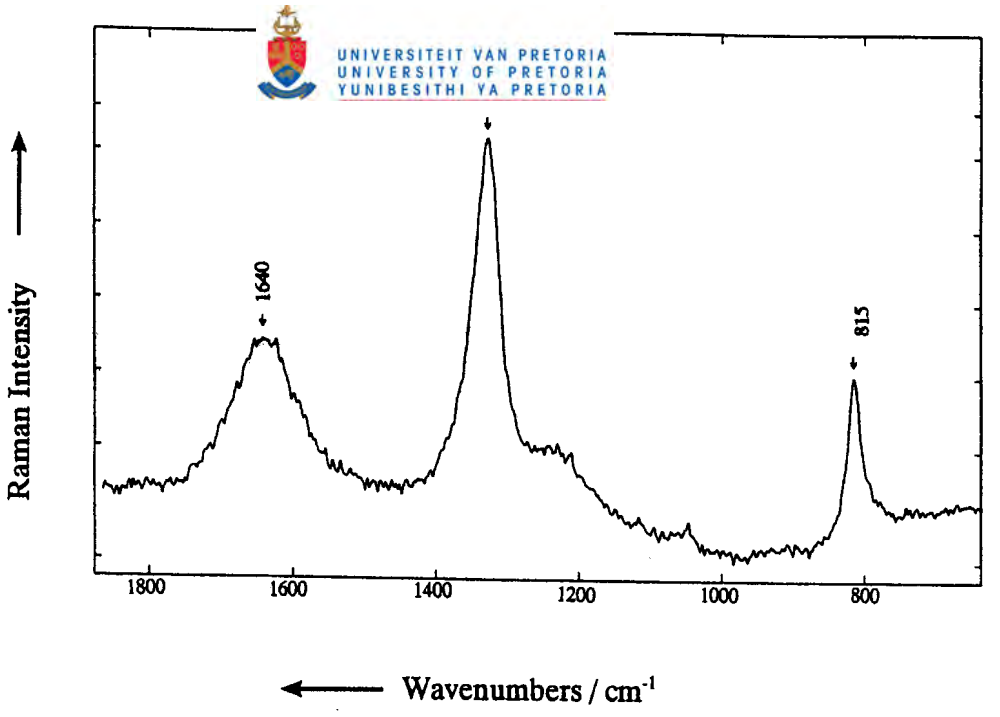

Figure 5.2.: **The Raman spectrum of 0.0625 mol.ℓ^{-1} nitrite in deionised water, showing the Raman active vibrations ν_s (NO_2) (around 1330 cm^{-1}) and δ (ONO) (at 815 cm^{-1}).**

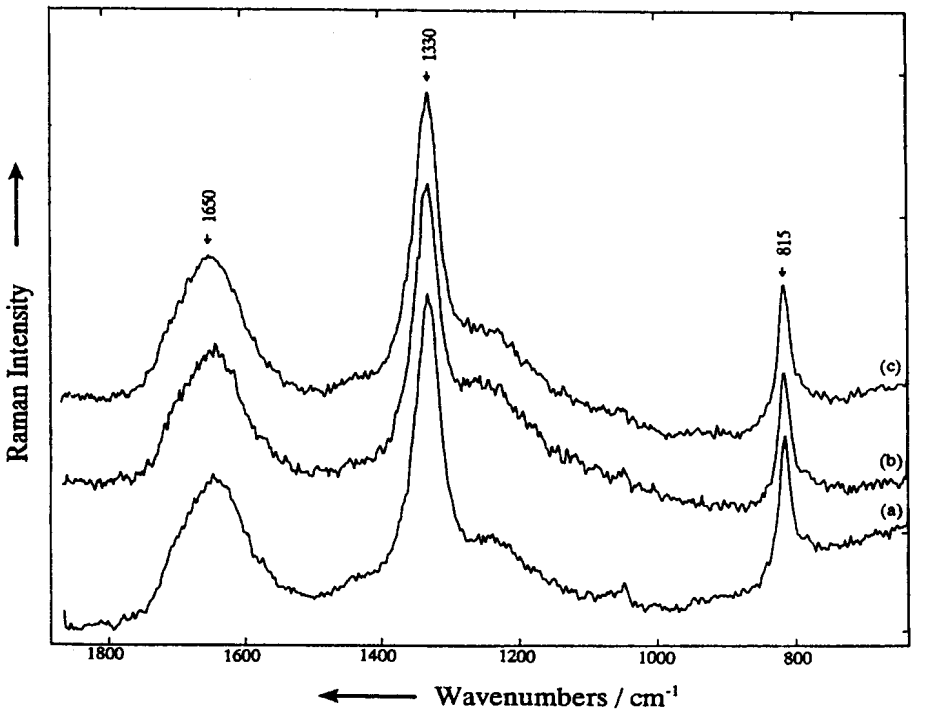

Figure 5.3.: **The Raman spectra of 1 mol.ℓ^{-1} NH_4Cl at pH 6 (a) after preparation, (b) after 1 day, and (c) after 4 days.**

The Raman spectra of 1 mol.ℓ^{-1} NH_4Cl at pH 6 and pH 4.8 behave more or less the same, because there is no change in spectra over several days of analysis. This is observed in figures 5.3 and 5.4. The same applies to the nitrite in 1 mol.ℓ^{-1} NH_4Cl at pH 3, but in this case the low intensity nitrate peak at around 1050 cm^{-1} appears after day 1 and remains for several days without a further increase in intensity even after day 9, as illustrated in figure 5.5.

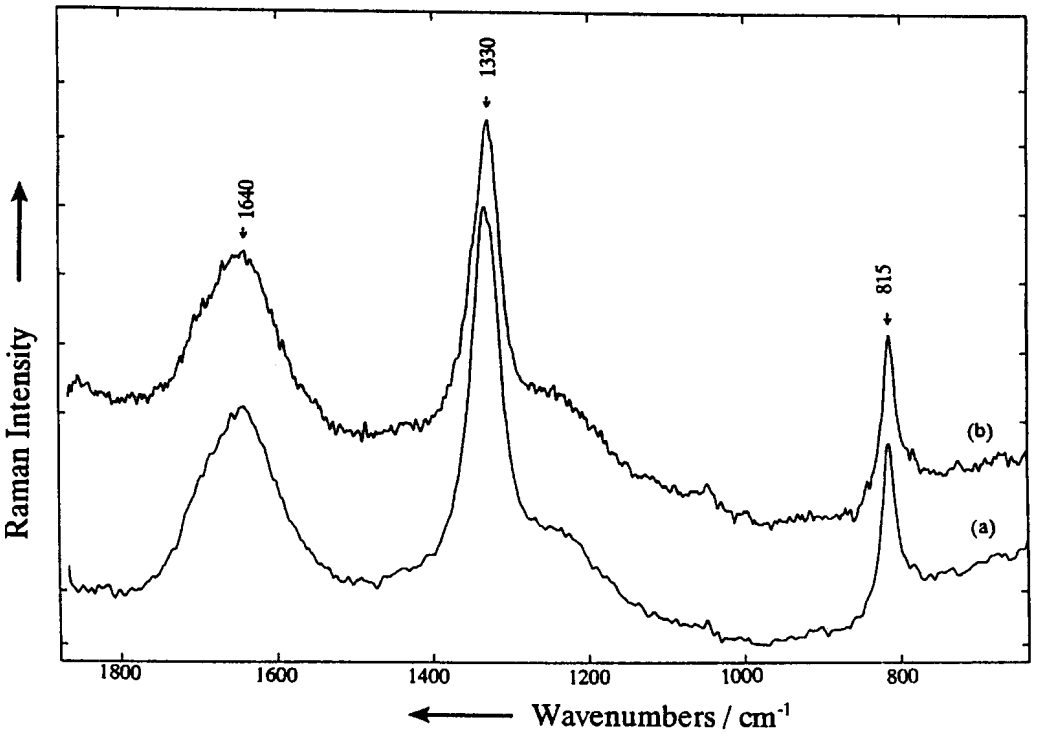

Figure 5.4.: The Raman spectra of 1 mol.ℓ^{-1} NH_4Cl at pH 4.8 (a) after preparation and (b) after 4 days.

Figure 5.6 shows the nitrite in the presence of a low concentration of acid, viz. 0.01 mol.ℓ^{-1} HCl. At t=0 both peaks of nitrite (1330 and 815 cm^{-1}) as well as the low intensity nitrate at 1050 cm^{-1} are observed. With time the peak intensity of nitrate increases with a simultaneous decrease in the peak intensity of the nitrite. The decomposition of nitrite proceeds very slowly here and it took several days (for example 4 days, figure 6c) for nitrate peak to reach a maximum value.

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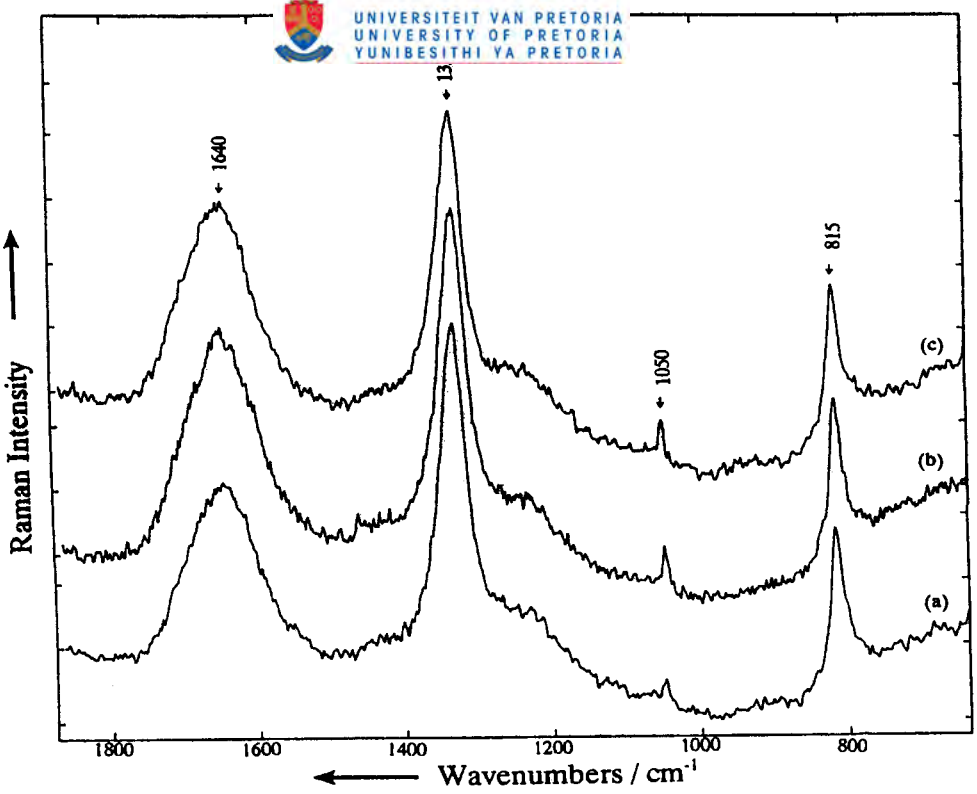

Figure 5.5.: **The Raman spectra of 0.0625 mol.ℓ^{-1} nitrite in 1 mol.ℓ^{-1} NH_4Cl at pH 3 (a) 1 day after preparation, (b) after 5 days, and (c) 9 days.**

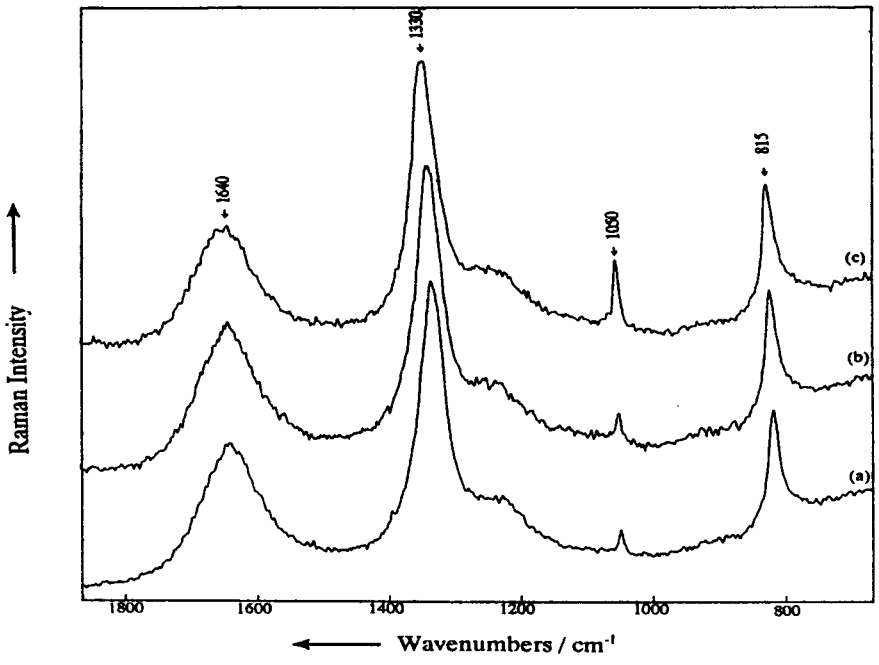

Figure 5.6.: **The Raman spectrum of nitrite in the presence of 0.01 mol.ℓ^{-1} HCl.**

Kinetic study

A nitrite sample in 1 mol.ℓ^{-1} HCl was used to study the kinetics of the reaction. Figure 5.7 shows the series of Raman spectra which were recorded to follow the decomposition of nitrite to nitrate. A spectrum was recorded at the beginning of the reaction when reactants were mixed at t = 0, and every subsequent 15 minutes until completion of the reaction.

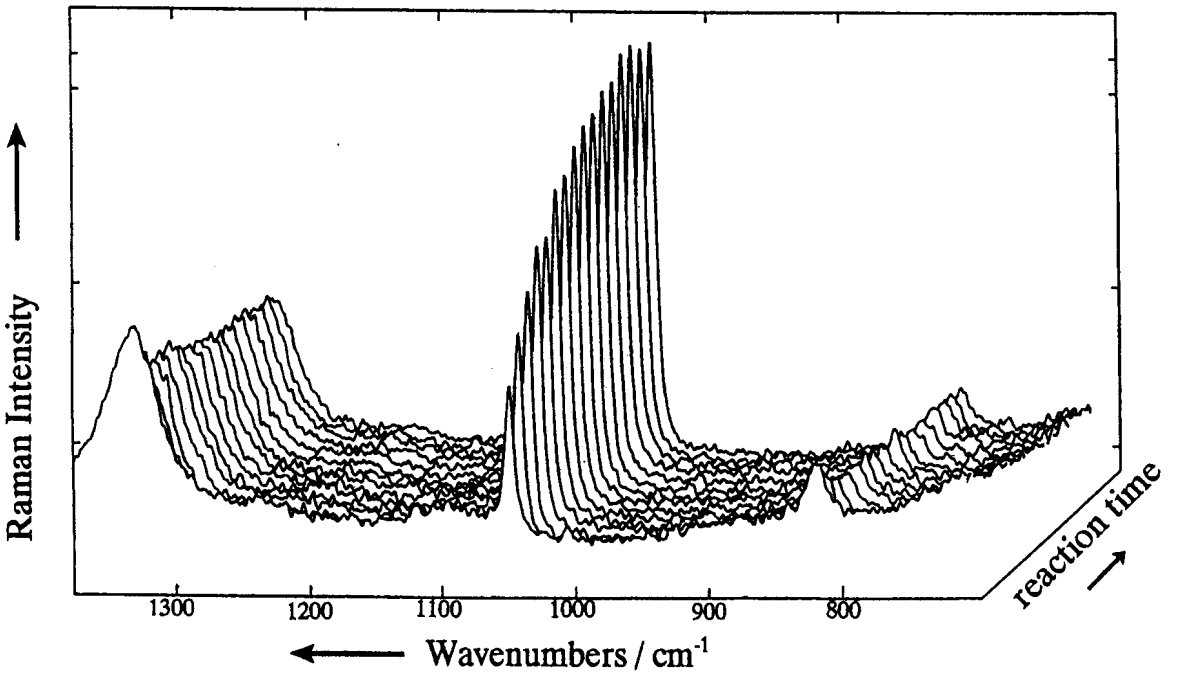

Figure 5.7.: The series of Raman spectra recorded between 700 and 1400 cm^{-1} to show the decomposition of nitrite to nitrate. The results obtained here were used for the kinetic study.

From figure 5.7 it can be seen that the intensities of the two bands representing NO_2^- decrease while that of the NO_3^- band increases with time. The absolute intensities of the various peaks are summarised in Table 1. From the data a graph could be plotted $\ln(I/I_0)$ vs. time for both the 1330

and 815 cm^{-1} bands of nitrite. The result is shown in figure 5.8 with a fitting of the data to a straight line. The slope of the graph is negative with respect to nitrite, resulting in k-value of 1.16 $\times 10^{-5}$ s^{-1} from the 1330 cm^{-1} peak and a corresponding value of 1.18 $\times 10^{-5}$ s^{-1} for the 815 cm^{-1} peak. The mean decomposition rate of 0.625 mol.ℓ^{-1} NO_2^- in 1 mol.ℓ^{-1} HCl can thus be described as 1.18 $\times 10^{-5}$ s^{-1}, with $t_{½}$ = 16 hours.

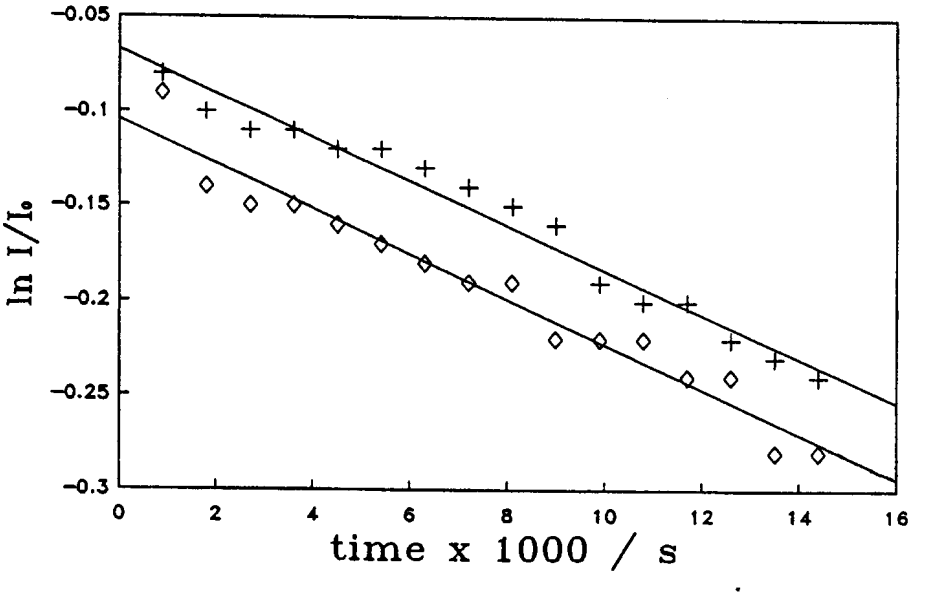

Figure 5.8.: The plot of ln(I/I$_0$)vs. Time in seconds, where I is the intensity of 1330 cm^{-1} (+) and 815-cm^{-1} (◊) positions in the Raman spectra.

5.10. Conclusions

We can conclude from these series of measurement that nitrite decomposes with time in acidic medium. The higher the concentration of acid the faster the decomposition of nitrite. Nitrite and nitrate are both potentially unstable, and all food and water samples should be analysed as soon as possible after collection. If it is necessary to store the samples prior to analysis, the effects of

the storage conditions on the stability of the analyte must be established in prior trials.

The chemical decomposition of nitrite can be reduced by storing aqueous samples under strongly alkaline conditions. This approach is not feasible for preserving solid samples like food, and it is not also suitable for drying, which will results in thermal decomposition of the analyte. In such circumstances it may be appropriate to store samples at freezer temperature. However, nitrite is not indefinitely stable under these condition, and the possible errors introduced by approach should always be investigated in preliminary studies.

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"Technology means the systematic application of scientific or other organized knowledge to practical tasks."

By: John Kenneth Galbraith

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CHAPTER 6

SPECTROPHOTOMETRIC DETERMINATION OF NITRITE IN FOODSTUFFS BY FLOW INJECTION ANALYSIS

6.1. Introduction

This chapter describes the spectrophotometric determination of nitrite in foodstuffs by flow injection analysis (FIA).

Nitrite determinations are very frequent in environmental analysis. There is currently considerable interest in the determination of nitrite and nitrate in water, soil and various foodstuffs. Nitrite does not commonly occur in nature, since it is either oxidised to the more chemically stable nitrate, or reduced by bacteria and other micro-organisms to ammonia. The role of nitrite as an important precursor in the formation of nitrosamines, many of which have been shown to be potent carcinogens, has been studied [1-2]. Thus, methods for the nitrite determinations in foods and other biological systems are becoming very important.

6.2. Choice of analytical techniques

The concept of flow injection analysis (FIA), introduced in 1975 by Ruzicka and Hansen [3], has been applied successfully to the determination of nitrite in water. Over recent years newer techniques such as HPLC [4-6], derivatisation GC [7-10], and chemiluminescence [11-13] have

also been applied with success for the determination of nitrite in samples. The sampling frequency of these techniques are however lower than flow injection, the systems are more complexed and analysis per sample also more expensive.

Numerous colorimetric methods have been proposed for the determination of micro-amounts of nitrite. The most promising method for the determination of nitrite seems to be a modified version [14 - 21] of the Shinn [22] reaction which is based on the well known Gries reaction. This is the reaction of nitrite with a primary aromatic amine to form a diazonium salt, which is then coupled with another aromatic compound to form an azo dye of which the absorbance is measured. A number of flow injection systems [23 - 26] have been developed using these modifications. In general the flow injection colorimetric technique gives results that agree well with other procedures.

Although the method seems to be free of interferences, there are a number of variables with which precautionary measures should be taken when trying to utilise its full intrinsic accuracy and precision. Among these are the composition of the nitrite standards, composition of the buffer (sample carrier) and reagent solutions, temperature, added salts and especially the pH of the buffer and the sample solutions.

Some routine laboratories [27] complained that problems were encountered when the FIA version of the Shinn reaction was applied to certain samples mainly from an acidic origin. It was clear from the conversations that the results obtained varied and it seemed that the experimental nitrite value obtained was lower than the value expected. It was very difficult to get to the root of the actual problem. An investigation was therefore launched to try and find a solution to the problem.

The problem was investigated and this chapter reports on the results obtained in this regard. Based on the results obtained, a method was proposed for the determination of nitrite in foodstuffs.

6.3. Experimental

All chemicals were of analytical-reagent grade and de-ionized water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used throughout. All solutions were degassed before measurements with a vacuum pump system and stored under a nitrogen atmosphere.

6.3.1. Reagents

Standard nitrite solution. Nitrite stock solution (expressed as sodium nitrite) was prepared by dissolving 1.00 g of oven dried sodium nitrite at 110°C in de-ionized water and diluting to 1ℓ. The solution was standardised against a 0.02 mol/ℓ permanganate solution, treated with a few drops of chloroform and kept in a refrigerator. Working standard solutions containing sodium nitrite were prepared by appropriate dilutions.

Colour reagent. Various colour reagents were prepared, and kept in a refrigerator.

Colour 1 was prepared by dissolving 40 g of sulphanilamide and 2 g of N-(1-naphthyl)-ethylenediammonium dichloride in 200 ml of 80%(v/v) phosphoric acid and diluting to 1ℓ with pure water.

Colour 2 was prepared by dissolving 20 g of sulphanilamide and 1 g of N-(1-naphthyl)-

ethylenediammonium dichloride in 200 ml of 80%(v/v) phosphoric acid and diluting to 1ℓ with pure water.

Colour 3 was prepared by dissolving 80 g of sulphanilamide and 4 g of N-(1-naphthyl)-ethylenediammonium dichloride in 200 ml of 80%(v/v) phosphoric acid and diluting to 1ℓ with pure water.

The sample carrier (buffer) was prepared by dissolving 60 g of NH_4Cl and 0.4 g EDTA in 1ℓ of de-ionized water with the pH adjusted to pH 3, pH 6, and pH 9 using 25%(v/v) NH_3 solution.

6.4. Equipment

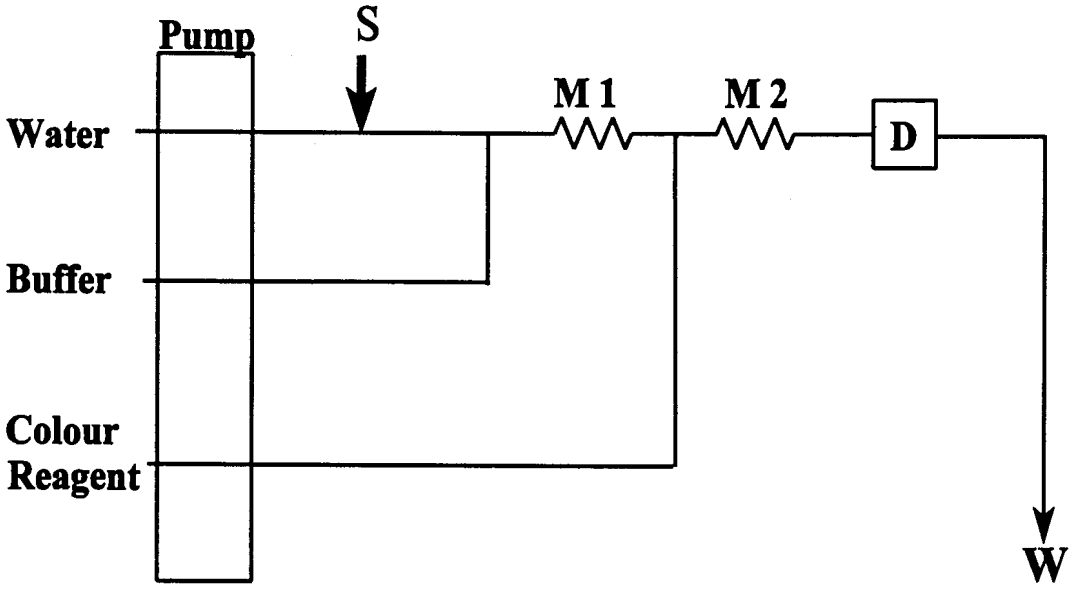

Figure 6.1.: Schematic outlay of the flow injection system used. S = sample; M = mixing coil (where M 1 = 100 cm and M 2 = 400 cm); D = detector; W = waste.

The flow injection manifold used is schematically shown in figure 6.1. The manifold consisted

of Tygon tubing (internal diameters of 0.76 mm) cut into the required lengths and wound around glass tubes with an o.d. of 10 mm. A Cenco peristaltic pump (operating at 10 rpm) was used for propelling the carrier and reagents. 100 $\mu\ell$ samples and standards were injected via a VICI Valco 10 port multi-functional sampling valve with two identical sample loops. The maximum absorbance was monitored at 540 nm in a UNICAM 8625 UV/VIS spectrophotometer equipped with a 10 mm Hellma-type flow-through cell (volume 80 $\mu\ell$).

6.5. Sample preparation

Cured meat samples, without sodium nitrite (used as blank) and spiked with 160 mg/kg nitrite expressed as sodium nitrite were obtained from a manufacturer. Samples (40 g of each sample) were finely ground using a mortar and each sample was transferred into 200 mℓ of hot doubly distilled de-ionised water. Different procedures were used for the extraction of sodium nitrite from these samples. This included cooking of the samples on a microwave oven and a hot plate for different time periods. Cold extraction was also done by leaving the 200 mℓ portions of the grounded samples on the laboratory bench under nitrogen atmosphere for 24 hours. The sample preparation procedure was repeated using 1 mol/ℓ NH_4Cl solutions adjusted to pH 3, 6 and 9 as extractants.

6.6. Results and discussion

6.6.1. Physical parameters

The physical parameters of an FIA affect the degree of dispersion and the zone penetration to be

obtained when the reagent and sample zones are propelled to the detector along the flow conduit. The following parameters were optimised, that is, flow rate, reaction coil diameter and sample volume to obtain optimum mixing of the zones and deliver an appreciable amount of the detectable species.

6.6.1.1. Effect of sample volume

The sample volume was optimized by varying it for a fixed reagent volume of 100 $\mu\ell$. The results are tabulated in Table 6.1. Linear range decreases for larger sample volumes. The decrease in linear range was due to high absorbance scale for the spectrophotometer to detect. The 100 $\mu\ell$ was chosen as the optimum sample volume

Table 6.1.: Effect of sample volume

Sample volume in $\mu\ell$	Relative peak height			
	Standard 1	Standard 2	Standard 3	Standard 4
50	0.82	1.72	3.38	6.91
100	1.12	2.30	4.57	8.98
140	1.34	2.63	5.34	--
200	1.70	3.51	7.16	--

Standard 1 = 25 mg/ℓ; Standard 2 = 50 mg/ℓ ; Standard 3 = 100 mg/ℓ; Standard 4 = 200 mg/ℓ.

6.6.1.2. Effect of flow rate and reaction coil

The contact time between the sample zone containing nitrite ion and the colour reagent is very important for the reaction to proceed sufficiently, this depends on the flow rate. The flow rate was

monitored over a period of three days using either the 200 cm or 400 cm reaction coil. The results are summarized in Table 6.2. The results tend to be more stable over a three day period when a 400 cm coil and 2.74 mℓ/min flow rate are used. The higher the flow rate the more stable are the results. Therefore the 400 cm reaction coil and the 2.74 ml/min were chosen as the optimum conditions for nitrite determination.

Table 6.2.: Effect of flow rate and reaction coil

Reaction coil length	Day	Peak height in mV				Flow rate in mℓ/min
		Standard 1	Standard 2	Standard 3	Standard 4	
400 cm	1	1.6957	3.3930	5.0883	6.7774	2.58
	2	1.3804	2.7588	4.1414	5.5218	
	3	1.5163	3.0323	4.5469	6.0678	
200 cm	1	1.7068	3.4116	5.1226	6.8295	2.58
	2	1.9394	3.8767	5.8172	7.7533	
	3	2.0378	4.0757	6.1120	8.1507	
400 cm	1	2.1576	4.3153	6.4730	8.6315	2.74
	2	2.0425	4.0902	6.1306	8.1730	
	3	2.2539	4.5101	6.7637	9.0169	
200 cm	1	1.6895	3.3800	5.0719	6.7648	2.74
	2	2.0785	4.1629	6.2364	8.3203	
	3	2.0244	4.0537	6.0756	8.0976	

6.6.2. Chemical parameters

The FIA system was optimised with respect to effect of the carrier stream pH and concentration of the colour reagent.

6.6.2.1. Effect of carrier stream pH

The effect of ammonium chloride was studied by adjusting the pH (that is, pH 3, pH 6, and pH 9) using 25% ammonia solution. The results are summarised graphically in figure 6.2. The pH 9 buffer was out of range. From the results we can conclude that pH 6 is the optimum pH to be used for nitrite determination.

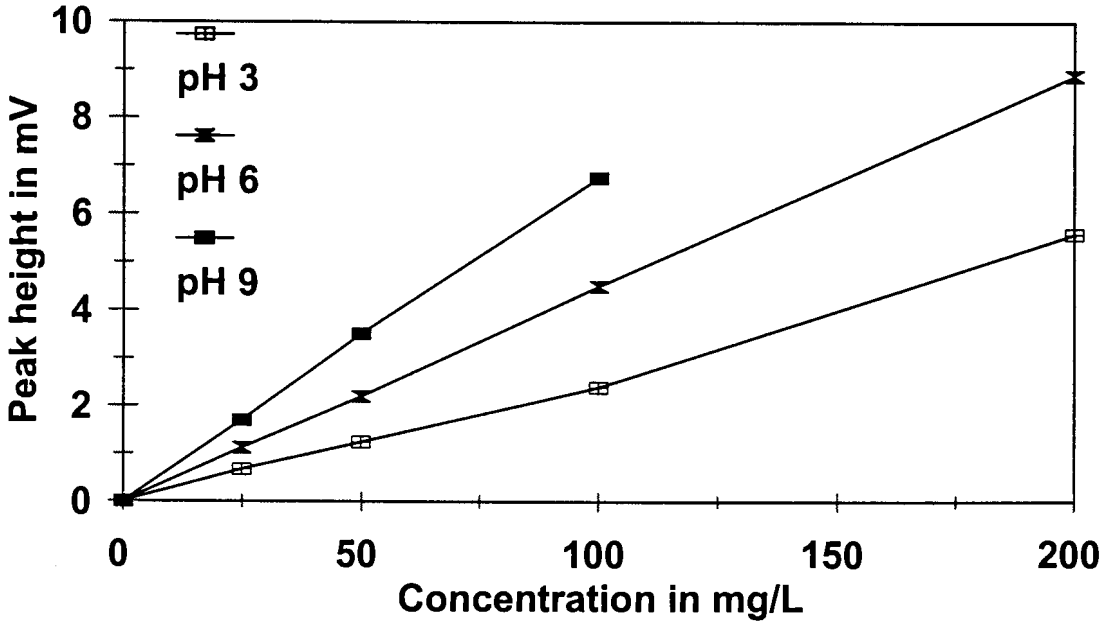

Figure 6.2.: Effect of ammonium chloride pH

6.6.2.2. Effect of the concentration of the colour reagent

The colour reagent affects the amount of detectable species that will be formed, therefore, it is important that reagent concentration is optimised especially for low nitrite concentration that are

difficult to detect. Three (3) different colour reagents were prepared as described in the experimental section. The results are tabulated in Table 6.3.. A decrease in the nitrite linear range was observed for the higher reagent concentration, that is colour 3, due to the absorbance of the coloured complex that become too high for the spectrophotometer to detect. Colour 1 was chosen as optimum because it gave the nitrite linear range and the best sensitivity for this linear range

Table 6.3.: Different types of the colour reagent

Colour reagent	Peak height in mV			
	Standard 1	Standard 2	Standard 3	Standard 4
Colour 1	1.1034	2.1868	4.4316	8.7272
Colour 2	0.3503	0.6625	1.3401	2.8124
Colour 3	1.8946	3.5832	7.8704	--

6.6.3. Nitrite determination in cured meat

The FIA procedure was first studied in detail to see if the origin of the problem was in the method. The flow injection parameters were varied and optimised. The precision of the FIA system confirmed previous results [26] obtained. Spiked water samples also gave an excellent recovery. The results obtained were consistent which proved that nothing was wrong with the optimised flow injection system and that the modified version of the Shinn reaction gave excellent results. The pH of the standard sodium nitrite solutions and water samples spiked with sodium nitrite in doubly distilled de-ionised water, different concentrations of hydrochloric acid and different concentrations of ammonium chloride at different pH values were then studied using the optimised FIA systems. Some of the results obtained were poor and the precision dropped dramatically in some cases. The results were not consistent and in some cases the nitrite

value decreased with course of time. It seemed from this investigation that the problem with weak results became more obvious when the pH of the samples dropped below 7. It was therefore clear that the complaints of some routine laboratories [27] were valid. Intensive studies of the stability of the nitrite ion under acidic and non-acidic conditions (related to sample conditions) with Raman spectroscopy revealed that the nitrite decomposes to NO_3^- and NO in acidic medium [28]. This further confirmed previous results that care had to be taken in the preparation of samples before FIA determination. It was therefore decided to study the behaviour of nitrite ion under different sample conditions.

The following different extraction procedures were studied. Hot extraction of the sodium nitrite was done by cooking grounded 200 mℓ sample portions on a microwave oven and a hot plate for different time periods. The hot samples were transferred into separating flasks and left under nitrogen atmosphere until all the fats on top and the solid pieces of Vienna had moved down to the bottom. The best extraction was obtained by cooking the portions for 5 minutes. Cold extraction was also done by leaving 200 mℓ portions of the grounded samples on the laboratory bench under nitrogen atmosphere for 24 hours. Four different samples, without sodium nitrite (used as blank) and four samples spiked with 160 mg/kg sodium nitrite were analysed. The results obtained for each sample was an average of ten runs. The sodium nitrite value in the blank samples without sodium nitrite was below 0.5 mg/kg and had no influence on the final results. The results obtained are outlined in Table 6.4.

TABLE 6.4: Vienna samples in pure water.

Sample number	Spiked amount of sodium nitrite as mg/kg	Samples left for hrs				Samples cooked for 5 minutes on the hot plate				Samples cooked for 5 minutes in the microwave			
		A	X	B	Y	A	X	B	Y	A	X	B	Y
1	160	22.47	14.0%	0.21	0.13%	54.07	33.8%	43.91	27.44%	150.59	94.1%	133.67	83.54
2	160	22.97	14.3%	0.18	0.11%	55.45	34.7%	42.18	26.36%	151.10	94.4%	136.27	85.17
3	160	22.17	13.9%	0.24	0.15%	54.80	34.2%	43.50	27.19%	149.30	93.3%	131.37	82.11
4	160	23.10	14.4%	0.22	0.14%	55.48	34.6%	43.71	27.32%	152.40	95.2%	132.90	83.06

A = Amount of sodium nitrite in mg/kg recovered when analysed immediately

X = Percentage of sodium nitrite recovered when analysed immediately

B = Amount of sodium nitrite in mg/kg recovered when analysed after 24 hours

Y = Percentage of sodium nitrite recovered when analysed after 24 hours.

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When the results of the three extraction conditions in Table 6.4 were compared, the samples left to stand on the laboratory bench for 24 hours gave an average percentage recovery of approximately 14.2%, the samples cooked on the hot plate for 5 minutes an average percentage recovery of 34.1% and the samples cooked in the microwave oven for 5 minutes an average percentage recovery of approximately 94.25% when analysed immediately. The percentage recovery of the samples cooked in the microwave oven were far much better than the samples cooked on the hot plate and the samples left to stand overnight on the laboratory bench. When the samples were analysed after 24 hours, the average percentage recoveries were 0.13% for cold sample extraction over 24 hours, 27.08% for the hot extraction on a hot plate and 83.47% for the hot extraction on a microwave oven. Although the hot extraction with a microwave oven still gave a percentage recovery of 83.47% when analysed after 24 hours, there is loss in the concentration of nitrite in all three extraction conditions when left before analysis. This means that nitrite decomposes with time even at mild acidic conditions [28]. The microwave hot extraction procedure gave the best results and was used for further extraction.

The main problem was however that it was not always possible to analysed the cured meat samples immediately and the storage of samples in different pH media was studied to see if it was possible to preserve the samples over a certain period of time. The whole sample preparation procedure described above was therefore also performed cooking polony samples in 1 mol/ℓ NH_4Cl solution with the pH adjusted to pH 3, 6 and 9 and also in pure water. Four different samples, without sodium nitrite (used as blank) and four samples spiked with 160 mg/kg sodium nitrite were again analysed. The results obtained for each sample was an average of ten runs. The sodium nitrite value in the blank samples without sodium nitrite was below 0.5 mg/kg and had no influence on the final results. The results obtained are given in Table 6.5.

TABLE 6.5: Polony samples in 1 mol/ℓ NH_4Cl with pH adjusted to 3, 6, 9 and in pure water.

Sample number	Spiked amount of sodium nitrite as mg/kg	pH 3 medium				pH 6 medium				pH 9 medium				Pure water			
		A	X	B	Y	A	X	B	Y	A	X	B	Y	A	X	B	Y
1	160	53.78	33.6%	41.33	25.83%	86.88	54.3%	77.35	48.34%	152.00	95.0%	152.01	95.0%	153.48	95.9%	133.46	83.4%
2	160	52.70	32.9%	42.10	26.31%	88.03	55.0%	75.33	47.08%	152.32	95.2%	152.26	95.2%	154.65	96.7%	136.14	85.1%
3	160	52.85	33.0%	43.08	26.93%	88.23	55.1%	72.28	45.18%	153.48	95.9%	153.36	95.9%	153.90	96.2%	133.67	83.5%
4	160	54.23	33.9%	42.55	26.59%	87.50	54.7%	75.68	47.30%	154.83	96.8%	154.86	96.8%	153.48	95.9%	134.10	83.8%

A = Amount of sodium nitrite in mg/kg recovered when analysed immediately

X = Percentage of sodium nitrite recovered when analysed immediately

B = Amount of sodium nitrite in mg/kg recovered when analysed after 24 hours

Y = Percentage of sodium nitrite recovered when analysed after 24 hours.

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The percentage recoveries of samples prepared in 1 mol/ℓ NH_4Cl with the pH adjusted to 3 and 6 in Table 6.5 are much less than expected when analysed immediately. The polony samples prepared in 1 mol/ℓ NH_4Cl solution with the pH adjusted to 3 gave an average percentage recovery of about 33.6% and the samples prepared in 1 mol/ℓ NH_4Cl solution with the pH adjusted to 6 resulted in an average percentage recovery of approximately 54.8% when analysed immediately. It is clear from the results that the percentage recovery for the samples prepared in a pH 6 medium was by far better than the samples prepared in a pH 3 medium when analysed immediately. This means that nitrite decomposed faster with an increase in acidic conditions. The nitrite concentration decreased to an average percentage recovery of about 26.4% for the pH 3 medium and to an average percentage recovery of approximately 47.0% for the pH 6 medium after 24 hours. It is clear from these results that acidic conditions had a definitive influence on the decomposition of nitrite in polony samples and that samples analysed under these conditions will not give accurate results.

When the results of the extraction conditions in 1 mol/ℓ NH_4Cl at different pH values and in pure water in Table 6.5 were compared, it followed that nitrite in 1 mol/ℓ of NH_4Cl solution with the pH adjusted to 9 and nitrite in pure water gave quite reasonable results when analysed immediately. Their respective average percentage recoveries are approximately around 96%, which was a good recovery when compared with extractions by the other two sample media used, i.e., nitrite in 1 mol/ℓ NH_4Cl with the pH adjusted to 3 and 6. Nitrite in pure water also decomposed after 24 hours by about 12.4%. It is therefore also obvious that pure water can not be used for sample preparation. The nitrite concentration decreased by less than 0.5% for samples in 1 mol/ℓ NH_4Cl with the pH adjusted to 9 when left for 24 hours and remain more or less the

same when left under nitrogen atmosphere for 24 hours.

The pH of the polony samples in pure water was measured as 5.2, revealing that preparation of polony samples in pure water tended to be acidic. This confirmed why decomposition of nitrite also took place when pure water was used. However, due to the weak ionic strength in pure water, the rate of decomposition was slower. This explained the reason why problems were encountered with some routine laboratories [27], when the FIA version of the Shinn reaction was applied for the determination of nitrite in foodstuffs.

The t-test (Null hypothesis) was applied to show whether the percentage recovery of 95.4% is accepted or not and also if the change in medium had any effect when polony samples were analysed immediately.

Sample medium	Mean (M) in mg/kg	Standard deviation (S)	Relative standard deviation
pH 9	153.16	1.28	0.84%
Pure water	153.88	0.55	0.36%

M_1 = mean of pH 9 medium

M_2 = mean of pure water medium

S_1 = standard deviation of pH 9 medium

S_2 = standard deviation of pure water medium

n = number of replicates

A comparison was done to see whether change in medium affected the results, using the

following equation:-

$$S^2 = \{ (n_1 - 1) S_1^2 + (n_2 - 1) S_2^2 \} / (n_1 + n_2 - 2)$$

$$S = 0.9704$$

There were 3 degrees of freedom so the critical value of $|t|$ was 3.18 (P = 0.05). The observed value of $|t|$ was 0.9704 which was less than the critical value, so the null hypothesis was retained. The null hypothesis adopted, was that the means of the results given by the two media are equal. There was no evidence that the change of the medium affected the recovery rate, when samples were analysed immediately. This is, however, not the case in most routine laboratories and preparation of samples in 1 mol/ℓ NH_4Cl with the pH adjusted to 9 should be preferred.

It was therefore decided to see whether the difference between the spiked value (μ) and the mean was significant. In this case μ = 160 mg/kg and the mean (M_1) = 153.10 mg/kg. The following equation was used:

$$t = (M_1 - \mu) \sqrt{n_1} / S_1$$

$$t = - 10.69$$

There were 3 degrees of freedom so the critical value of $|t|$ was 3.18 (P = 0.05). The observed value of $|t|$ was - 12.19 which was less than the critical value so the null hypothesis was retained. The null hypothesis adopted, was that the true value and the mean are equal. It therefore follows that the preparation procedure of samples in 1 mol/ℓ NH_4Cl with the pH adjusted to 9 is suitable for the flow injection determination of nitrite in foodstuffs.

6.7. Conclusions

Great care should be exercised when nitrite samples are analysed. A sudden loss of nitrite occurred without apparent reasons even when the samples were prepared in alkaline medium. Nitrite samples should therefore always be kept under nitrogen atmosphere during preparation. Nitrite samples must always be analysed immediately after preparation. These results indicate that inorganic nitrite can be determined reliably in cured meats by FIA under alkaline medium. Time and pH of samples must always be taken into consideration when working with nitrite samples.

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"If I have seen further it is by standing upon the shoulders of Giants."

By: Sir Isaac Newton

CHAPTER 7

SIMULTANEOUS SPECTROPHOTOMETRIC DETERMINATION OF NITRATE AND NITRITE IN FOODSTUFFS/WATER BY FLOW INJECTION ANALYSIS

7.1. Introduction

This chapter describe the simultaneous spectrophotometric determination of nitrate and nitrite in foodstuffs and water samples by flow injection analysis.

Nitrate and nitrite play an important role in the nitrogen cycle involving the complex interaction of different ecosystems of the biosphere. Both nitrate and nitrite are present in food and water, and it is by these sources that humans are exposed to these ions. Nitrate is necessary for plant growth. According to Christy et al [1], probably more than 90% of the nitrogen absorbed by plants is in the form of nitrate. Nitrate is chemically stable throughout the relevant range of the pH. It can be reduced to nitrite when is in contact with metals such as occurs during the cooking of food in aluminium utensils (WHO, 1977) [2]. Nitrite is very unstable, particularly at acidic pH values [3] at which it can disproportionate to yield nitrate and nitrogen oxide and/or react with many components of foods including amines, phenols, and thiols. Nitrite gives cured meat its characteristic colour and flavour and it is important in the control of bacteria, particularly Clostridium botulinum.

The acute toxic effects of large dose of nitrite are well documented, the significance of relatively small concentrations of nitrite to adults is judged mainly in the light of the possible role of nitrite as the precursor of the carcinogenic N-nitroso compounds (NNC). There is evidence that high intra-gastric nitrite concentration correlates with an increased risk from stomach cancer (Hartman, 1982) [4]. The reduction of nitrate to nitrite in the gastric lumen is an important source of nitrite for the formation of NNC. It has been suspected for some years that the effect of exposure to nitrate and nitrite may cause human cancer (Mirvis, 1983) [5].

7.2. Choice of analytical techniques

The study of nitrate metabolism has been significantly hampered by the lack of simple methods for nitrate determinations. Several methods for such determinations are now used commonly but impose restrictions that make the analysis time consuming and tedious. Nitrate concentrations are of importance in natural waters for many reasons, e.g. their importance in eutrophication [6] and their responsibility for methaemoglobinaemia [7-8] . The various methods at present employed for the determination of nitrate in water fall into one of the five (5) main categories:

(a) The reduction of nitrate to ammonia [9-10].

(b) Photo-induced reduction of nitrate to nitrite [11].

(c) Direct spectrophotometry [12-13].

(d) Potentiometric methods using ion-selective electrode method [14].

(e) Reduction of nitrate to nitrite [15-17].

Thus there is a need for a quick, accurate and precise method for nitrate and nitrite determination. The cadmium reduction method has been successfully applied by a number of authors to the determination of nitrate in a wide range of water samples.

Since its inception in 1975 [18], flow injection analysis has been applied successfully to the simultaneous determination of nitrate and nitrite in water using various techniques [19-24]. In most of the procedures nitrate is reduced to nitrite with a copperised cadmium. In the flow injection procedure an injected nitrite zone is diazotised with sulphanilamide and the product is coupled with N-(1-naphthyl)ethylene diammonium dichloride to form a highly coloured azo dye. The resulting aminoazo compound has a molar absorptivity of about 5×10^4 ℓ. mol^{-1}. cm^{-1} at 543 nm, the wavelength of its absorption maximum.

Most authors [25-27] worked on different factors like the effect of sample pH, various cadmium reductors, flow rate, pH of the salt, temperature, etc. This research focus on the following factors:

(1) Effect of copperised and acidified reductor column.

(2) Life long of the reductor column.

(3) Length of the reductor column.

(4) The stability of the nitrite ion in the presence of nitrate.

(5) Comparison between Standard addition method and the normal calibration techniques to see whether there is any matrix interferences in the food sample.

7.3. Experimental

7.3.1. Reagents

All chemicals were of analytical-reagent grade and de-ionized water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used throughout. All solutions were degassed before measurements with a vacuum pump system and stored under a nitrogen atmosphere. For the buffer solution, 60.0 g of ammonium chloride and 0.4 g of EDTA were dissolved in 1 ℓ of de-ionized water; the pH of this solution was adjusted to 8.87 with diluted (25% v/v) ammonia solution. The colour reagent solution was prepared by dissolving 80 g of sulphanilamide and 4 g of N-(1-naphthyl)-ethylenediammonium dichloride in a 200 mℓ of 85% phosphoric acid and diluting to 1 ℓ with de-ionized water. This solution was stored in a refrigerator.

Nitrate stock solution was prepared by dissolving 6.07 g of an oven dried sodium nitrate (for 1.5 hours at 110°C) in de-ionized water and diluting to 1 ℓ. The solution was treated with a few drops of chloroform and kept in a refrigerator for preservation. For the nitrite stock solution 4.92 g (dried for 1.5 hours at 110°C) of sodium nitrite was dissolved in de-ionized water and made up to 1 ℓ. It was standardised against a 0.02 mol/ℓ permanganate solution, treated with a few drops of chloroform and stored in a refrigerator. Working standards of solutions containing both nitrate and nitrite were prepared by appropriate dilutions.

The following certified reference materials (CRMs) were used for verification. Nitrate single element standard, concentration is 200 μg/mℓ the matrix is water; date September 1997 and

expiration date: March 1999. Lot Number: A7035032. Item number: ICA204-1

Nitrite single element standard, concentration is 200 μg/mℓ the matrix is water; date September 1997 and expiration date: March 1999. Lot Number: A7045029. Item number: ICA203-1

All the concentration of the final solutions were verified by against NIST SRM#. The accuracy of both standards were certified to be $\mp$0.5 of the stated value for eighteen months from the date of shipment. The CRMs were bought from EM Science, 480 S. Democrat Road, Gibbstown, New Jersey 08027.

7.4. Equipment

7.4.1. Flow injection manifold

The flow injection manifold used is schematically shown in figure 7.1. The manifold consisted of Tygon tubing (inside diameters of 0.76 mm) cut into the required lengths and wound around glass tubes with an o.d. of 10 mm. A Cenco peristaltic pump (operating at 10 rpm) was used for propelling the carrier and reagents. 100 $\mu\ell$ samples and standards were injected via a VICI Valco 10 port multi-functional sampling valve with two identical sample loops. The maximum absorbance was monitored at 540 nm in a UNICAM 8625 UV/VIS spectrophotometer equipped with a 10 mm Hellma-type flow-through cell (volume 80 $\mu\ell$). A digital pH meter (Zeiss, W. Germany) model MIA 3000 was used to measure the pH of the solutions and that of the sample carrier.

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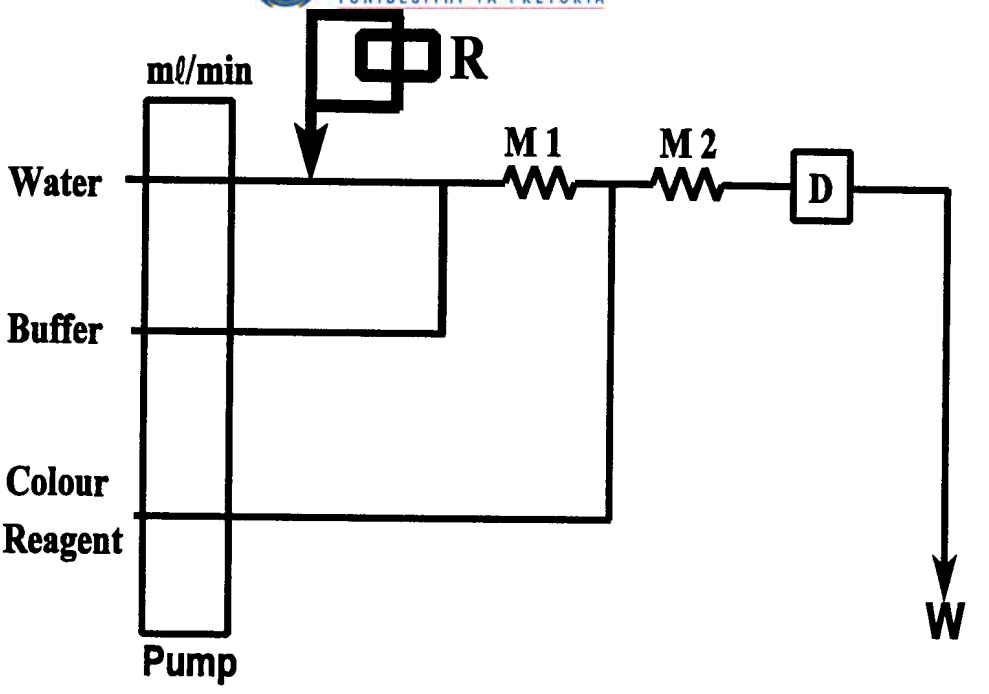

FIGURE 7.1.: **Flow injection manifold for the simultaneous determination of nitrate and nitrite in foodstuffs. S = sample; M = mixing coil (where M 1 = 100 cm and M 2 = 400 cm); D = detector; W = waste; R = reductor column.**

7.4.2. Reductor columns

The reductor columns (Figure 7.2) were made of glass vary in length (i.e., 35 mm, 60 mm and 80 mm) but of the same internal diameter of 10 mm filled with cadmium granules (Merck, with particles of diameter 0.3-1.5 mm). The particles were held by a glass frit at both ends so that they don't block the FIA system. The cadmium granules were prepared by washing with acetone for a few minutes, 2 mol/ℓ HCl, water and methanol, dried *in vacua* and kept over silica gel in a desiccator. About 8 g is needed for an 80 mm column. Both copperised and acidified cadmium were used to reduce nitrate to nitrite. The copperised cadmium was prepared by pumping a 0.1 mol/ℓ copper sulphate through the column for about 10-20 minutes and rinsed with de-ionized

water. The acidified cadmium was prepared by pumping 0.1 mol/ℓ HCl through the column and rinsed with de-ionized water. The reduction column was regenerated by passing either the copper sulphate solution or hydrochloric acid solution for 10-20 minutes and it can be stable for over 36 hours.

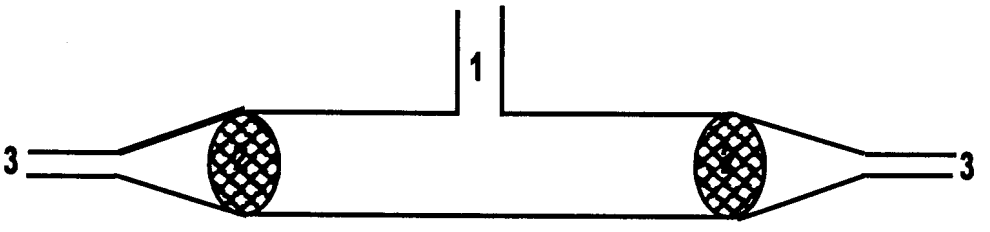

FIGURE 7.2.: Geometry of the cadmium reductor column. 1= Filling port; 2 = Glass frit/filter; 3 = Inlet/outlet tubes.

7.5. Sample preparation

Cured meat samples, without sodium nitrite and sodium nitrate (used as blank) and spiked with 160 mg/kg nitrite and nitrate expressed as sodium nitrite and sodium nitrate were obtained from the manufacturer. Samples (40 g of each sample) were finely ground using a mortar and each sample was transferred into 200 mℓ of hot de-ionized water. The sample was cooked on a microwave oven for five minutes.

Water samples were received from Water Research Institute. Each sample was diluted to an appropriate dilution (the working range of the flow system was between 0.02-400 μg/ℓ). The concentration of samples vary between the range 0.1-22 mg/ℓ. The samples were spiked with the known amount of nitrate to check the percentage recovery. Nitrite standards were ran as samples to prove that the whole of nitrate was reduced.

7.6. Results and discussion

The flow injection system was optimised as reported in the previous publication [28].

7.6.1. Reduction efficiency test

In order to test the reduction efficiency of the various forms of cadmium, it was necessary to obtain performance characteristics for a theoretical 100% reduction yield. This figure was achieved by omitting the reduction step and using the nitrite standards (In-house Reference Materials) and the nitrite Certified Reference Material. A series of nitrite standards ranging from 0-400 $\mu g/\ell$ were ran as samples. The results below (Table 7.1.) is the concentration of nitrite expressed as peak height in volts. The results is an average of fifteen (15) runs per sample or standard.

Table 7.1.: Reduction efficiency test results for nitrite

Concentration of nitrite in $\mu g/\ell$	Peak height/V for Certified Reference Material	Peak height/V for In-house Reference Material
0.00	0.0023	0.0030
100	1.4591	1.4498
200	2.9082	2.9014
300	4.3773	4.3691
400	5.8564	5.8601

The graph plotted is a straight line through the origin (Figure 7.3.). There is no variation between

the certified reference material (CRM) and in-house reference material (IRM) results. The relative standard deviation of the above results is 1.25%.

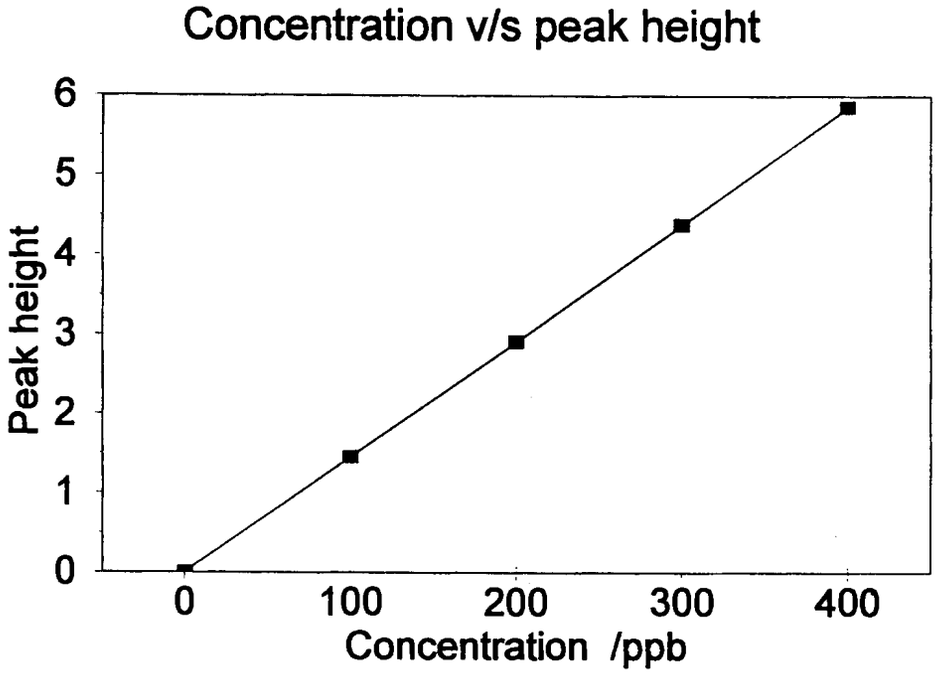

Figure 7.3.: Reduction efficiency test graph of nitrite standards.

7.6.2. Comparison of the three reductor columns

The columns were made of glass and have the same diameter but different lengths, i.e., column 1, 2, and 3 had lengths 35 mm, 60 mm, and 80 mm, respectively. The columns were packed with cadmium granules prepared as stated in the experimental section. The results are summed up in the form of Table 7.2 below. From Table 7.2 results it was concluded that the longer the reductor column the more efficient is the reduction of nitrate to nitrite. Reductor column three (R3) seems

to be the more ideal for the determination of micro amount of nitrate in both the water and the food samples. The other factor which was studied was the effect of the pH of sample carrier on the reduction of nitrate to nitrite. The lower the pH of the sample carrier the lower is the recovery of nitrate. Previous authors did make mention of the effect of pH of sample carrier. The best working pH is above 8.00, at pH less than 8 the recovery will be less. It was also concluded that reductor column three (R 3) will be used for the remainder of the experiment. The relative standard deviations for R 1, R 2, and R 3 are 3.94%, 3.22%, and 1.88% respectively.

Table 7.2.: Comparison of the lengths of the three reductor columns

Type of reductor	pH of sample carrier	Concentration of nitrate expressed as nitrite in $\mu g/\ell$				
		0.00	**100**	**200**	**300**	**400**
Reductor one (1)	3.797	0.0013	0.9137	1.8274	2.7500	3.6551
	6.226	0.0005	0.9525	1.9051	2.8575	3.8098
	9.050	0.0012	1.0342	2.0679	3.1026	4.1376
Reductor two (2)	3.797	0.0011	1.2277	2.4556	3.6833	4.9108
	6.226	0.0009	1.2451	2.4902	3.7401	4.9805
	9.050	0.0015	1.2815	2.5631	3.8501	5.1262
Reductor three (3)	3.797	0.0011	1.3096	2.6194	3.9300	5.2384
	6.226	0.0012	1.3245	2.6511	3.9735	5.2897
	9.050	0.0007	1.4268	2.8536	4.2900	5.7171

7.6.3. Comparison of the two cadmium types

Both the granulated and the coarse powder give more or less the same results at pH above 8.05. Further comparison was carried out between the acidified and the copperised cadmium. Table

7.3 below tabulate the results of both cadmium, i.e., acidified and copperised, using the cadmium granules. (NB. R 3 was used in all the results tabulated in Table 7.3.

The acidified cadmium reductor column experienced a loss of 9.17% in reduction over a period of 24 hours whereas the copperised one experience a loss of 63.5% in reduction over the same period. Further studies were carried out to see what happens if the reductor columns are re-pumped after 24 hours. The percentage reduction did improve after the columns were re-pumped for four (4) hours. But as time prolonged the reduction efficiency start to decline. The reduction efficiency is between 90 -100% after the re-pumped of the reductor column. The acidified reductor column is preferred because of its long shelf-life. It is advisable to re-pump the reductor column after use so that it can be re-used again, rather than getting rid of the cadmium because it is expensive to purchase it.

TABLE 7.3.: Results obtained using an acidified and copperised cadmium reductor columns

Type of reductor	Time	Concentration of nitrate expressed as nitrite in $\mu g/\ell$				
		0.00	**100**	**200**	**300**	**400**
Copperised cadmium	Day one	0.0003	1.4232	2.8499	4.2696	5.6928
	Day two	0.0011	0.5188	1.0398	1.5564	2.0752
Acidified cadmium	Day one	0.0009	1.4294	2.8588	4.2772	5.7176
	Day two	0.0014	1.2993	2.6000	3.8979	5.1972

7.6.4. Determination of nitrate in water samples.

Table.7.4 below tabulate the results of water samples analysed using the acidified cadmium. Each results is an average of ten (10) runs. The percentage recoveries of both the standard method and the FIA method give more or less the same percentage recoveries. The percentage recoveries vary from 93.8%-101% for both the methods. From the above results we can conclude that nitrate can be determined successfully with the reduction of nitrate to nitrite using cadmium reduction column.

Table 7.4.: Determination of nitrate expressed as nitrite in water samples

Sample number	Spiked amount of nitrate in $\mu g/\ell$	Standard method in $\mu g/\ell$	Percentage recovery of nitrate	FIA method in $\mu g/\ell$	Percentage recovery of nitrate
1	308	301	97%	305	99.0%
2	112	113	101%	111	99.1%
3	125	120	96.0%	125	100%
4	172	169	98.0%	170	98.8%
5	304	303	99.7%	290	95.4%
6	65	61	93.8%	62	95.4%
7	80	76	95.0%	78	97.5%
8	82	91	98.9%	88	95.6%

The t-test with multiple samples (paired by differences) [29] was applied to compare whether the two methods for nitrate determination differs significantly at the 95% confidence level. The mean of the tabulated differences is $\bar{x}_D = 0.625$ and the sample standard deviation is $S_D =$

5.704. For the null hypothesis we assert that the two methods agree, that is, the population mean difference is zero, H_0: $\mu_D = 0$. The alternative hypothesis $\mu_D \neq 0$, this imply the two tailed test. There are eight (8) determinations, therefore $\nu = 7$.

At the 5% significance level $t_{0.05,7} = 2.365$. The critical t-values are therefore ± 2.365. Finally $t_{calculated}$ was computed:

$$t_{calculated} = \bar{x}_D/S_D \times\sqrt{N} = 0.310$$

$t_{calculted.}$ lies within the critical range. At the 5% significance level we accept the null hypothesis that the two methods give the same results.

7.6.5 Simultaneous determination of nitrate and nitrite in cured meat

All the food samples were prepared as stated in the experimental section. The following sets of results are based on the analyses carried out using the technique of standard addition method (SAM) and the normal calibration curve method (CCM). The cadmium reductor column was rinsed with acid after use and before the analysis and rinsed with double-deionised distilled water for two-three hours every day. The results are tabulated in table 7.5. The percentage recoveries of both nitrate and nitrite vary from 93.2% to 99.8% for both techniques. The percentage recoveries of SAM is higher than that of the CCM which is 96.1%- 99.8% for SAM and 93.2% -96.5% for CCM. The reason for higher recoveries for SAM is because the matrix is the same in both the sample and the calibration standards.

Table 7.5.: Simultaneous determination of nitrate and nitrite in cured meat samples

Sample number	Spiked amount of sodium nitrate/ nitrite as mg/kg	Standard addition method (SAM)				Calibration curve method (CCM)			
		Sodium nitrate		Sodium nitrite		Sodium nitrate		Sodium nitrite	
		A	X	B	Y	C	S	D	T
1	160	158.17	98.9%	156.33	97.7%	154.11	96.3%	152.67	95.4%
2	160	155.07	96.9%	158.59	99.1%	150.48	94.1%	156.05	97.5%
3	160	159.66	99.8%	155.00	96.9%	156.27	97.7%	149.11	93.2%
4	160	156.22	97.6%	155.19	97.0%	151.23	94.5%	151.98	95.0%
5	160	158.15	98.8%	157.18	98,6%	156.81	98.0%	156.35	97.7%
6	160	153.74	96.1%	154.34	96.5%	155.01	96.9%	154.58	96.6%

A = Amount of sodium nitrate in mg/kg recovered using SAM.

X = Percentage of sodium nitrate recovered using SAM.

B = Amount of sodium nitrite in mg/kg recovered using SAM.

X = Percentage of sodium nitrate recovered using SAM.

C = Amount of sodium nitrate in mg/kg recovered using CCM.

S = Percentage of sodium nitrate recovered using CCM.

D = Amount of sodium nitrite in mg/kg recovered using CCM.

T = Percentage of sodium nitrite recovered using CCM.

The t-test (Null hypothesis) was applied to show whether the two techniques, i.e., SAM and CCM give the same results at the 95% confidence level. For the null hypothesis we assert that

the two methods give the same results, i.e., $\bar{x}_1 = \bar{x}_2$. The alternative hypothesis $\bar{x}_1 \neq \bar{x}_2$, this imply the two tailed test. There are twelve (12) determinations, therefore $\nu = 10$.

Method/technique	Mean ($\bar{x}$) in mg/kg	Standard deviation (S)	Relative standard deviation
Standard addition (SAM)	156.84	2.2165	1.44%
Normal calibration curve (CCM)	154.00	2.6132	1.70%

$\bar{x}_{1s}$ = Mean of nitrate for SAM.

$\bar{x}_{2c}$ = Mean of nitrate for CCM.

S_{S1} = Standard deviation for SAM.

S_{C2} = Standard deviation for CCM.

At the 95% confidence level $t_{0.05,10} = 2.228$. The critical t-values are therefore $\mp$ 2.228. Finally $t_{calculated.}$ was computed:

$$\mp t_{calculated.} = (\bar{x}_{1s} - \bar{x}_{2c})/S_p \times [(\sqrt{N_1} \times N_2 / N_1 + N_2)] = 2.030$$

$$\text{Where, } S_p^2 = \{(N_1 - 1)S_{S1}^2 + (N_1 - 1) S_{C2}^2\}/(N_1 + N_2 - 2) = 2.4230$$

Therefore $t_{calculated}$ lies within the critical range, i.e., $t_{calculated}$ is less than the $t_{tabulated} = 2.228$. At the 95% significance level we accept the null hypothesis and reject the alternative that the two

techniques give the same results. Similarly for nitrite is $t_{calculated} = 2.040$ which is less than the $t_{tabulated} = 2.228$, so therefore the null hypothesis is retained and the alternative is rejected. We can conclude that the SAM and the CCM give more or less the same result at the 95% confidence level for both nitrate and nitrite determination in cured meat.

7.7. Conclusions

The idea of monitoring the experiment over a longer period was to prove that nitrite is stable in the presence of nitrate and also to show that the cadmium reductor column can be re-usable over a longer period as long as you keep on rinsing the reductor column with the appropriate solution.

The acidified cadmium reductor column is preferred because of its long-life. The longer the reductor column the more efficient is the reduction stage. The standard addition method give a higher yield than the normal calibration curve method. The proposed method can be successfully applied for the simultaneous assay of nitrate and nitrite in foodstuffs/water.

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"Man's mind stretched to a new idea never goes back to its original dimensions."

By: Oliver Wendel Holmes

CHAPTER 8

Exploring the quantitative determination of phosphate in fertilisers and cured meat using flow injection analysis and Raman spectroscopy

8.1. Introduction

This chapter describe quantitative determination of phosphate in fertilisers and cured meat using flow injection analysis and Raman spectroscopy.

Phosphorus constitutes only approximately 1% of the total mass of the human body (mostly in the form of hydroxy apatite in bones and teeth). Orthophosphate forms soluble complexes with many cations, and this plays an important role of increasing the bioavailability of those cations. Phosphates are useful in food systems in that they stabilise colloidal suspensions, a characteristic that determines certain uses of phosphates in the diary industry [1]. They are also used for colloidal dispersions (in water treatment, < 2 mg/ℓ) of sodium polyphosphate to prevent formation and growth of calcium carbonate crystals from hard water, which might cause clogging of pipes. Contamination of water with phosphate from fertilizers has been extensively studied for its potential effects on aquatic enrichments [2]. Although eutrophication of even larger bodies of water may occur in the presence of phosphate and nitrogen contamination, this effect seems to be absent when phosphate levels only are introduced into the water. Long term exposure to low phosphate levels has not affected plankton or animals, although short term exposure to high

phosphate levels have caused severe environmental damage .

8.2. Choice of analytical methods

The determination of inorganic phosphate and pyrophosphate is important in the field of environmental and food analysis. There are several methods available for the determination of phosphate in water. The spectrophotometric procedure is the most popular technique. The spectrophotometric procedure is based on the formation of 12-molybdophosphate followed by reduction to heteropoly blue species [3]. Recently most authors used a spectrophotometric method in which a molybdophosphate aggregate is formed with Malachite Green [4-5]. Spectrophotometric procedures are complicated due to the formation of colour complexes, which might hinder the analyte of interest. Other techniques such as thin liquid chromatography [6], paper chromatography [7], HPLC [8], ion chromatography [9], and gas chromatography [10] were used for the extraction and determination of phosphate in various samples.

Raman spectroscopy has been applied to the qualitative and quantitative analysis of inorganic, organic, and biological systems [11]. The technique is mainly used for structural and qualitative analysis. Quantitative analysis by Raman spectroscopy has not yet been exploited widely. This lack of use has been due largely to the high cost of the instrument relative to that of absorption instrumentation. Limitations of Raman spectroscopy shares with other emission techniques the handicap of being a single-beam technique and this lead to uncorrected variations in source, sample, and optics. Another limitation is the "Raman scattering" phenomenon which is weak as compared to a process such as absorption of radiation. The major limitation is that of strong fluorescence from many samples. This limitation can be alleviated by chemical or physical

manipulation of the sample. The chemical or physical manipulation can be achieved by addition of quenchers, clean-up of the sample, or burn-out of the fluorescence by preliminary exposure to high-intensity radiation. The fluorescence limitation has received considerable attention [12-15].

The advantage of Raman spectroscopy is that it provides vibrational spectra that are rich in highly reproducible detailed features, providing the possibility of highly selective determinations. Raman spectroscopy is superior to infrared (IR) for spectroscopy investigating inorganic systems because aqueous solutions can be employed, the low frequency spectral region is easily obtained, glass or quartz cells can be employed.

The quantitative analysis of Raman spectroscopy has been exploited as far-back as 1971 [16]. The basics of quantitative analysis is described, including the use of internal and external standards to compensate for instrumental and sample variations. Raman spectroscopy was also used for routine analysis in determination of pollutants in natural water samples [17-18]. During the last two decades the quantitative analysis of Raman spectroscopy has grown very fast. Applications have included the determination of phenols, sulfur oxyanions, pharmaceuticals, antitumour, aromatic amines, azo dyes, and ethanol in fermentation broth [11]. Recently FT Raman spectroscopy of minerals was reported [21], one of the analyte of interest was phosphate. Aminzadeh [22] also reported the fluorescence bands in the FT-Raman spectra of hydroxy apatite and other relevant calcium phosphates. This chapter report on the quantitative determination of phosphates in cured meat and fertilisers. The idea of using protein free filtrate (PFF) and acid digestion method to eliminate fluorescence interferences by proteins in cured meat was also explored.

8.3. Experimental

8.3.1. *Reagents*

All reagents used were of analytical grade and ultrapure water, from a Modulab system (Continental Water Systems, San Antonio, TX) was used as a solvent. All glassware was soaked in 25% nitric acid for about two days and leached with ultra pure water and dried before being used.

Standard phosphate solution, 50000 mg/ℓ Potassium dihydrogen phosphate (KH_2PO_4) was oven dried to constant mass and dissolved in 1ℓ of ultra pure water. Working standard solutions were prepared fresh daily from the stock solution.

8.3.2. *Equipment*

Raman spectra were recorded in the macro sample chamber of an XY Dilor multichannel spectrometer. The 514 nm line of an Ar^+ laser made by Coherent was used as an exciting line with a power of 500 mW and the resolution was 2 cm^{-1} during all recordings. Acquisition time and laser power were varied during experiments. Samples were in glass tubes, (with diameter of 6 mm, thickness of 1 mm, and the length of 67 mm), and the spectra recorded in a backscattering geometry.

Flow injection manifold used was similar to the one used in previous chapters.

8.4. *Sample preparation*

5.00 g of commercial fertilisers (Wonder Planting and Vegetables Fertilisers) purchased from the local supermarket was dissolved in a 200 mℓ of pure water. The fertiliser samples were prepared as follows:

Sample 1: Fertiliser grains dissolved in 200 mℓ hot water.

Sample 2: Fertiliser grains, grind using a mortar dissolved in 200 mℓ hot water.

Sample 3: Fertiliser grains dissolved in 200 mℓ cold water.

Sample 4: Fertiliser grains, grind using a mortar dissolved in 200 mℓ cold water.

Sample 5: Fertiliser grains dissolved in 200 mℓ water digested in a microwave for 5 minutes.

Sample 6: Fertiliser grains, grind using a mortar dissolved in 200 mℓ water digested in a microwave for 5 minutes.

All the above samples were allowed to stand for 24 hours, then filtered using the Whatman 11.0 cm filter paper. Both the filtrate and the precipitate were analysed for phosphates. Vienna sausage samples (40 g of each Vienna sausage sample) were finely grind using a mortar and each sample was spiked with 200 mℓ of phosphate standards ranging from 50000 - 1000 mg/ℓ, and then digested in a microwave oven for 5 minutes. The samples were filtered and then analysed. Tap water, sea water, toothpaste and table salt solution were also analysed.

8.5. Results and discussion

The influence of site and factor group symmetry is removed in solution and therefore the Raman

spectra of phosphate ions in solution differ from that in the solid. Figure 8.1 represents the series of phosphate standards in purite water ranging from 50000-1000 mg/ℓ. The total symmetric Raman stretching vibration of phosphate ions in solution is observed around 876 cm^{-1} as is indicated on the figure. This can be compared to the totally symmetric Raman-active stretching vibration of phosphate (KH_2PO_4) solid occurs at around 914 cm^{-1} (figure 8.6).

The other two peaks which are visible, i.e. the broad 800 cm^{-1} and the sharp 968 cm^{-1} bands are typical of the Raman spectrum of the glass used for sample holder, are shown in figure 8.2. The 876 cm^{-1} band was chosen for phosphate determination and the 800 cm^{-1} glass peak was used as an internal standard because of its stability.

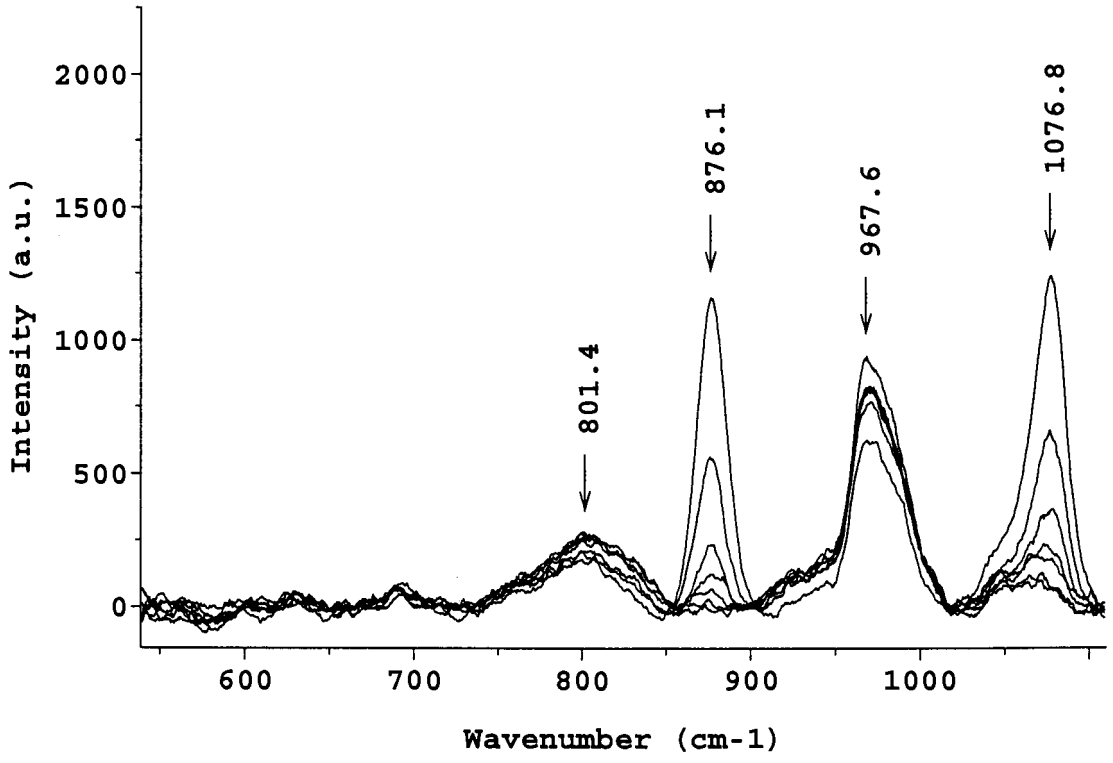

Figure 8.1.: The series of Raman spectra of phosphate standard solutions showing the Raman-active vibrations $\nu_s(HPO_4^{2-})$ around 876 cm^{-1}.

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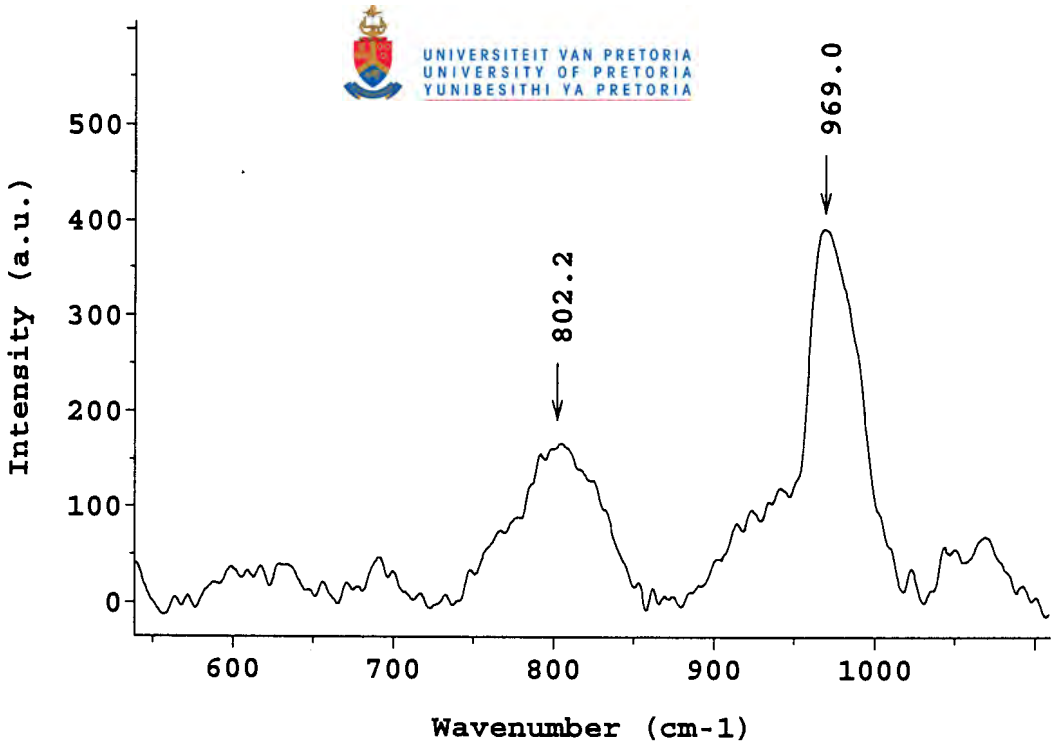

Figure 8.2.: **Raman spectrum of empty glass sample holders showing the peaks at 800 cm^{-1} and 968 cm^{-1}.**

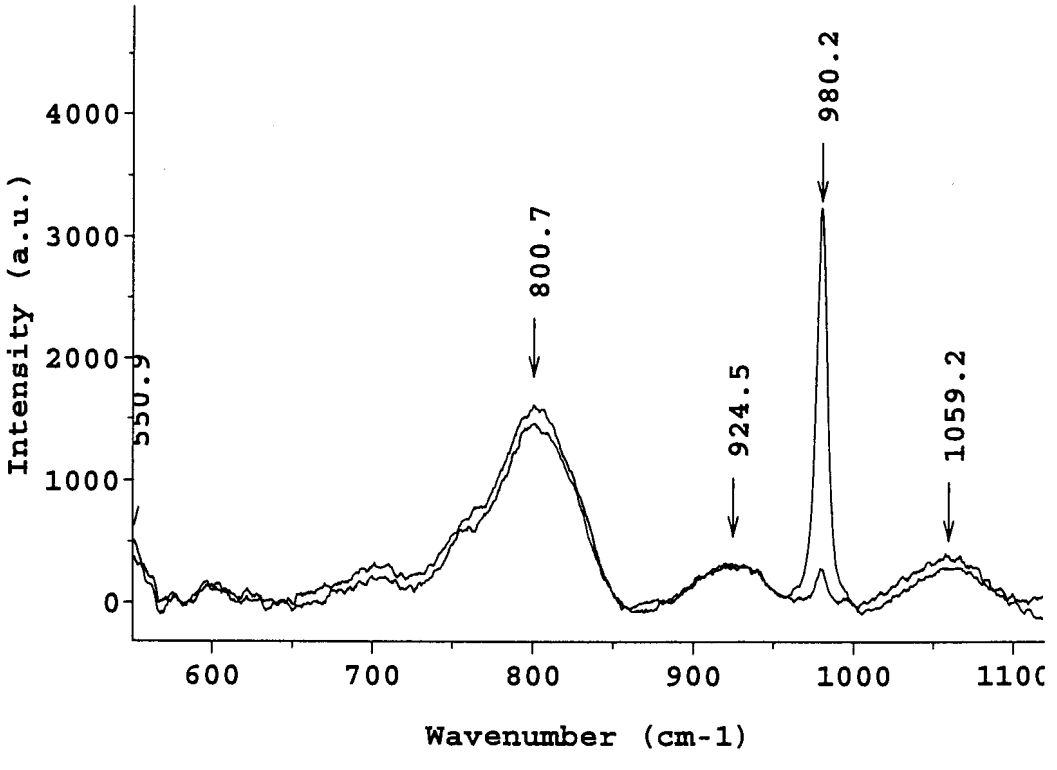

Figure 8.3.: **The series of Raman spectra of sea water and table salt showing the Raman-active vibrations $\nu_s(PO_4^{3-})$ around 924 cm^{-1}.**

Figure 8.3 shows Raman-spectra of the sea water and the table salt solution which are more or less the same, as in both the very broad PO_4^{3-} band occurs around 924 cm^{-1} . The two glass peaks are also visible, at 800 cm^{-1} and 980 cm^{-1} respectively.

From the spectra used in figure 8.1, the absolute intensities of the two peaks, i.e., 800 cm^{-1} (for glass) and 876 cm^{-1} (for phosphate standards), were recorded as integrated intensities with the baseline automatically subtracted. The absolute intensities of the various phosphate standard peaks are summarised in Table 8.1.

Table 8.1.: Absolute values of intensities for phosphate standards.

Conc. in mg/ℓ	Intensity (I) of HPO_4^{2-} at ≈ 876 cm^{-1}	Intensity (I_s) of glass at ≈ 800 cm^{-1}	Ratio (I/I_s)
50 000	1174	267	4.39
25 000	562	256	2.20
12 500	281	258	1.09
7 000	157	257	0.61
4 000	95	271	0.35
2 000	48	280	0.17
1 000	26	287	0.09

From the data in Table 8.1, a graph of concentration v/s intensity ratio (I/I_s), could be obtained {where I is the intensity of the $\nu_s(HPO_4^{2-})$ around 876 cm^{-1} and I_s the intensity of glass (internal standard peak) at 800 cm^{-1}}. The straight line obtained here is shown in figure 8.4. The analytical curve prepared with a series of standards with 0, 1000, 2000, 4000, 7000, 12500, 25000, and 50000 mg/ℓ phosphate gave a correlation coefficient of 0.9999 and limit of detection of 250 mg/ℓ by least square procedure, regression line equation, which was used to calculate the concentration

of samples, is y = 0.000088x - 0.00228.

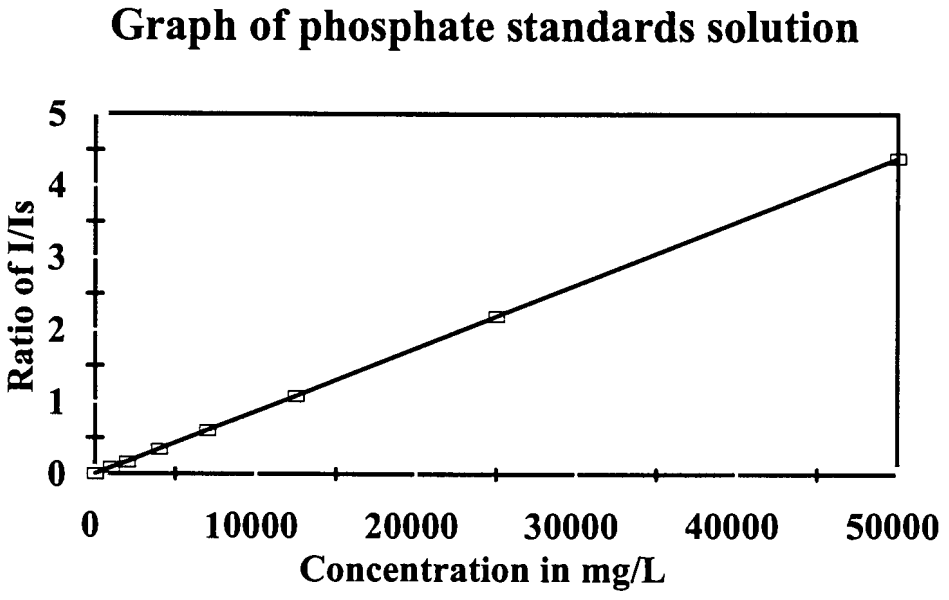

Figure 8.4.: Graph of I/I_s vs. Concentration in mg/ℓ, where I is the intensity of the $v_s(HPO_4^{2-})$ around 876 cm^{-1} and I_s the intensity of glass (internal standard peak) at 800 cm^{-1}.

8.5.1. Determination of phosphate in Vienna sausage samples using Raman spectroscopy

Vienna sausage samples were spiked with phosphate standard as stated in the experimental section. The results are presented in Table 8.2. A comparison of the results of Tables 8.1 and 8.2, shows that the signal of phosphate was suppressed due to high protein and fat content present in Vienna sausage samples. Proteins in biological fluids interfere with many analysis and must be removed non-destructively.

Raman spectra of proteins often result in fluorescence which originates from the aromatic amino acids which occur in proteins. Fluorescence can, of course, be a serious impediment to the observation of Raman bands of such compounds. One way of reducing fluorescence is by chemical or physical manipulation of the sample, eg, addition of quenchers, clean-up of sample, or burn-out by preliminary exposure to high intensity radiation.

Table 8.2: Absolute values of intensities for spiked Vienna sausage samples

Spiked Vienna samples in mg/ℓ	Intensity (I) of HPO_4^{2-} at ≈ 876 cm^{-1}	Intensity (I_s) of glass at ≈ 800 cm^{-1}	Ratio (I/I_s)
x + 50 000	543	260	2.09
x + 25 000	276	258	1.07
x + 12 500	140	258	0.54
x + 7 000	77	264	0.29
x + 4 000	49	283	0.17
x + 2 000	25	278	0.09
x + 1 000	--	287	--

With the Vienna sausage samples having a high protein content, it was necessary to remove the protein non-destructively. This was achieved by the method of protein free filtrate (PFF) as described by Christian [19]. With the PFF method a measured volume of sample is usually treated with a measured volume of reagent. Following precipitation of the protein the sample was filtered through a Whatman (11 cm) filter paper without washing. An aliquot of the PFF was analysed for phosphate content. However the PFF did not improve the signal.

The acid digestion was then applied as described by Christian [19]. After the acid digestion method the signal of phosphate disappear completely, the only visible peak being a sharp peak

of nitrate around 1044 cm^{-1}, which is typical in that region [20]. The results obtained from the Raman spectrum in showed that the presence of nitric acid hinders the determination of phosphate. A different medium (acetate-acetic buffer, pH 6.0) was used to digest the Vienna. Figure 8.5 is the Raman spectrum of acetate-acetic buffer (pH 6.0) solution in the absence of phosphate, showing the Raman bands of glass. This proves that the Raman bands of the buffer solution does not interfere with those of the phosphate ion. However after digestion with the buffer there was still fluorescence which means that the buffer is not a good masking agent for proteins, fats and other ingredients causing fluorescence in Vienna sausage samples.

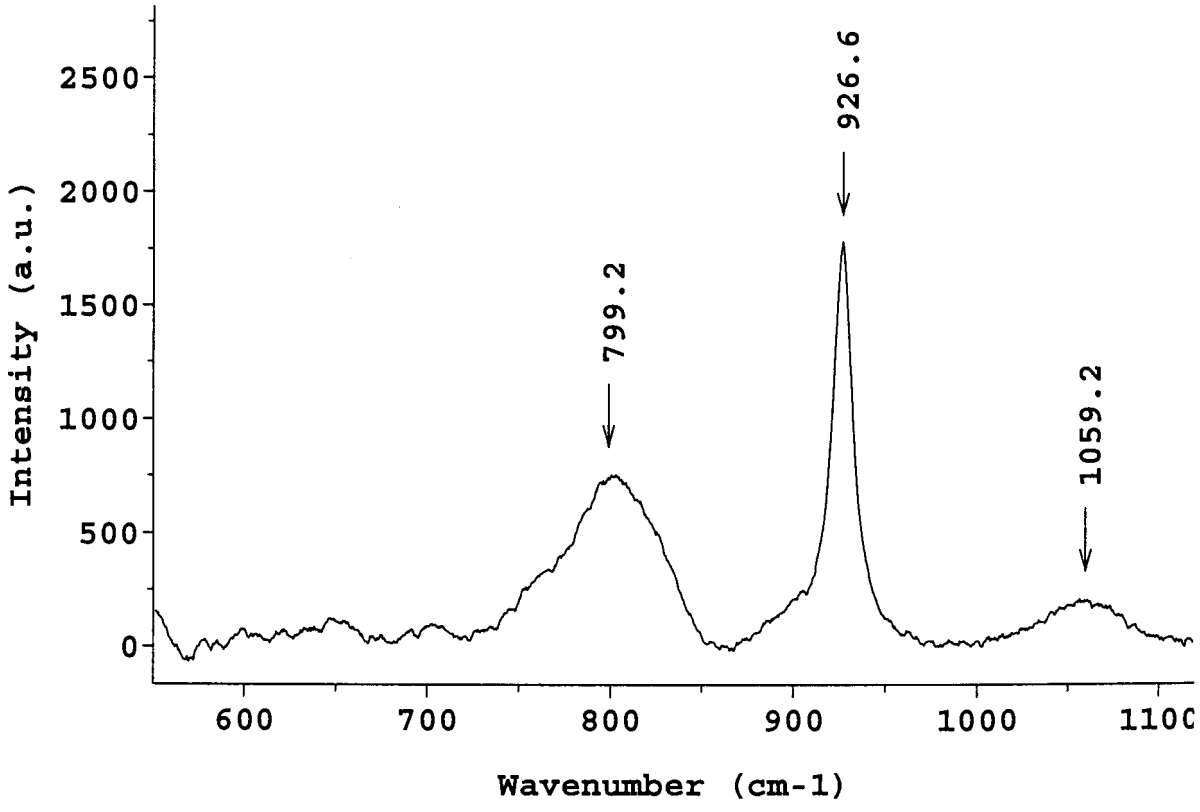

Figure 8.5.: Raman spectrum of acetate-acetic buffer in glass, showing the peaks at around 800 cm^{-1} and 968 cm^{-1}. Typical of glass, no buffer peaks.

8.5.2. Determination of phosphate in Vienna sausage samples using FIA

Phosphate in Vienna sausage samples was determined by a spectrophotometric method involving flow injection coupled with solvent extraction, for which the results are presented in Table 8.3.

Table 8.3.: Phosphate in Vienna sausage samples using FIA

Sample identification	Day	Concentration in mg/ℓ			Z
		X_1	X_2	Y	
$\bar{x}_1$	1	55.93	66.00	25.72	94.5%
$\bar{x}_2$	2	55.12	70.09	27.08	99.5%
$\bar{x}_3$	3	55.66	67.36	25.31	93.0%
$\bar{x}_4$	4	56.07	68.72	26.94	99.0%

$\bar{x}$ = is an average of five repetitive measurements.

X_1 = Concentration of Vienna sausages samples obtained using normal calibration method (NCC).

X_2 = Concentration of Vienna sausages samples obtained using standard addition method (SAM).

Y = Standard solution (27.22 mg/ℓ) run as sample.

Z = Percentage recovery of standard solution (27.22 mg/ℓ).

The ion associate formed between molybdophosphate and Malachite Green (MG) [4-5] was extracted into ethanol and the absorbance was measured at 660 nm. The carrier stream was 0.1 mol/ℓ sulphuric acid and the reagent stream contained ammonium molybdate, MG and sulphuric acid. The sampling rate was 45 samples per hour. The detection limit was 0.0638 mg/ℓ of

phosphate and the % RSD were 2.45% and 1.175% for both normal calibration technique (NCC) and standard addition method (SAM). Phosphate was determined satisfactorily by the proposed FIA method because the percentage recoveries of a spiked sample vary from 93.0% to 99.5%. The standard addition method gave a higher yield because the matrix effect is the same in both standards and the samples. The samples were analysed over a four day period and they were very stable.

Statistical comparison between NCC and SAM

A comparison of the normal calibration curve method (NCC) and the standard addition method (SAM) was performed to see whether the two methods give the same results at the 95% confidence level. The t-test (Null hypothesis) was applied to show whether the two techniques, i.e., SAM and CCM give the same results at the 95% confidence level. For the null hypothesis we assert that the two methods give the same results, i.e., $\bar{X}_1 = \bar{X}_2$. The alternative hypothesis $\bar{X}_1 \neq \bar{X}_2$, this imply the two tailed test. Number of observation (N) is eight (8), therefore degrees of freedom (v) = 6.

Method/technique	**Mean ($\bar{X}$) in mg/ℓ**	**Standard deviation (S)**	**Relative standard deviation (%RSD)**
Standard addition method (SAM)	68.04	0.1291	2.58%
Normal calibration curve (NCC)	55.66	0.0310	0.76%

$\bar{X}_1$ = Mean of phosphate for SAM; $\bar{X}_2$ = Mean of phosphate for CCM; S_1 = Standard deviation for SAM; S_2 = Standard deviation for CCM.

At 95% confidence level $t_{0.05,6}$ is of 2.447. The critical t-values are therefore ∓ 2.447. Finally $t_{calculated}$ was computed:

$$\mp t_{calculated} = (\bar{x}_1 - \bar{x}_2)/S_P \times [(\sqrt{N_1} \times N_2)/(\sqrt{N_1} + N_2)] = 13.10$$

$$\text{Where, } S_P^2 = \{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2\}/(N_1 + N_2 - 2) = 0.0088$$

Therefore $t_{calculated}$ lies within the critical range, i.e., $t_{calculated}$ is less than the $t_{tabulated} = 2.447$. At the 95% significance level we accept the null hypothesis and reject the alternative that the two techniques give the same results. We can conclude that the SAM and the CCM give more or less the same result at the 95% confidence level.

8.5.3. Determination of phosphate in fertilisers using Raman spectroscopy

The totally symmetric stretching vibration in the Raman spectra of solid phosphate (KH_2PO_4) is around 914 cm^{-1}. Some similarities exist between the solid phosphate (KH_2PO_4) band and that of solid fertiliser (see figure 8.6). In both the bands occur between 913-918 cm^{-1}. In solution these bands occur between 870-877 cm^{-1} as shown in figure 8.1.

Table 8.4 present the results of fertiliser samples analysed. The samples were analysed before and after filtering. The precipitate was analysed to determine whether any traces of phosphate remained. No traces of phosphate could be found in the precipitate, so it can be assumed that all of the phosphate was dissolved in purite water. There was no change (no loss of the analyte) even after the samples were analysed after two days. The method of sample preparation did not alter

the phosphate content in fertilisers as all the samples were left to stand on the bench for 24 hours before analyses. Raman spectroscopy was used successfully for the quantitative determination of phosphate in fertilisers and tap water. The percentage recoveries range from 91.2-97.9%. The only handicap seems to be in food samples due to the high fat and protein content. Here FT-Raman could prove more successful.

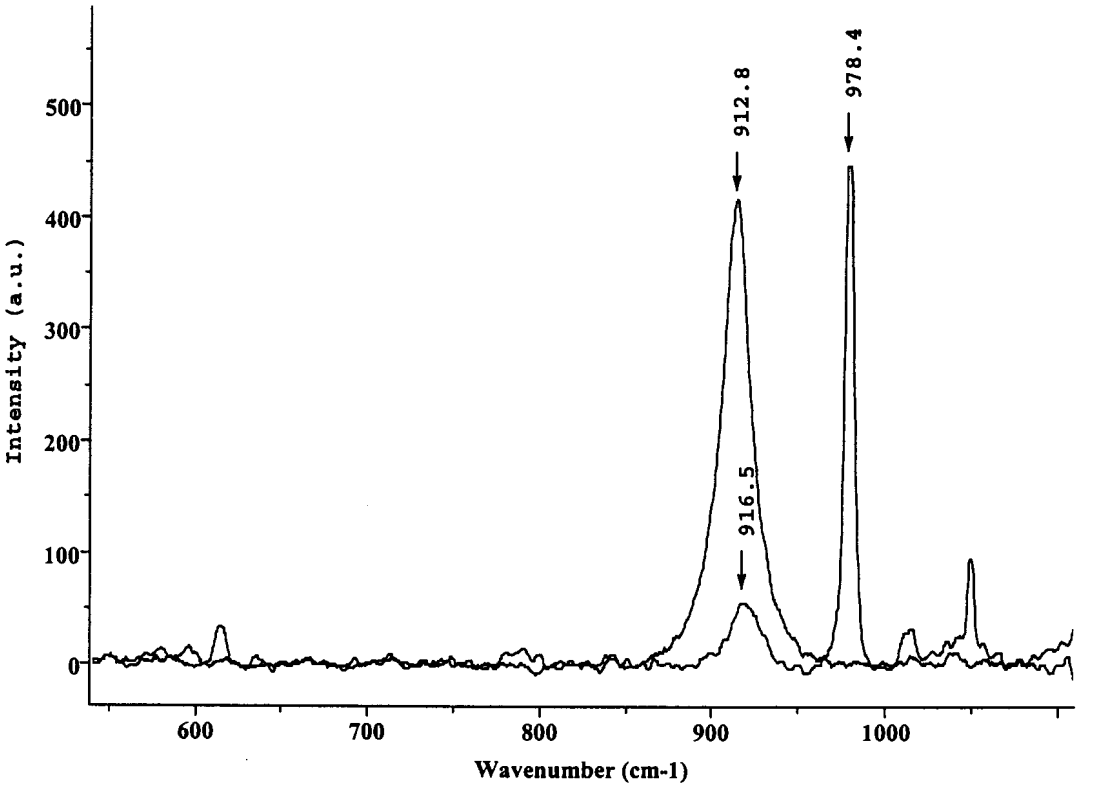

Figure 8.6.: Raman spectrum of phosphate solid and fertiliser solid showing the HPO_4^{3-} stretching vibration around 916.5 cm^{-1}.

Table 8.4.: Determination of phosphate in fertilisers

Sample number	Spiked amount of phosphate in mg/ℓ	Amount of phosphate recovered in mg/ℓ	Percentage recovery of phosphate
1	8500	8208	96.6%
2	8500	7867	92.6%
3	8500	7753	91.2%
4	8500	7980	93.9%
5	8500	8321	97.9%
6	8500	8208	96.6%
Tap water	3500	3435	98.1%

8.6. Conclusions

Raman spectroscopy was used successfully for the quantitative determination of phosphate in fertilisers. Precautions need to be taken when acid is used to digest the sample because this might results in the loss of the analyte or the analyte reacting with the acid itself. Food samples are very difficult to determine using conventional Raman spectroscopy because of fluorescence which hinders the analyte of interest. It is advisable to give a lot of attention on sample preparation when food samples are to be analysed, and FT-Raman analysis is advisable.

8.7. References

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"It is the customary fate of new truths to begin a heresies and to end as superstitions."

By: Thomas Huxley

CHAPTER 9

MICROANALYSIS OF NICKEL IN VIENNA SAUSAGES BY SPECTROPHOTOMETRY/FLOW INJECTION ANALYSIS

9.1. Introduction

This chapter describes the microanalysis of nickel in the filtrate of Vienna sausages by spectrophotometry/flow injection analysis.

Metallic nickel has been known for about 200 years, but interest in its biochemical and toxicological activities is fairly recent [1]. Nickel is found in many ores. It also occurs free in meteorites. Most stable form of nickel is Ni^{2+}. Nickel is not highly toxic, but its compounds have produced toxic effects in humans and other animals. Nickel carbonyl is the most hazardous of all industrial exposures to nickel [2]. Trace amounts of nickel in water (< 200 $\mu g.\ell^{-1}$), air ($1 \times 10^{-10} \mu g.\ell^{-1}$), and meat (±0.2 mg/kg) are not considered as toxic [1]. Nickel is used in stainless steel and other corrosive resistant alloys, provide electrode less coating, replace silver in coins, catalyst for hydrogenation of organic compounds. Ordinary human diet is 0.3-0.5 mg nickel per day. Total nickel intake varies with the types of food that are consumed [3]. Acidic foods take-up nickel from cooking and storage vessels. However, since it is poorly absorbed, nickel ingested from foods is relatively non-toxic.

9.2. Choice of analytical techniques

Analysis results of trace elements in nutrients are produced on a large scale to estimate the dietary intake of nutritive and toxic elements and to decide on the acceptance or rejection of foodstuffs [4]. Obviously data for such purposes must be accurate; if not, the consequences could be serious for public health and for economy. For the determination of trace elements in drinking water and foodstuffs GFAAS, ICP-AES, ICP-MS and AAS are commonly used. Colorimetric procedures have been used for the determination of trace elements.

The colorimetric procedure for the determination of nickel is based on its reaction with Dimethylglyoxime (DMG) in an organic solvent at a pH around 7.5 [5-11] and detected at 475 nm . There are quite few publications that have exploited the use of 4-(2-pyridylazo) resorcinol (PAR) [9-11] as colour forming reagent in spectrophotometric determination of nickel.

Most authors [12-14] have worked on spectrophotometric determination of nickel in steel using flow injection analysis (FIA). FIA has increasingly been recognised as an important tool in various fields such as clinical chemistry, food science, environmental pollution and industrial control. The method offers distinct advantages in reproducibility, flexibility, sample throughput and cost performance. Since its inception in 1975 [15], there are more than 8000 publications to date. Not withstanding the profusion of spectrophotometric methods for FI determination of metal cations, there is scarcity of nickel methods [16-17].

This chapter proposed a spectrophotometric microanalysis of nickel in Vienna sausages using DMG as the chromogenic reagent. The acid digestion method was used for cured meat sampling.

The following factors were optimised: effects of buffer pH, carrier stream, mixing coil length, reagent concentration, and flow rate.

9.3. Experimental

9.3.1. Reagents

All reagents used were of analytical grade and ultrapure water, from a Modulab system (Continental Water Systems, San Antonio, TX). All glassware was soaked in 25% nitric acid for about two days, leached with ultrapure water and dried before being used. All solutions were degassed before measurements with a vacuum pump system and stored under nitrogen atmosphere.

DMG of concentration 0.01 mol/ℓ was used as a ligand for nickel determination. Buffer solution, 0.2 mol/ℓ acetate buffer of pH 6.4 (that is, 0.3 ml of 0.20 mol/ℓ acetic acid and 9.70 ml of 0.2 mol/ℓ sodium acetate) was prepared. This solution is stable up to 2 weeks. Citrate complex forming stream was prepared as 0.08 mol/ℓ tri-sodium citrate and 0.02 mol/ℓ borax adjusted to pH 9.12 with hydrochloric acid. EDTA masking stream was prepared as 0.05 mol/ℓ ethylene-diaminetetra-acetic acid di-sodium salt.

Nickel standard solution, a 1.09989 Titrisol Nickel standard 1.000 g ± 0.002 g Ni (Nickel(ii)-chloride in water) was provided by Merck. Working standard solutions were freshly prepared daily by appropriate dilutions. Vienna sausage samples contained in tin and plastic containers were bought from the local supermarket.

9.3.2. Equipment (flow injection manifold)

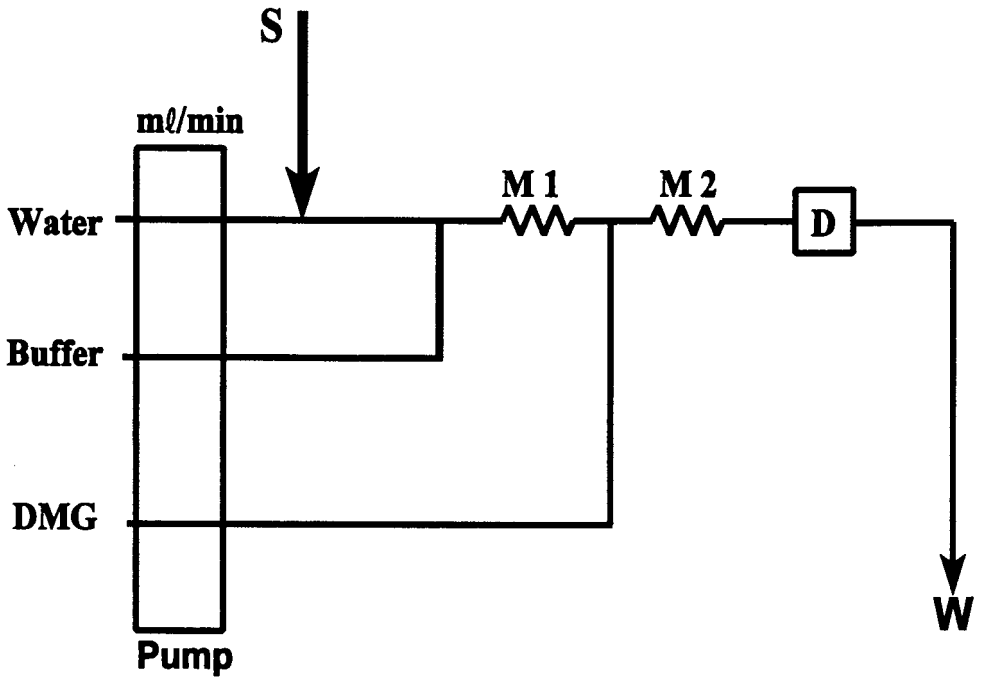

FIGURE 8.1.: **Flow injection manifold. S = Sample; M = Mixing coil, where M1 = 100 cm and M2 = 200 cm; D = Detector; W = Waste; Tube i.d. = 1.44 mm.**

The flow injection manifold used is schematically shown in figure 8.1. The manifold consisted of Tygon tubing (inside diameters of 1.44 mm) cut into the required lengths and wound around glass tubes with an o.d. of 10 mm. A Cenco peristaltic pump (operating at 10 rpm) was used for propelling the carrier and reagents. 100 $\mu\ell$ samples and standards were injected via a VICI Valco 10 port multi-functional sampling valve with two identical sample loops. The maximum absorbance was monitored at 475 nm in a UNICAM 8625 UV/VIS spectrophotometer equipped with a 10 mm Hellma-type flow-through cell (volume 80 $\mu\ell$). A digital pH meter (Zeiss, W. Germany) model MIA 3000 was used to measure the pH of the solutions and that of the sample carrier.

9.4. Sample preparation

Vienna sausage from tin container was preserved in brine solution (sodium chloride solution). Both the solution and the sausage were analysed for nickel content. Vienna sausage samples (40 g of each Vienna sausage sample) were finely grind using a mortar and each sample was transferred into 200 mℓ of hot doubly distilled de-ionised water. The sample was cooked on a microwave oven for five minutes. The samples were filtered and then analysed. They were spiked with the known amount of nickel to check the percentage recovery of nickel. After filtering the sample was treated using the acid digestion method [19].

With the acid digestion method, a mixture of nitric acid and hydrogen peroxide was used to digest the Vienna sausage filtrate. 200 mℓ sample was boiled with nitric acid on a hot bath until free of nitrous oxide fumes. The sample was evaporated to about 100 mℓ and hydrogen peroxide was added to the mixture to destroy last traces of organic matter. The sample was then evaporated further to about 20 mℓ and then cooled. The sample was transferred to a 50.00 mℓ volumetric flask and was made up to the mark with de-ionized water. The sample was then analysed for nickel content.

9.5. Results and discussion

The procedure describe by Shpigun *et al.* [9] was exploited and it ended up blocking the flow system due to the high concentration of iodine. The Tygon tubes were dyed by the iodine solution. As a results it was very difficult to detect whether the instrument was reading the nickel or the iodine itself. The reason of using iodine was to oxidised interfering ions like iron. It was

decided to omit the iodine solution step to see whether it would be possible to detect nickel. The results presented on the optimisation of the instrument prove that it is possible to determine nickel with less interference from foreign ions.

The dilute ammonium solution was used as a buffer. Ammonia solution causes some interference with the nickel because elements like cobalt in large amounts interfere with nickel in an ammonical solution [18]. Nickel (II) forms a very strong complex (blue colour) with NH_3 in the basic medium by the following reaction:

$$Ni(OH)_2\downarrow + 6NH_3 \rightarrow [Ni(NH_3)_6]^{2+} + 2OH^-$$

When samples were analysed using ammonia as a buffer, double peaks were observed which are much sharper than when the acetate buffer was used and the baseline was very unstable. The first peak might be due to nickel binding with DMG and the second one might be ammonia binding with nickel because ammonia and nickel DMG complex absorb on the same wavelength. The peak height values were less than those of the acetate buffer, almost halve.

Nitrate, nitrite, and phosphate are substances that are present in high amount (± 160 mg/ℓ) in cured meat and are likely to interfere with nickel. The effect of these substances were investigated as follows: the samples were spiked with a higher concentration (250 mg/ℓ) of nitrate, nitrite and phosphate which resulted in broad peaks. The concentration of the three compounds below 200 mg/ℓ does not have an effect on nickel determination. Chromium at concentrations greater than 0.1 mg/ℓ also interferes, but its interferences can be eliminated by using a buffer solution. Some researchers [12-14] used a soluble tartrate or citrate to prevent

interference from iron (III), aluminium, cobalt, or bismuth. The sodium acetate rather than the ammonium acetate buffer was used to eliminate interferences from cobalt, zinc, or manganese which are also present in cured meat.

9.5.1. *Optimisation*

The system was optimised with respect to effect of carrier stream, flow rate, mixing coil length, sample volume, reagent concentration and effect of pH of buffer solution.

9.5.1.1. *Effect of carrier stream*

Water, dilute nitric acid with varied concentrations (0.05, 0.5, 1.0, and 1.5 mol/ℓ), and dilute ammonia solutions were used as carrier streams. The dilute ammonia was eliminated due to its competition with DMG for the nickel ion complexation. Table 9.1 is the results of dilute nitric acid concentration v/s peak height analysed over a period of three days. The peak height decreases with time and the base line was unstable. Water was used as a carrier stream due to its stable peak height and base line over the same period.

Table 9.1.: The effect of different concentrations of dilute nitric acid and water on nickel determination.

Carrier stream	Days	Peak height in mV				
		Blank	100 μg/ℓ	200 μg/ℓ	300 μg/ℓ	400 μg/ℓ
1.5 mol/ℓ	1	0.0009	0.6572	1.2986	1.9623	2.6300
	2	0.0007	0.6138	1.2301	1.8417	2.4555
	3	0.0011	0.5420	1.0756	1.6184	2.1684
1.0 mol/ℓ	1	0.0006	0.7975	1.6121	2.3927	3.1838
	2	0.0010	0.7609	1.5076	2.2830	3.0441
	3	0.0008	0.6759	1.3496	2.0278	2.7041
0.5 mol/ℓ	1	0.0006	0.9316	1.8587	2.7957	3.7301
	2	0.0004	0.8825	1.7702	2.6490	3.5314
	3	0.0009	0.6821	1.3599	2.0461	2.7285
0.05 mol/ℓ	1	0.0005	1.2558	2.4989	3.7724	5.0243
	2	0.0007	1.2422	2.5000	3.6521	4.8937
	3	0.0007	1.1934	2.3859	3.5803	4.7720
Water	WX	0.0005	1.3035	2.6158	3.9062	5.2097

WX = water was stable over the three day period.

9.5.1.2. *Effect of sample loop internal diameter (volume)*

The length of the sample loop was 11 cm, the internal diameter (I.D.) of the sample loop was varied. The bigger the diameter the higher is the peak height. The 1.14 mm internal diameter was chosen as the optimum (Figure 9.2) so that it can be possible to detect micro amount of nickel.

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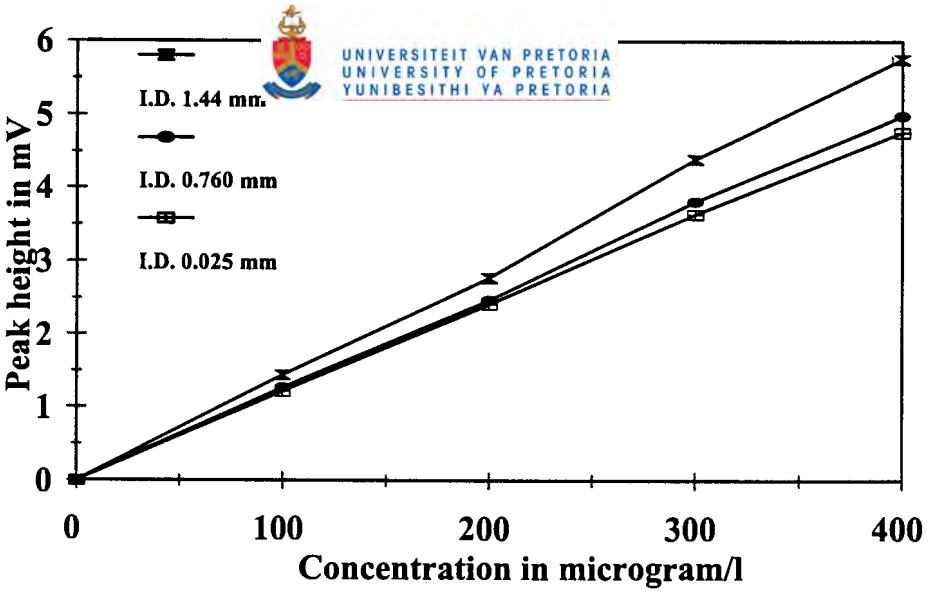

FIGURE 9.2.: **Effect of sample loop internal diameter (I.D.) on nickel determination**

9.5.1.3. *Effect of flow rate and the reaction coil (mixer coil)*

The various flow rates were monitored using either the 100, 200 or 400 cm reaction coils. The results are tabulated in Table 9.2. The 200 and 100 cm reaction coils gave sharp peaks, whereas the 400 cm reaction coil gave broader peaks. From the results it shows that it was little variation in peak heights between the 100 and 200 cm mixer coils. The 200 cm coil was chosen ahead of the 100 cm coil because it is the best for the sample and the reagents to mix thoroughly before detection. There was also little variation between the 4.02 and 4.22 ml/min flow rates. The 200 cm mixer coil and 4.22 ml/min flow rate were chosen as the optimum operating conditions.

Table 9.2.: Effect of flow rate on nickel determination.

Reaction coil length	Peak height in mV				Flow rate in ml/min
	100 μg/ℓ	200 μg/ℓ	300 μg/ℓ	400 μg/ℓ	
100 cm	1.2629	2.5312	3.7834	5.0601	3.24
	1.2783	2.5697	3.8351	5.1129	4.02
	1.2810	2.5626	3.8434	5.1235	4.22
200 cm	1.2778	2.5563	3.8333	5.1111	3.24
	1.2878	2.5712	3.8634	5.1509	4.02
	1.2880	2.5832	3.8721	5.1600	4.22
400 cm	1.1665	2.3329	3.4994	4.6661	3.24
	1.1697	2.3404	3.5101	4.6788	4.02
	1.1710	2.3412	3.5112	4.6800	4.22

9.5.1.4. *Effect of DMG concentration*

The concentration of DMG was varied over a three day period, the results are presented in Table 9.3. The lower the concentration the more stable are the results over three days. By using of a high concentration of DMG FIA system tend to be blocked after three days of analysis because of the precipitate (complex between Ni^{2+} and DMG) that is forming. Another way of avoiding the precipitation is either to flush the system with water after each run, which is time consuming. The results are also not stable which let us to choose the 0.01 mol/ℓ concentration as the optimum working concentration.

Table 9.3.: Effect of the concentration of DMG on nickel determination

Concentration of DMG	Day	Peak height in mV			
		100 μg/ℓ	200 μg/ℓ	300 μg/ℓ	400 μg/ℓ
0.01 mol/ℓ	1	1.3655	2.7331	4.0863	5.4617
	2	1.3589	2.7208	4.0934	5.4593
	3	1.3675	2.7345	4.1006	5.4656
0.03 mol/ℓ	1	1.4310	2.8643	4.2953	5.7331
	2	1.4618	2.9165	4.3854	5.8500
	3	1.4015	2.8100	4.2045	5.6124
0.10 mol/ℓ	1	1.4885	2.9803	4.4572	5.9457
	2	1.5035	3.0077	4.5107	6.0139
	3	1.5382	3.1064	4.6150	6.1530
0.25 mol/ℓ	1	1.5673	3.1350	4.7023	6.2702
	2	1.6015	3.2103	4.8048	6.4073
	3	1.5882	3.1759	4.7603	6.3482

9.5.1.5. *Effect of the buffer pH*

The pH of the buffer was varied to see whether there is any effect. The results are presented graphically in Figure 9.3. The base line was not stable for both the pH 3.42 and pH 4.63 acetate buffer. The pH 6.4 was chosen as the optimum pH for the determination of nickel, because it gave a higher absorbance reading and also the base line was very stable.

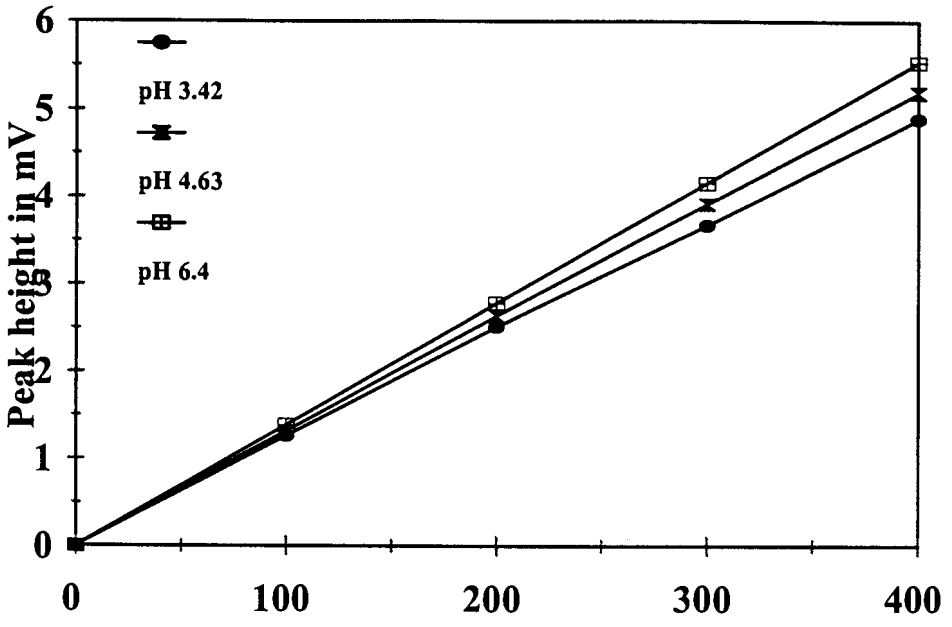

FIGURE 9.3.: **Effect of acetate buffer pH on nickel determination**

9.5.2. *Determination of nickel in cured meat*

Nickel was determined in meat using the optimum conditions as investigated under optimisation. The optimum conditions used for determination are the pH 6.4 buffer solution, 0.01 mol/ℓ DMG, flow rate of 4.22 ml/min and 200 cm mixer coil, the internal diameter of loop length of 1.14 mm, and water as a carrier stream.

The Vienna sausage samples were prepared as stated on the experimental section and then filtered. The three samples, that is, sample in plastic container (PS), sample in tin container (TS), and the brine (BS) sauce (used to preserve the Vienna sausage in tin container) were analysed directly for the determination of nickel. The standard addition method was also applied to match the matrix interferences. With the direct determination two peaks were observed. The first one

which was very small appears 20-22 seconds and the second (much bigger and sharper) appears at around 28-29 seconds. The first peak might due to proteins or fat which might interfere with the analyte of interest and the second peak is that of nickel complexing with DMG. There is a lot of fats and proteins in foodstuffs.

It was decided that the sample be digested in acid as described in the experimental section to get rid of all the fats and proteins which might interfere with analyte of interest. After acid digestion method the three peaks were observed. The first peak was a negative peak and the other two were positive peaks, at 20 and 28 seconds respectively. The baseline was also unstable and the nickel peak disappear with time. Standard addition method was applied to the acid digest samples but the peaks remains. Baseline was very unstable, as the experiment progress the smaller peak increases and the bigger peak decreases. The acid digest method is quite popular in the absorption spectrometry to eliminate all the interfering compounds.

The interference might be due to transition metals like iron which are present in Vienna sausages. EDTA and citrate were added to the sample containing nickel before the measurement of absorbance to mask the interference of the transition metal ions like iron. As a result the method was very selective for nickel. Only one peak was visible, this proves that iron was the one which was interfering with the nickel.

From the data in Figure 9.3 using the results of pH 6.4 acetate buffer, a graph (Figure 9.4) was plotted of concentration versus peak height. The calibration curve prepared with a series of standards with 0.00, 100, 200, 300, 400 $\mu g/\ell$ nickel gave a correlation coefficient of 0.9999 and limit of detection of 0.101 $\mu g/\ell$ by least square procedure, regression line equation, which was

used to calculate the concentration of samples, is

$$H = 0.0139C - 0.00922$$

where H is peak height in mV, and C is concentration of nickel in $\mu g/\ell$.

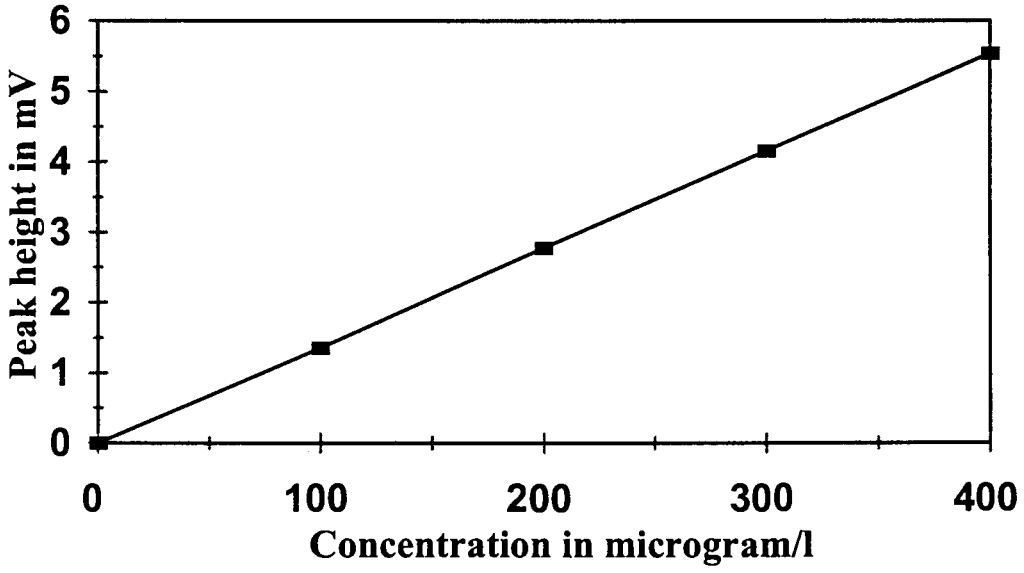

FIGURE 9.4.: Calibration curve of nickel standard solutions

The results of both AAS and FIA are summarised in Table 9.4. From both methods it was found that concentration of nickel is higher in tin container than in plastic container. This is a result of the release of nickel from the can itself. There was also a micro amount (about 15 $\mu g/\ell$) of nickel in brine solution detectable by FIA. The FIA method was applied successfully because the percentage recoveries of a spiked sample (Y = 200 $\mu g/\ell$) vary from 92.94% to 97.02%. FIA method gave a higher yield than the AAS method.

Table 9.4.: Determination of nickel in Vienna sausage samples

Sample number	Concentration of nickel in $\mu g/\ell$							Percent recovery of Y
	AAS			FIA			Y	
	BS	TS	PS	BS	TS	PS		
1	--	278.00	214.42	15.74	290.41	220.19	190.34	95.17%
2	--	286.38	221.61	15.66	287.79	217.54	192.75	96.38%
3	--	295.92	225.26	15.50	301.03	223.92	185.89	92.94%
4	--	282.92	219.54	16.07	294.76	230.05	194.03	97.02%
5	--	281.59	223.69	15.83	280.85	232.72	188.64	94.32%

BS = Brine sample solution; PS = Plastic container samples; TS = Tin container samples;

Y = 200 $\mu g/\ell$ nickel standard solution run as sample.

9.5.3. Statistical comparison between the AAS and FIA methods

The comparison was done between the AAS and the FIA (for TS samples) to see whether the two methods give the same results at the 95% confidence level. The statistical results are tabulated in Table 9.5.

Table 9.5.: Statistical calculations

Sample type	Mean ($\bar{x}$) in $\mu g/\ell$ of Ni^{2+}		Standard deviation (S) of mean		Relative standard deviation (%RSD)	
	AAS	FIA	AAS	FIA	AAS	FIA
TS	284.96	290.97	6.8209	7.5566	2.40%	2.60%
PS	220.90	224.88	2.3821	6.4224	1.91%	2.86%

For the Null hypothesis we assert that the two methods give the same results, i.e., $\bar{x}_{1TS} = \bar{x}_{2TS}$.

The alternative hypothesis $\bar{x}_{1TS} \neq \bar{x}_{2TS}$, this implies the two tailed test. There are ten (10)

determinations, therefore ν = 8. At the 95% confidence level $t_{0.025} = 2.306$, therefore the critical t-values are ± 2.306. Finally $t_{calculated}$ was computed:

$$\pm t_{calculated} = \frac{\bar{x}_{1TS} - \bar{x}_{2TS}}{S_p} \times \sqrt{\frac{N_{1TS} \cdot N_{2TS}}{N_{1TS} + N_{2TS}}} = 1.320$$

Where

$$S_p = \sqrt{\frac{S_{1TS}^2 (N_{1TS} - 1) + S_{2TS}^2 (N_{2TS} - 1)}{N_{1TS} + N_{2TS} - 2}} = 7.918$$

Therefore $t_{calculated}$ lies within the critical range, that is, $t_{calculated}$ = 1.320 is less than the $t_{tabulated}$ which is equal to 2.306. At 95% confidence level we accept the Null hypothesis and reject the alternative that the two methods give the same results. Similarly for PS samples $t_{calculated}$ = 1.1583 which is less than the $t_{tabulated}$ = 2.306, so therefore the Null hypothesis is retained and the alternative is rejected. We can conclude that both the AAS and FIA methods give the more or less the same result at the 95% confidence level for both the PS and TS samples.

9.6. Conclusions

The proposed DMG nickel complex method is the best method for the determination of nickel in cured meat. The determination of nickel in foodstuffs can be difficult procedure in obtaining recovery of the total nickel through digestion procedure only. Sample preparation requires the use of oxidising acids (like nitric acid) for oxidising sugar, fats, proteins etc.. From the research findings we can conclude that the acid digestion method is the best to eliminate interferences from compounds like proteins. A lost in the analyte of interest might be encountered if all the

transition metals are not masked. Transition metals (for example, iron) that are likely to be present in foodstuffs must be masked with masking agents like EDTA for the method to be very selective for nickel determination. After acid digestion method the sample was masked with EDTA and citrate which resulted in higher recovery of nickel in Vienna sausages. From statistical analysis it was concluded that both the AAS and FIA method gave the same results at the 95% confidence level.

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"The frontiears are not east, west, north or south, but wherever a man fronts a fact".

By: Henry David Thoreau

CHAPTER 10

A NEW BIENZYMATIC AMPEROMETRIC SENSOR FOR PROTEINS ASSAY IN MILK

10.1. Introduction

This chapter describes a new bienzymatic amperometric sensor for protein assay in milk.

Proteins are indispensable components of living matter. They function as enzymes that are responsible for the metabolic process of the organism and also as structural components [1]. They also serve as antibodies. The four main elements that are found in proteins are nitrogen, carbon, hydrogen, and oxygen. There are also some additional elements in proteins, like sulphur and phosphorus. The three main principal proteins found in milk are casein, lactalbumin and lactoglobulin. These proteins are very important by contributing as essential amino acids for normal health and growth [2].

10.2. Biosensors

Biosensors comprise an analyte selective interface in close proximity or integrated with a transducer, whose function it is to relay the interaction between the surface and analyte either directly or through a chemical mediator [3]. The nature of the transducer and the transduced

parameter will depend on the type of bioanalytical event concerned with detection of the analyte. Three kinds of transducers can be used for the construction of electrochemical biosensors, viz, amperometric, potentiometric and piezoelectric. Potentiometric biosensors give a lower sensitivity whereas the amperometric sensors gives a high sensitivity. Piezoelectric biosensors gives a higher sensitivity, but at the same time their selectivity decreases significantly.

The first biosensor can probably be attributed to Clark and Lyons [4] who described an '**enzymatic electrode**' using glucose oxidase as a selective biorecognition molecule for glucose. The enzyme was held next to a platinum electrode in a membrane sandwich. The Pt electrode polarized at +0.6 V vs SCE, respond to hydrogen peroxide, produced by the enzyme reaction:

$$Glucose + O_2 \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

This lead to the development of the first glucose analyser for the measurement of glucose in whole blood. Amperometric biosensors satisfy many of the requirements for clinical assays, environmental monitoring or process control. Such sensors offer excellent sensitivity, fast response, selectivity toward electro active species, miniaturization and low cost.

Despite the obvious benefits, biosensor technology has been slow to penetrate the food industry. The potential advantage of using biosensors for food analysis is a rapid, specific quantification without the need for extensive sample preparation. Rapidity is accomplished by coupling the biological component to a transducer to obtain an immediate result. Specificity is achieved through the reaction of an analyte with a particular component selected from a range which occur naturally, examples are enzymes, antibodies, lectins and DNA [5].

10.3. Choice of analytical methods

Proteins determination is of great interest in diverse fields such as food science, biochemistry, clinical investigation, and in several research fields. The very complex structure of proteins made their characterization very difficult.

Most of the methods recommended for proteins assay are laborious and not accurate enough. They are based on ion-exchange chromatography [6], HPLC [7], capillary zone electrophoresis [8-9], matrix assisted laser desorption ionization-time-of-flight MS (MALDI-TOF-MS) [10-11], and immunoassay [12].

Biosensors offers excellent sensitivity, fast response, selectivity towards electroactive species, possibility of miniaturization, low cost, and quantification without the need for extensive sample preparation [13]. Therefore, food analysis is considered one of the major fields where biosensors technology can be exploited to obtained analytical information in order to evaluate the quality, composition and freshness of the food [14].

This chapter proposed the use of an bienzymatic amperometric sensor without and with flow injection analysis (FIA) for proteins assay in milk. The sensor is based on the immobilization of two enzymes: carboxypeptidase A and L-aminoacid oxidase (L-AAOD) in carbon paste. The reaction that are taken place in the presence of the two enzymes [15] are:

$$\text{proteins} \xrightarrow{\text{carboxypeptidase A}} \text{L - amino acids}$$

$$\text{L - amino acids} + O_2 + H_2O \xrightarrow{\text{L-AAOD}} \text{2 - ketoacid} + NH_3 + H_2O_2$$

$$H_2O_2 \xrightarrow{650\ \text{mV}(Pt/Ag-Ag/Cl)} O_2 + 2H^+ + 2e^-$$

10.4. Experimental

10.4.1. Bienzymatic Amperometric (BEA) Sensor Design

Paraffin oil (Fluka, Switzerland) and graphite paste (1-2 micron, synthetic, Aldrich) were mixed in a ratio 1:4 (w/w) followed by the addition of 100 μℓ enzymatic solution (carboxypeptidase A (EC 3.4.17.1 Type I from bovine pancreas) and L-amino acid oxidase (L-AAOD) (EC 1.4.3.2. Type I: Crude Dried Venom from Crotalus adamanteus, Sigma):1.4 mg L-AAOD/mℓ in carboxypeptidase A solution). A plastic peak was filled with the graphite-paraffin oil paste leaving an empty space of 3 to 4 mm in the top part to be filled with carbon paste that containing the enzymes. The diameter of the sensor was 3 mm. Electric contact was obtained by inserting a silver wire into the carbon paste. The electrode tips were gently rubbed on fine paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-001, Orion) before use. The biosensor was stored dry at 4°C.

10.4.2. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in connection with a PGSTAT 20 and software (Eco Chemie version 4.4) was used for all chronoamperometric (E = 650 mV) measurements. A glassy carbon electrode and a calomel electrode served as the counter and reference electrodes in the cell.

10.4.3. Flow injection system

The electrodes were incorporated into the conduits of a flow injection system (Figure 10.1) with basic design similar to that previously described [16-18]. A Carle microvolume two-position sampling valve (Carle No. 2014) containing two identical sample loops was used. Each loop has a volume of 30 μℓ. A Cenco peristaltic pump operating at 10 rev/min supplied the carrier streams to the manifold system. Tygon tubing (0.51 mm i.d.) was used to construct the manifold; coils were wound round suitable lengths of glass tubing (15 mm o.d.). As carrier a solution of 1 mol/L NaCl solution was used. The sample was injected in a phosphate buffer (pH = 7.00) stream. A 55-s cycle sampling time was used, giving the system a capacity of about 72 samples/h.

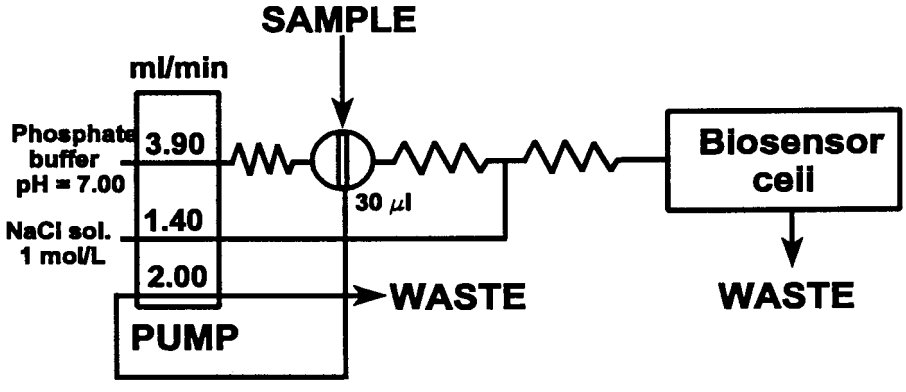

Figure 10.1.: Schematic diagram of the FIA system used for the determination of proteins

Device control was achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa). The FLOWTEK [19] software package (obtainable from MINTEK) for computer-aided flow analysis was used throughout for device control.

10.5. Reagents and materials

Protein standard solution (500 mg/ℓ) was supplied by Sigma (St. Louis, USA). Phosphate buffer (pH = 7.00) was supplied by Merck (Darmstadt, Germany). De-ionized water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used for all solutions. Milk - full cream and low fat as well as yogurt - Yogi Sip were supplied from a Pick' n Pay food store, Pretoria, South Africa.

A stock protein solution of 100 mg/ℓ was prepared by dilution of the standard protein solution (500 mg/ℓ) provided by Sigma. The proteins solutions necessary for the calibration graph, were prepared from stock protein solution by serial dilutions. All the solution used in the measurements without FIA, were buffered using phosphate buffer (pH = 7.00).

10.6. Sample preparation

10.6.1. Milk samples

2.94 mℓ of milk were diluted to 1000 mℓ with de-ionized water. Small volumes (of μℓ range) were added to 10 mℓ de-ionized water (when the measurement was done using a FIA system) or to 10 mℓ phosphate buffer (pH = 7.00) solution (when the measurement was done in the absence of FIA).

10.6.2. Yogurt - Yogi Sip samples

The volume taken from Yogi Sip to prepare the sample was 3.22 mℓ. All the other steps of sample preparation are similar with those followed by milk samples.

10.7. Results and discussion

10.7.1. Electrode response

The response of the electrode was determined using a chronoamperometric technique (E = 650 mV), using Pt and saturated calomel electrodes as auxiliary and reference electrodes, respectively at a pH = 7.00 (phosphate buffer).

The calibration equation obtained for the amperometric biosensor without the flow injection analysis (FIA) system is as follows:

$$\log I = 4.68 - 9.20 \log c \; ; \; r = 0.9845$$

where I is the intensity of the current (I in μA), and c is the concentration of the proteins (c in μmol/ℓ).

The limit of detection is low: 1.5 μmol/ℓ and the linear concentration range is between 1.8 and 2.8 μmol/ℓ. The response time is 90 s.

When the amperometric biosensor was used as detector for FIA, the following calibration equation was obtained:

$$H = -7.98 + 3.38\ c\ ;\ r = 0.9967$$

where H is the peak height, and c is the concentration of protein (c in µmol/ℓ). The limit of detection was 1.5 µmol/ℓ. The slope (3.38 nA / µmol/ℓ) and working concentration range (2.00 - 3.25 µmol/ℓ) were changed due to the non-equilibrium working conditions in FIA systems, as previously described [16].

The electrode response displayed good stability and reproducibility over the tests for one week, with and without a FIA system (a RSD less than 0.01 % was recorded).

10.7.2. pH Influence on the response of the bienzymatic amperometric (BEA) sensor

At pH = 7.00 both enzymes recorded maximum activity. It is very important to have the same pH value (7.00) for all the solution used in the measurements, because the intensity of current strongly varied with the pH, when this enzymes are used for electrodes construction [20-21].

10.7.3. Selectivity of the bienzymatic amperometric (BEA) sensor

The selectivity of the biosensor was checked using the mixed solutions method, over glucose, lactose and galactose - as main components in milk that can interfere. Amperometric selectivity coefficients were determined following the method proposed by Wang [22]. In the evaluation

the concentration of the interfering ion used, was selected to be four times higher than for the protein. All the amperometric selectivity coefficients were lower than 10^{-4}. That means that these compounds do not interfere. Inorganic cations such as Na^+, K^+, Ca^{2+} also do not interfere in the analysis of proteins.

10.7.4. Analytical applications

The biosensor proved to be useful for the protein assay in milk and milk products (like yogurt) in both cases: with and without FIA. A recovery test was done using the new amperometric bienzymatic sensor. The results obtained for the test: 99.98 ± 0.02 % (in the absence of FIA) and 99.87 ± 0.01 % (in the presence of FIA), proved the suitability of this sensor to be used for the assay of proteins.

Table 10.1.: Determination of proteins in milk and milk products using bienzymatic amperometric biosensor with and without FIA.

Type of milk product	Spiked amount of protein in grams	Recovered protein in grams	
		Without FIA	With FIA
Low fat milk	3.40	3.37	3.37
Full cream milk	3.40	3.39	3.39
Yogi sip	3.10	3.07	3.09

The content in proteins was determined for two types of milk {low fat and full cream (3.4 g proteins/100mℓ milk) produced in South Africa} as well as for an yogurt {Yogi Sip (3.1 g proteins/100 mℓ milk) produced in South Africa}. The results presented in Table 10.1 show a very good agreement between the two methods proposed for proteins assay (when the

amperometric bienzymatic sensor it is used with and without FIA) and the content in proteins given on the milk and yogurt boxes. For all the milk products, the average recovery was higher than 99.00 % with a RSD less than 0.08 %: 99.06 ± 0.07 % in the absence of FIA, and 99.73 ± 0.03 % in the presence of FIA.

10.8. Conclusions

This chapter presents a very simple method for total protein assay in milk and milk products. The method is based on a new bienzymatic sensor based on the immobilization of L-carboxypeptidase and L-amino acid oxidase in carbon paste.

The main advantages of the proposed system are: very simple sample preparation (dilution of milk products), possibility of on-line monitoring of proteins, high reliability of analytical information, rapidity, simplicity of construction and operation, low cost of analysis. An advantage of the FIA system is the possibility of on-line monitoring of proteins in milk.

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"The principal mark of genius is not perfection but originality, the opening of new frontiers."

By: Arthur Koestler

CHAPTER 11

CONCLUSIONS

11.1. Introduction

There is a need for an instrument that can offer distinct advantages in reproducibility, cost effective, lower detection limits, on-line monitoring with a high sample throughput, flexibility, and automation. FIA was complying moderately with the above mentioned requirements. FIA has been successfully applied in all major fields of analytical chemistry, environmental, food and clinical analysis due to its versatility. A variety of detectors were used extensively with the FIA to fulfill some of the requirements mentioned above. In this study the best FIA systems for the determination of nitrogen compounds, phosphates and nickel as process analysers are investigated. Also included is the kinetic study of nitrite using Raman spectroscopy.

11.2. Kinetic study of the decomposition of nitrite

Raman spectroscopy was employed as a means of determining the rate of decomposition of nitrite to nitrate in acid samples. Nitrite decomposes with time in acidic medium, with an increase in decomposition rate as the concentration of acid increases. The mean decomposition rate of 0.625 mol/ℓ nitrite in 1 mol/ℓ HCl is 1.18×10^{-5} s^{-1}, with $t_{½}$ = 16 h. This rate explains the problems encountered when the FIA version of the Shinn reaction is applied to certain nitrite samples mainly from acidic origin. Nitrite is potentially unstable, and all food and water samples

should be analysed as soon as possible after collection. The chemical decomposition of nitrite can be reduced by storing aqueous samples under strongly alkaline conditions.

11.3. Nitrite determination method

Nitrite samples are digested in a microwave oven, treated with a 1 mol/ℓ NH_4Cl solution at pH 9 (all under nitrogen atmosphere) and are immediately analysed. Nitrite is diazotised in the FIA system with N-(1-naphthyl)ethylenediammonium dichloride to form a coloured azo dye, which is measured at 540 nm. The average recovery of nitrite was approximately around 96%, which was a good recovery when compared with the extractions by the other two sample media used, i.e., nitrite in 1 mol/ℓ NH_4Cl with the pH adjusted to 3 and 6. The detection limit is 0.036 mg/g for sample injections of 400 $\mu\ell$. The sampling rate is about 50 samples per hour and the relative standard deviation is 0.67%. These results indicate that inorganic nitrite can reliably be determined in cured meats by FIA under alkaline medium. Time and pH of samples must always be taken into consideration when working with nitrite samples.

11.4. Simultaneous spectrophotometric determination of nitrite and nitrate

Two types of cadmium (i.e., cadmium coarse powder and cadmium granules) were used to reduce nitrate to nitrite. Both cadmium gave practically the same yield. From the experimental results we can conclude that nitrite is stable in the presence of nitrate and also the cadmium reductor columns can be reusable over a longer period as long as the reductor column is rinsed with the appropriate solution. The percentage recoveries of both nitrate and nitrite vary between 93.8% to 101%. The proposed FIA method can be successfully applied for the simultaneous

assay of nitrate and nitrite in foodstuffs and water.

11.5. Quantitative determination of phosphate

Comparison was done between FIA and Raman spectroscopy in quantitative determination of phosphate in fertilisers in Vienna sausages. It was very difficult to use the conventional Raman spectroscopy for food samples because of florescence which hinders the analyte of interest. Phosphate in Vienna sausages was determined satisfactorily with the proposed FIA method because the percentage recoveries of the spiked sample vary between 93.0% to 99.5%. Raman spectroscopy was used successfully for the quantitative determination of phosphate in fertilisers.

11.6. Microanalysis of nickel

The FIA method was developed for the spectrophotometric determination of nickel in Vienna sausage. DMG in acetate-acetic acid buffer at pH 6.4 was used as a chromogenic reagent. DMG tend to form complexes with a lot of transitional metals, so it is very important that the method is selective for nickel. Transition metals that are likely to be present in foodstuffs were masked with EDTA and citrate which resulted in higher recoveries of nickel in Vienna sausages. The FIA results were compared with the AAS results using statistics, both methods gave practically the same results at the 95% confidence level. The proposed DMG nickel complex method is the best method for the determination of nickel in foodstuffs.

11.7. Proteins assay in milk

A new method was proposed which uses a bienzymatic amperometric sensor with and without FIA for proteins assay in milk. The results obtained for the test showed a recovery of 99.98% (without FIA) and 99.87% (with FIA), which proves that this sensor is suitable for the assay of proteins in milk. The main advantages of the proposed system are: very simple sample preparation, on-line monitoring of proteins, rapidity, simplicity, low cost. The advantage of the FIA system is the possibility of on-line monitoring of proteins in milk.

"It is the characteristic of wisdom not to do desperate things."

By: Henry David Thoreau

APPENDIX

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

APPENDIX A

PUBLICATIONS AND PRESENTATIONS

Publications

1. J.F. van Staden, M.A. Makhafola, and D. de Waal, (1996) *Applied Spectroscopy* **50**, 991-994 .

2. J.F. van Staden and M.A. Makhafola, (1996) *Fresenius J. Anal. Chem.* **356**, 70-74.

3. J.F. van Staden and M.A. Makhafola, (1999) *S.A. Journal of Chem.* **52**(1), 49-54.

Presentations

1. A study of the influence of certain factors on the spectrophotometric determination of nitrite in flow injection systems.

   M.A. Makhafola and J.F. van Staden

   Analytika 94: Stellenbosch, South Africa. 8 -13 December 1994.

2. Theoretical backround on estimation of nitrogen compounds by continuous flow systems.

   M.A. Makhafola

   University Seminars: University of Pretoria, Pretoria, South Africa. 22 August

1995.

3. Spectrophotometric determination of nitrite in foodstuffs by FIA.

M.A. Makhafola and J.F. van Staden

South African Chemical Institute 33rd. Convention: Cape Town, South Africa. 29 January - 2 February 1996.

4. Spectrophotometric determination of nitrite in foodstuffs by FIA.

M.A. Makhafola and J.F. van Staden

Colloquium Spectroscopicum Internationale XXXI: Melbourne, Australia. 20-26 September 1997.

5. Determination of heavy metals in cola drink by ICP.

M.A. Makhafola and T.W.A. Sepeng

Analytika 98: Midrand, South Africa. 11 -14 October 1998.

6. Spectrophotometric determination of phosphate in cured meat by FIA.

M.A. Makhafola and J.F. van Staden

Analytika 98: Midrand, South Africa. 11 -14 October 1998.

7. Kinetic study of the decomposition of nitrite to nitrate in acid samples using Raman spectroscopy.

M.A. Makhafola, J.F. van Staden and D. de Waal

Colloquium Spectroscopicum Internationale XXX: Ankara, Turkey 5-10 September

1999.

8. Microanalysis of nickel in Vienna sausages by spectrophotometry/FIA.

M.A. Makhafola and **J.F. van Staden**

8th International Conference on Flow Analysis: Warsaw, Poland. 25-29 June 2000.

9. A new bienzymatic amperometric sensor for protein assay in milk.

M.A. Makhafola, R.I. Stefan, and J.F. van Staden

8th. International Meeting on Chemical Sensors 2000 (IMCS 2000): Basel, Switzerland. 02-05 July 2000.

10. Determination of phosphate in cured meat and fertilisers: Comparison between FIA and Raman spectroscopy.

M.A. Makhafola and J.F. van Staden

Colloquium Spectroscopicum Internationale XXXII:Pretoria, South Africa. 8-13 July 2001.

APPENDIX B

METHOD CONSTRUCTION

B1. Introduction

One of the major developments in analytical chemistry during the last century has been the appearance of commercial automatic analytical systems, which provide analytical data with a minimum of operator intervention [1]. All the experimental work done in this project was fully or partially run by computer. The computer software used for this project is called the FlowTEK programme which was developed for flow injection sequential injection analysis by Marshall and van Staden [2]. The full detail of the operation of the FlowTEK programme is outlined in the operation manual [3]. The flow injection system consists of a peristaltic pump, injection valve and UV/VIS spectrophotometric detector. All three are controlled by computer based on the FloTEK programme. Before any experimental work could be carried out the FlowTEK programme need to be programmed for the smooth running of the flow system. The following are the steps followed to set up the FlowTEK program.

B1.1. Set-up for the detector

The UV/VIS spectrophotometry gave maximum output at zero absorbance. The detector was set-up as follows:

From the main menu select *Setup (S)*. From the *Setup* menu select *Detectors (D)*. Follow the questions as asked and answer them appropriately. The questions will be asked in the following manner:

Command	***Answer***	
Enter number of detectors	1	
Enter signal transformation for detector	1	For inversion of the signal
Enter analog input point for detector	1	Indicate the position where the detector is coupled to the distribution box.

In the distribution box, the signal from the detector was set to be amplified 100× and the baseline was set by using the adjusting screw. The direct conversion of the signal could be monitored by using the hotkey F4.

B1.2. Set-up for the pump and injection valve

The peristaltic pump and the injection valve were set-up by using a method. The method was set-up in the following manner:

From the main menu select *Method (M)*. From the *Method* menu select *Type device (T)*. Answer the following questions and commands:

Command	***Answer***	
Enter number of devices	3	The pump and the injection valve
Enter type device 1	GP	Gilson pump
Enter digital output point for GP	1	Indicates the position of connection of GP on the distribution box.

Enter type device 2	IV	Injection valve
Enter digital output point for IV	3	
Enter type device 3	IV	
Enter digital output point for IV	5	

After completing the above steps, the screen will be divided into three horizontal columns. Each column represent one of the devices entered in the method construction. There is a yellow in each of the columns, this is an indication that the Gilson pump is in the "off" position. The next step is to set-up the experimental time. Select *Exp time (E)* in the *Method* menu and answer the following questions:

Command	***Answer***
Enter time to start data collection	90
Enter experiment time	160

The next step is to deactivate the ***Num Lock*** key so that specific actions of the devices can be entered. After deactivating the Num Lock key, a small green coloured square will be flashing in the bottom right corner of the computer screen. If you hit any one of the arrow keys the cursor will appear. Use the cursor to the column of the device in question and align it with time for change of a specific event. Move the cursor to the top column (indicated GP on the right hand side of the screen. Align it with time 0 and press enter. The time is now marked. The next step is select *Insert (I)* from the *Method* menu and answer the following questions:

Command	***Answer***	
Enter event (FRO)	F	F-forward; R-reverse; O-off

Events can be deleted by activating the Num Lock key. After activating the Num Lock key t red

square will flash on the bottom right hand corner of the computer screen. The next step is to insert an event (like injection or load) by selecting the *Insert (I)* from the *Method* menu. After selecting *Insert (I)* event then answer the following questions:

Command	***Answer***	
Enter device No.	2	
Enter time of event	0	
Enter event (IL)	I	I-inject; and L-load

After the method is constructed, the next step is to save the method, this is done by choosing the *File (F)* on the *Method* menu and answer the following questions:

Question	***Answer***
Save, Retrieve or Erase (SRE)	S
Enter method file name	c:\Temp1\data\Cuopt1.met (met = method)

Once the above steps are completed the constructed method would appear on the screen as indicated in Figure B.1. The method is now ready to be used or built in a procedure.

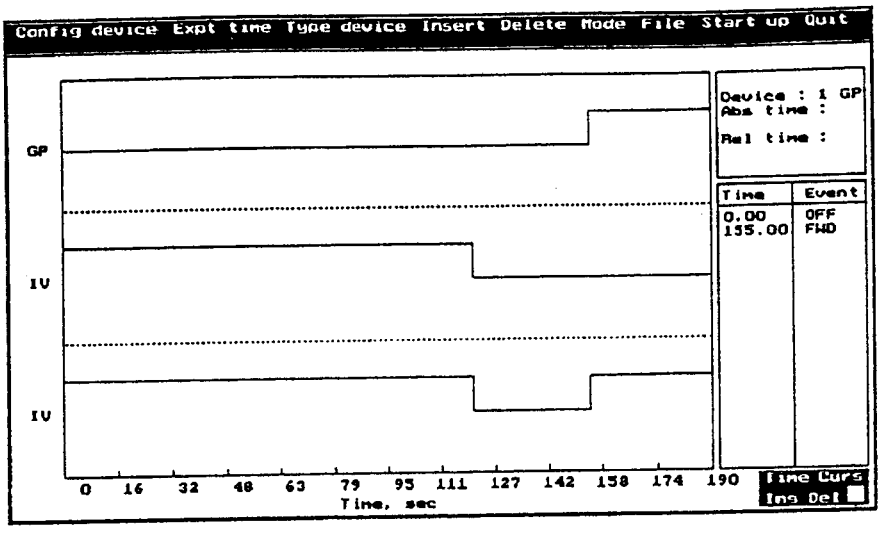

Figure B.1: Method screen after completion of a method

B1.2.1. Building a procedure

The method can be built into a procedure so that it can be used with other methods or for repetitions of the same method. The procedure is built by doing the following steps:

Return to *Main* menu then select *Repeated (R)*. From the *Repeated (R)* menu select *Build Proc (B)* and then answer the following questions:

Command	***Answer***
Enter procedure file name	c:\Temp1\data\Cuopt1.pdr (pdr = procedure)
Enter procedure or method file name	c:\Temp1\data\Cuopt1.met
Enter number of repetitions	1
Enter procedure or method file name	Wait
Enter time to wait (in sec)	60
Enter procedure or method file name	c:\Temp1\data\Cuopt1.met
Enter number of repetitions	1

Once the building procedure steps are completed the Figure B.2 will appear on the computer screen. The procedure can be built according to the number of repetitions of the cycle required.

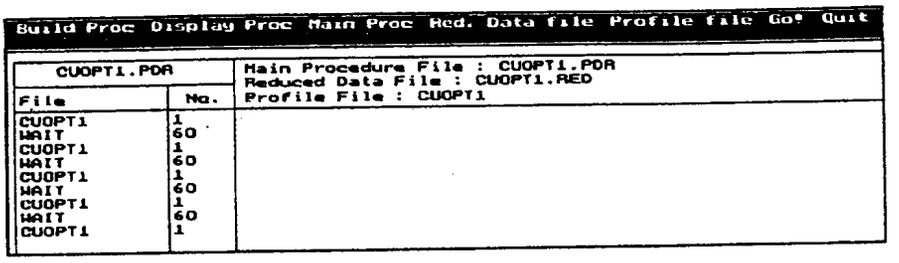

Figure B.2: Repeated screen after the building of a completed procedure

The definition of a procedure can be terminated by pressing the ***ESC*** key. After pressing the ESC key, then the procedure must be selected. The selected procedure will be used to execute the experiment. From the *Repeated* menu select the *Main Proc (M)* and answer the following questions (command):

Command	***Answer***
Enter main procedure file name	c:\Temp1\data\Cuopt1.pdr

Go back to the *Repeated* menu then select the *Red.Data file* menu . The *Red.Data file* selects a reduced data file for saving all the experimental information. Once you are through answer the following questions:

Command	Answer
Enter reduced data file name	c:\Temp1\data\Cuopt1.red

The option *Profile file* on the *Repeated* menu selects the file root name for storing the profile data. The name extension gives the experimental number. To start running the experiment select the option *Go! (G)* on the *Repeated* menu. Press **ESC** key to abort the main procedure.

B1.2.2. The calibration menu set-up

Return to the *Main* menu and then select *Calib (C)*. Select *Setup (S)* on the *Calib.* menu and then respond to the following commands:

Command	***Answer***
Enter no of calibration standards	5
Enter no of replicates for each standard	2
Enter concentration units	mg/ℓ
Now enter the different concentrations	0; 2; 4 etc.
Enter reduced data file name	c:\Temp1\data\Cuopt1.red
Press **ESC** for manual input	ESC

Select *Pk param (P)* on the *Calib.* menu and respond to the following commands:

Command	***Answer***
Enter peak parameter (HAWT)	H (H-height; A-area; W-width)
Std to edit (1-5; 0 for all; ESC)	0
Enter the calibration model (LQTEHA)	L (L-linear)

The response can also be entered manually and it is also possible to enter the response via the reduced data file. It is easier to do it manually because the program cannot discriminate against any shooters out. Save the calibration file by selecting *File (F)* on the *Calib.* menu.

Command	Answer
Save, Retrieve or Erase	S
Enter calibration file name	c:\Temp1\data\Cuopt1.cal

After the calibration set-up is completed it will appear like Figure B.3 on the computer screen.

All the device description can be viewed by selecting the *Notepad* menu from the *Main* menu. The *Notepad* menu consists of two pages. Figure B.4 shows the notepad screen for the experiment carried out.

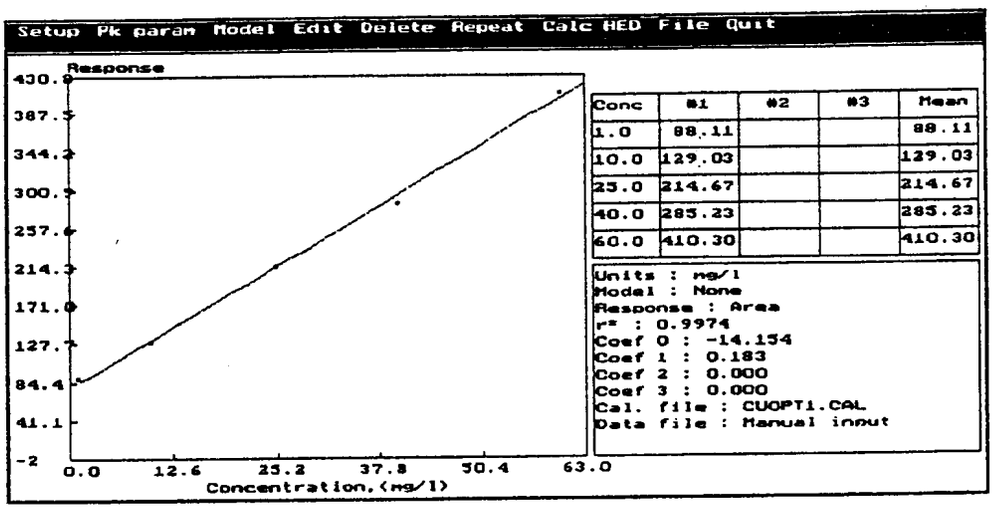

Figure B.3: Computer screen showing the calibration set up

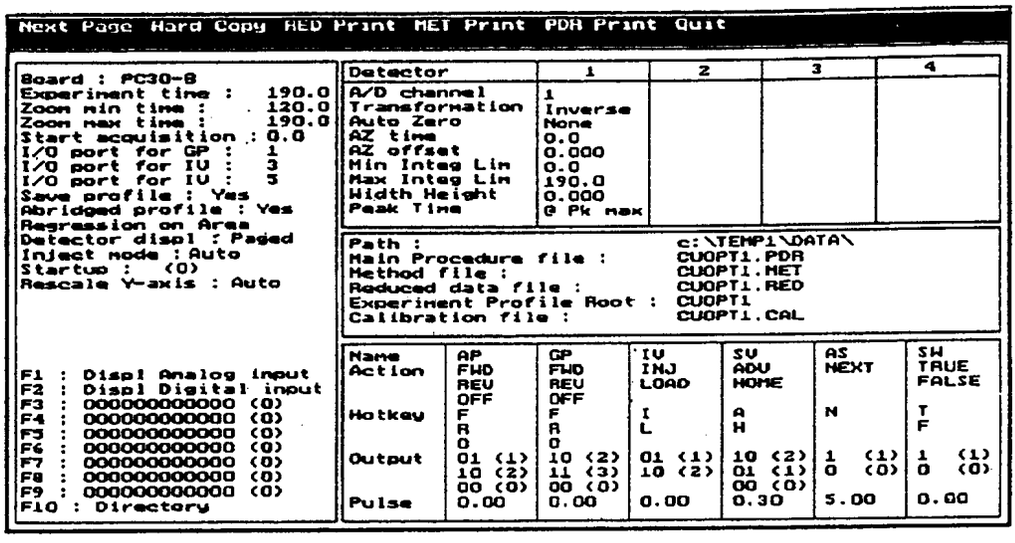

Figure B.4: Notepad screen

B.2 References

1. D.A. Skoog, F.J. Holler, and T.A. Nieman, *Principles of Instrumental Analysis*, 5th. Edition, Saunders College Publishers: New York, 1998.

2. G.D. Marshall and J.F. van Staden, **Anal. Instrum.**, **20**, 79 (1992).

3. *FlowTEK References Manual*, Device Control and Data Acquisition Software Version 1.1, Mintek, 1993.

"Science is not technology, it is not gadgetry, it is not some mysterious cult, it is not a mechanical monster. Science is an adventure of the human spirit. It is essentially an artistic enterprise, stimulated largely by curiosity, served largely by disciplined imagination, and based largely on faith in the reasonableness, order, and beauty of the universe".

BY: WARREN WEAVER