Selected applications of sequential injection analysis

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I declare that the dissertation, which I hereby submit for the degree MSc at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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SYNOPSIS

Sequential injection analysis (SIA) that was developed in 1990 is a simple, versatile and automated technique based on precise aspiration of small volumes of reagents and samples into a single channel. It is economical in terms of reagent consumption and waste generation. SIA is applied to food, environmental and pharmaceutical samples. SIA parameters and the chemical variables are optimized and figures of merit are reported. Spectrophotometer has been used as a detector in the analysis of formaldehyde (HCHO), ascorbic acid and ferric iron (Fe (III)). A stopped flow method was adapted to SIA spectrophometric technique. Enhanced sensitivity was obtained using a stopped flow method as compared to a non-stopped flow method.

The SIA spectrophotometric determination of Fe (III) through complex formation with the thiocyanate and the Tiron reagents has been studied in

chapter 3. The two methods were compared based on convenience, sensitivity, simplicity, reliability and on general application. Both methods are simple, employ readily available reagents and have lower detection limits and shorter analysis time of 72 samples per hour. The thiocyanate method enabled the determination of Fe (III) in the linear range of 2 to 50 mg/ℓ with a detection limit of 0,2 mg/ℓ whereas the Tiron method provided a linear range of 1 to 50 mg/ℓ with a detection limit of 0,1 mg/ℓ.

Chapter 4 presents the application of SIA spectrophotometric method for the determination of formaldehyde in wastewater. The method is based on the inhibition of brilliant green-sulfite reaction by formaldehyde. The kinetic method was implemented by stopping the flow when the complex reaches the detector and the measurements were taken as the reaction proceeds. The SIA method was compared with the chromotropic acid method and the paired *t*-test was used to determine whether the results obtained by the two methods differ significantly. Good sensitivity with a detection limit of 0,06 mg/ℓ and a wider linear range of 1-3 mg/ℓ was achieved.

Chapter 5 describes the indirect determination of ascorbic acid in pharmaceutical samples, sweets and beverage (orange juice). This method is based on reduction of Fe (III) to ferrous iron (Fe (II)) by ascorbic acid under UV-light irradiation and the reaction of generated Fe (II) with 1,10-phenanthroline. The flow was stopped in the holding coil for a defined time to ensure that complete reduction process take place under irradiation. Photochemical reaction methods improved the sensitivity with detection limit of 0,06 mg/ℓ. Good precision was obtained with relative standard deviation of 1,3 %. The method compared well with the N-bromosuccinimide titration method.

The versatility and simplicity of SIA technique makes it suitable to different requirements of various analytical problems.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Adequate food and water supply is a national priority for its citizens because they are our most important elements of life. Not only is the quantity needed; the quality is also of the greatest importance.

Water is regarded as pure only if the chemicals it contains are at sufficiently low concentrations not to impair human health [1]. Surface and ground water are collected and treated to provide drinking water. Pollutants from domestic, agricultural and industrial sources mostly affect quality. Some pollutants are introduced during drinking water processing [2]. The main problem with the pollutants is that they may be present at very low concentrations that need highly sensitive instruments to be detected.

Formaldehyde is of particular concern. According to the United State Environmental Protection Agency (EPA) and World health organization (WHO) it is a potential carcinogen even at low concentration levels [3,4]. Waste water from industries contains a variety of organic compounds, which have undesirable health effects if not removed before being discharged to the source water. Formaldehyde is commonly found in industrial wastewaters and often finds its way to the surface water. Without prior removal of this compound, it is impossible to produce a good quality of water from surface water because detection methods used during treatment may not be perfect to detect this compound [5]. Iron contamination is also of concern. Iron is essential for proper functioning of all living cells [1] and plays an important role in ecological system. However, it is a common problem in ground water especially in rural areas where well hand pump system is used to extract the

water. During extraction iron from hand pump tend to dissolve because causing high concentration of iron water. This kind of water results in poor taste and discoloration of clothes. Iron quantity present in food and drinking water is usually low in relation to human needs.

Food is defined as a natural product that can nourish the body and contributes to growth or physiological functions of the body. The primary constituents of foodstuff that are essential to life are water, minerals, fats, proteins, carbohydrates and vitamins. This chemical composition can cause deficiencies and toxicity when present at a certain level [6]. Vitamin C is an essential nutrient for human and it may be present in food naturally or it may be added during processing and supplied as an additional medicament to pharmaceutical preparations. Because it participates in many different biological processes accurate knowledge of its contents is needed for safety of consumers.

The effects that these analytes have on environment and human health has led to a rise in number of assays in both fields (food, pharmaceutical and environmental) especially for routine quality control analysis and to monitor them even at their lowest concentration. Fast, sensitive and economical methods have been adapted to meets this demands. Flow systems have been used for fast and automated sample manipulation and to avoid labour-intensive manual operations. Flow systems compared to manual procedures have advantages of on-line reagents addition and dilution and they are less subjected to the interferences due to less input from the operator.

Flow injection analysis (FIA) [7] has been used successfully as a laboratory techniques and a versatile tool for enhancement of instrumental analysis. This technique simplified the analysis. Several studies have been performed on the spectrophotometric determination of formaldehyde (HCHO) [8], ascorbic acid

[9] and Fe (III) [10] coupled to FIA. However, FIA has its own disadvantages: (1) reagent consumption is high due to continuous moving of the pump, (2) physical configuration of the manifold is required for each application and (3) complexity of the manifold when multi-reagents are employed.

Sequential injection analysis (SIA) introduced in 1990 [11-13] is regarded as a variant of FIA that addresses its drawbacks. It is economical (efficient use of reagent and minimization of waste), computer controlled and can be configured to perform most operations of FIA with minimal physical configuration of manifold thus making it simple and versatile by performing multitasks in a single channel. Computerized data analysis and automation of SIA make its method even more interesting.

Various detection methods for the determination of the above analytes have been used such as fluorimetry, potentiometry, flame atomic absorption spectroscopy and voltametry. Spectrophotometric technique has been widely used owing to its advantages. This technique is sensitive, fast, convenient and applicable to routine analysis. In this work spectrophotometric detector is used to monitor the coloured complexes.

In analytical problems information on the concentration of the particular component in the sample is required. But it might be difficult to detect the component maybe because it is present in complex matrix containing many potential interfering substances or because the chemical structure is not compatible with suitable means of detection. The solution to the interference problem is to allow the component of interest to take part in chemical reaction such that one of the products is easily detected. Equilibrium technique which allows the reaction to proceed to equilibrium before making measurement is more common. In contrast to equilibrium technique is kinetic/stop flow

technique in which the rate of reaction is related to the concentration of the analyte. Advantage of stop flow technique over equilibrium technique includes increased selectivity and shorter analysis time. Using SIA system reaction time can be adjusted inside and outside the detector during stop-flow period and can be increased without increasing a reaction coil and the dispersion. Stop flow method was also used in this work.

The aim of this work is to apply the principles of SIA coupled with spectrophotometer to environmental, food and pharmaceutical samples, to demonstrate the ability of SIA. Due to the simplicity with which stopped flow maybe performed with SIA, the stopped flow SIA methods were adapted in chapter 4 and 5. The selected are formaldehyde in wastewater, ascorbic acid in pharmaceutical samples, sweets and orange juice, and iron (III) in tap, mine and ground water.

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CHAPTER 2

LITERATURE SURVEY

2.1 INTRODUCTION

The appearances of commercial automatic analytical systems have been one of the major developments in analytical chemistry, especially in the industries that were faced with the workload of analysing a large number of samples. This introduction brought many advantages, replacement of highly skilled with unskilled workers; hence reduction in the cost of salary payments and also protection of the environment where an automated system can monitor the pollution level. This also saves time, where a large number of samples can be analysed with minimum intervention and replacement of manual operations.

Automatic systems have undergone many changes since their introduction from batch methods to continuous methods and the changes were brought about in order to meet the demands of industrial laboratories. In batch methods, the same manual stages (addition of reagents, dilutions, mixing and heating) take place through mechanical processes and it has been described as a mechanized version of manual operation.

It is necessary to monitor the process stream continuously in process industry and this is usually done by a process analyser. The drawbacks of manual methods were early recognised and the attention was focused on continuous analysers that eliminate the need for stepwise measurement, addition and processing of samples and reagents. Process analysers were developed from automated laboratory equipment, modified and improved to last for a long period under hazardous conditions [1].

The major breakthrough of replacement of manual operations was in 1957 [2], when Skeggs introduced the concept of segmented flow analysis (SFA), which was commercialised by Technicon Analyser. SFA is based on the segmentation of the flow by air bubbles to minimize cross contamination of adjacent samples. Samples are aspirated sequentially into the channel. Air bubbles place limitations on SFA because they cause the system to pulsate, they are compressible and they were not tolerable during the detection step. This later evolved into flow injection analysis (FIA).

FIA was assembled from simple, inexpensive and readily available components and provided a simple way of automating many wet chemical analyses. It has been accepted as both an analytical tool for serial analysis as well as an enhancement of chemical sensors. The application of FIA has been limited by the continuous use of reagents and multi-channel manifolds required for each reagent added. This complexity is not good for process analysis [3-5].

The major requirements for process analysis are simplicity, robustness and reliability. Sequential injection analysis (SIA) technique, fulfilling these requirements was designed. SIA is economical in terms of reagent consumption and waste generation and it is a convenient method by means of which sample manipulation can be automated. This analyzer can execute different operations and has been applied in fields as diverse as environmental, pharmaceutical, food and industrial processes.

2.2 SEQUENTIAL INJECTION ANALYSIS

Sequential injection analysis (SIA) is a flow methodology that became an answer to the complex chemistry and its main features are versatility, robustness and simplicity. Introduced in 1990 by Ruzicka and Marshall [6],

its popularity has grown tremendously and Hansen called it the second generation of FIA [7]. The concept of SIA is based on sequential aspiration of sample and reagent solutions into the channel hence the name sequential injection. In order to understand its origin, an overview on FIA is presented.

FIA is an analytical tool conceived in Denmark in 1975, coined jointly by both Ruzicka and Hansen [8]. Since then it has gained wide acceptance in industries and analytical laboratories at large, as a fast approach to chemical analysis. A large number of monographs and research papers have been published [9-11]. FIA is based on injecting the sample into a nonsegmented moving stream and then detecting the signal resulting from the combination of chemical and physical interaction between reagent and analyte [8,9]. FIA has three principles and the key principle is the reproducible dispersion of the injected sample zone into a moving carrier stream.

Dispersion is a parameter proposed by Ruzicka [9], which is used to characterise the passage of the sample through the system. Dispersion is defined as the dilution undergone by the sample volume injected into the flowing stream, and it is denoted by

$$D = C^0 / C^{\max} \qquad (2.1)$$

In the above formula D is the dispersion coefficient at the peak maximum produced whereas C^0/C^{max} is the ratio between concentration before (C^0) and after (C^{max}) transport through a given system.

Different sample processing such as dilutions, pre-concentration and extraction [12-15] have been integrated into this methodology. The absence of air segments in the flow stream and sample manipulation has been the success of the FIA system. FIA has proven to be useful not only in analysis but also in

solution handling. However, the increased application of process analysis has been hampered by the use of a complex manifold required. This was a major reason for the development of simple and single-line flow system called SIA.

Some of the principles of SIA are similar to those of FIA i.e. controlled dispersion and reproducible timing. The SIA manifold (Figure 2.1) includes a pump capable of moving forward and in reverse, a multi position selection valve, reaction and holding coil, pump tubes and a detector flow cell. Furthermore, the concept of SIA is based on the sequential aspiration of carrier solution, sample and reagent solution.

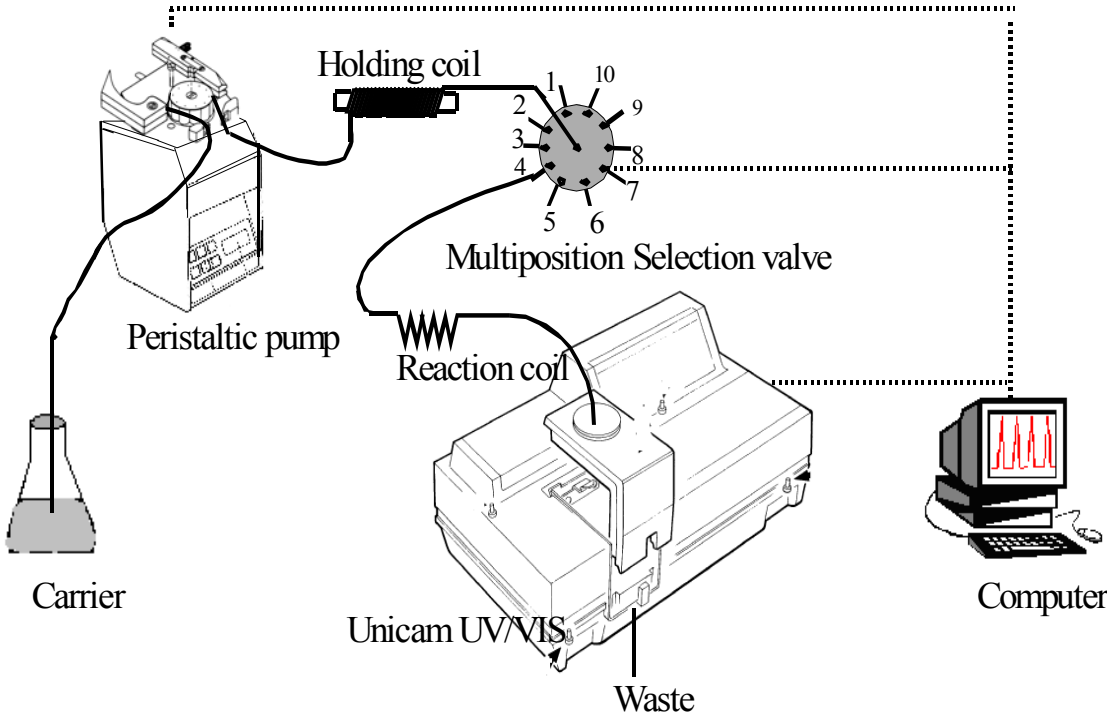

Figure 2.1 SIA System

After the steps of zone sequencing, during which the sample and reagents zones are stacked in the holding conduit adjacent to each other (Figure 2.2 a), the selection valve is switched to the port leading to the detector so that the stacked zones are propelled through the valve and reaction coil (Figure 2.2 b) into the detector producing measurable response while they mutually inter-disperse [16]

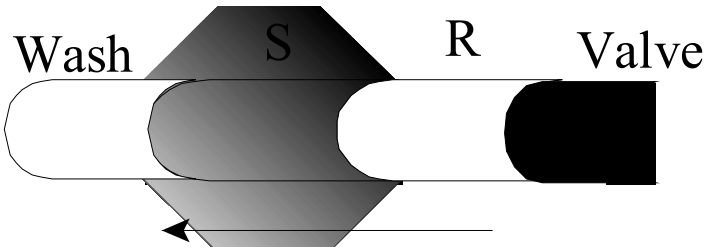

Figure 2.2a Sample (S) and reagent (R) zones stacked in holding coil

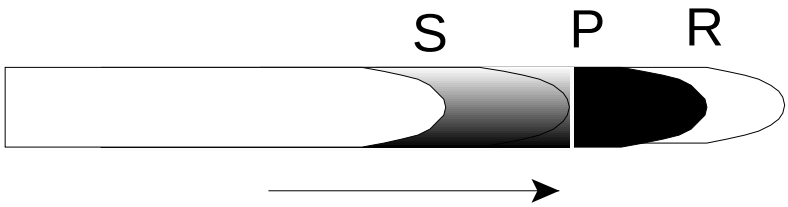

Figure 2.2b Sequenced zones of sample (S) and reagent (R) interdisperse during flow reversal forming detectable product (P)

As the central streamline in a conduit travels at twice the mean flow velocity, the zone will penetrate each other creating a complex concentration gradient (Figure 2.2 c) forming a region within which the analyte is being transformed into a detectable product.

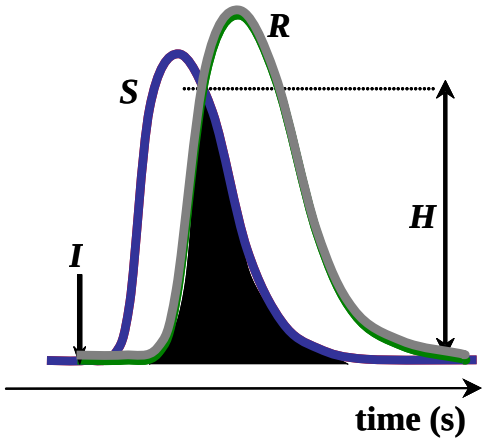

Figure 2.2 c Concentration profile as seen by the detector. S-sample, R-reagent, H-peak height and I-injection point.

Due to its simplicity and convenience with which sample manipulation can be automated, SIA has been viewed as a technique that has great potential for online measurements [17].

The potential of the SIA technique has been demonstrated by using a dye as a coloured and detectable sample [18-20]. Ruzicka *et al.* [6] tested his own theory on the assay of thiocyanate and indicated the feasibility and reproducibility of chemical analysis using the stopped flow method. Its application came as early as 1991 when Ruzicka and Gubeli [20] determined protolytic enzyme using a kinetic based method. Guzman *et al.*, [21] using fluorimetric detection determined an enzyme called factor thirteen. This enzyme plays an important role during the final stages of blood coagulation. Taylor [22] described a method for the determination of the bromine number by flow injection titration using sequential injection with sinusoidal flow.

Gubeli and co-workers [18] described the spectrophotometric and fluorescence based methods on chloride and phosphate determination and provided the guidelines for optimization of the analysis conditions. Other improvements were also considered, McCormack *et al.* [23] and Botha *et al*. [24] coupled SIA with mixing chambers, Ivaska and Ruzicka [25] compared the peristaltic and piston pumps for propelling the flow. Baron *et al*. [12] expanded the application by testing the variables such as dilutions and calibration for both single and multi zone analysis.

The success of the above-mentioned experiments depends on zone penetration (overlap) and dispersion, which are vital operations of SIA. Together they determine the sensitivity of the method and the relative signal to be recorded. They need to be adjusted to suit the intended measurements. They also promote the desired degree of penetration of samples and reagents.

2.3 ZONES PENETRATION AND DISPERSION

Proper zone sequencing leads to good zone penetration and maximum sensitivity. The concept of zone penetration is shown in Figure 2.3 and is defined by:

$$P = \frac{2W_0}{W_S + W_r} \qquad (2.2)$$

Where W_0 is the baseline width of zone overlap and W_S and W_r are the baseline width of the sample and reagent respectively. When P = 1, complete zone overlap is obtained, P = 0 zero zone overlap and partial overlap for P ranging from 0 to 1.

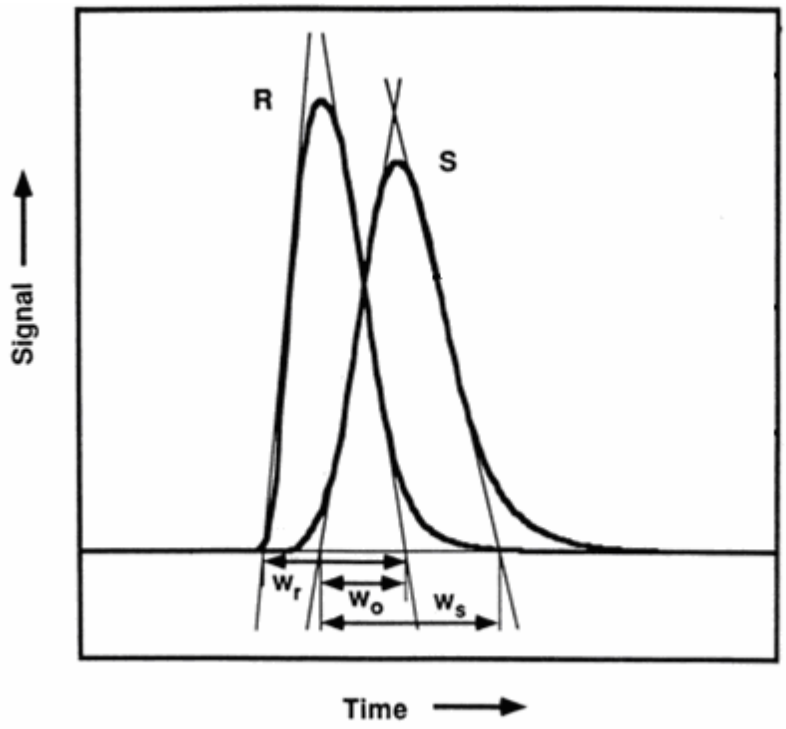

Figure 2.3 Description of zone penetration

Although zone penetration was found to be the key parameter of SIA [6], dispersion is also important as a guideline for system design [18]. Because SIA is based on dispersion its design follows the established rules for

providing limited (D=1-2), medium (D=2-10) or large (D>10) dispersion such as FIA [25].

Limited dispersion of D, ranging between 1 and 2 is achieved by using the shortest tube length L. It is used when the original sample composition needs to be measured as in conductivity measurements.

Medium dispersion, 2<D<10 is normally used for determination such as spectrophotometry, colorimetry and fluorimetry. In medium dispersion D, one or more of the reagents are employed and mixed with the sample solution in order to allow a detectable product to be formed. More importantly, sufficient mixing and time must be allowed before the desired product reaches the detector.

A large dispersion value of D>10 are obtained whenever a mixing chamber is used to dilute the highly concentrated samples. The alternative way of achieving diluted samples is by injecting small sample volumes. However some samples may be too concentrated, falling outside the detector range. When this is the case, a mixing chamber is required for dilution. Dispersion as a physical process is affected by several factors and it is described by a number of theoretical models. The overall dispersion is better described by the expression that take into account both convective and diffusion transport which contributes to the dispersion [3]. Mathematical expression is given by:

$$D\left(\frac{\partial^2 C}{\partial x^2}+\frac{\partial^2 C}{\partial r^2}+\frac{1}{r}\frac{\partial C}{\partial r}\right)=\frac{\partial C}{\partial t}+\upsilon_0\left(1-\frac{r^2}{a^2}\right)\frac{\partial C}{\partial x} \qquad (2.3)$$

The left hand term correspond to diffusion transport, where D is the dispersion coefficient, the terms with in the brackets account for axial and radial diffusion where C is a concentration that depends on r (tube radius) and

x is a tube length. The first right hand term correspond to a build up of matter and the other to convective transport where u_0 is the total tube radius and (*a*) the linear flow rate. There are several factors that affect dispersion.

2.4 FACTORS AFFECTING DISPERSION

2.4.1 Flow reversal

After aspiration steps, the valve is selected to the detector position and the flow is reversed. This flow reversal creates a composite zone in which sample and reagent zones penetrate each other due to axial and radial dispersion [27]. The unique feature of SIA is that mutual dispersion of reactants is favoured by flow reversal in the system [28]. The influence of flow reversal and length of the step was studied by several authors [6,18,29] using bromothymol blue as a dye reagent and both reported that the broadening of zones is dependant on the travelled length (L) and on the number of flow reversals (N), but it is the length of steps which causes zone penetration more effectively than N because L is proportional to zone dispersion whereas N has little influence.

Increase of L increases the intermixing of adjacent zones while increase in N promotes their homogenization [6,30]. Gubeli *et al*. and Ruzicka concluded that the first single flow reversal (necessary to flush the system) and its length L which is most effective in providing mutual zone penetration, and the use of multiple flow reversal which increases overall dispersion will be restricted for difficult solution handling tasks [18].

2.4.2 Sample and reagent volume

Sample and reagent volume strongly affect dispersion and is important in optimising the sensitivity of the system. The effect of sample volume and reagent has been demonstrated by using bromothymol blue dye (BTB). Two measurements were made. In the first instance the dye represented sample

solution and for the second measurements the dye solution represented the reagent zone. Water was used as a carrier. Gubeli [18] and van Staden [24] showed that the increase in $S_{1/2}$ ($S_{1/2}$ is the sample volume necessary to reach D^{max} =2 in any flow system irrespective of the systems geometry and flow type) value, results in the decrease in dispersion and increase in peak height and sensitivity of measurement. Ruzicka *et al* [31] indicated that changing the injected sample volume is a powerful way to change dispersion.

Gubeli [18] also showed that an increase in volume in the same proportion is not an effective way of improving sensitivity, and "injecting at least twice as large a reagent zone volume as the sample zone volume, while keeping the volume of the sample zone less than or equal to $0{,}5S_{1/2}$ allows the optimum conditions for sequential injection single reagent based chemistry to be met."

2.4.3 Reaction tube geometry

Studying the tube geometry is very important since it has an effect on dispersion and sensitivity on the application. The influence of mixing chamber reactors and tubing geometry (straight, coiled, "knitted") was studied by Marshall *et al*. [31] and van Staden [24]. The study by Marshall *et al.* shows that straight tubes results in greater axial dispersion coiled less axial dispersion and knitted gives the least axial dispersion of them all.

Unlike in FIA, where coiled (tube is coiled around a rod) and knitted (loop of tube is pulled through the existing loop forming a series/chain) reactors are preferred because they promote mixing in radial direction and reduce axial dispersion, which leads to lower sampling frequency, In SIA, axial dispersion promotes zone penetration; hence sensitivity therefore straight reactors are more favourable [31]. In van Staden's work, greater dispersion was experienced with an increase in mixing chamber volume in agreement with

the work of Ruzicka and Hansen [32] that a mixing chamber generates larger dispersion and yields lower measurement sensitivity than other channels.

2.4.4 Length and diameter of reaction coil

Inner diameter and length of reaction coil has more influence on the dispersion. Longer length results in higher residence time and increases the dispersion [28]. Marshall *et al* [31] noted that a smaller inner diameter causes back pressure and the back pressure is related to pump speed. Therefore the pump speed should be considered for the smaller inner diameter. Increase in the inner diameter decreases the axial dispersion, which is desirable by the SIA to improve zone penetration. In SIA, zones are first introduced into the holding coil, so by the time, the product zone reaches the reaction coil, it would have obtained a certain degree of dispersion. Therefore a smaller length and inner diameter are needed to minimize dispersion [24].

2.4.5 Flow rate

Various researchers have previously studied the influence of the flow rate on dispersion [24,28 and 31]. Marshall *et al.* [31] indicated that the flow rate is proportional to pump speed; therefore, changing the pump speed can cause the changes in the flow rate to vary. With the use of the sinusoidal flow pump in their studies, they found that a wide range of flow rates was obtained at a fixed pump speed. Van Staden *et al.* [24] used a peristaltic pump and reported that by changing the pump speed and pump tubing inner diameter (i.d.) the flow rate can be regulated. All the previously mentioned researchers came to one conclusion, namely that an increase in flow rate, decreases the dispersion thus causing an increase in peak height. But Cladera *et al.* [28] noted that the higher flow rates could hinder the correct identification of the peak maximum if the peak is too narrow at the maximum acquisition point.

2.5 ADVANTAGES AND DISADVANTAGES OF SIA

SIA offers very different possibilities with a series of advantages and disadvantages in relation to FIA. The manifold is more simple and universal. The major advantage of SIA is its ability to perform numerous complex chemistries coupled with various modes of detection without reconfiguration of the flow manifold [6]. In FIA, the peristaltic pump is usually permanently in motion, the analytical measurements being carried out or not, giving rise to excessive reagent consumption, whereas in SIA the system only performs when measurements are required, thus the reduction in consumption of both samples and reagents is high in relation to FIA [33].

Regardless of the number of solutions (sample, reagent, standards and wash solution) SIA uses only a single valve, a single pump and a single carrier stream hence a simple and robust technique [20]. An essential component in the operation of SIA is a computer fitted with an interface board /card and software for the control of the timing and the operation of the pump and valve as well as data acquisition [5]. SIA has proved to be the technique, which can be designed to operate in a multi-parametric way that is of special interest when considering the design of environmental monitors [33].

Another important advantage of SIA is the versatility that the multi-position valve provides [12,21]. Each port is dedicated to a specific purpose and the combination of sample, standards, reagent and detector around the valve is easily modified to suit a particular analysis.

In spite of these advantages, the SIA system presents a series of disadvantages if compared with the FIA system, analysis frequency being the most important. SIA aspiration of solutions into holding coil takes time, and then sampling frequency is halved compared to the conventional FI system.

Secondly, the control of the whole SIA system by incorporated computer is compulsory since the sequencing, injection and data acquisition depends on it [18,33]. However, the positive aspect of the technique makes it an attractive tool for the development of automated analyses.

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CHAPTER 3

COMPARISON OF TIRON AND THIOCYANATE METHODS FOR THE DETERMINATION OF IRON (III)

3.1 INTRODUCTION

Iron is the second most abundant metal and fourth abundant element in earth's crust. It is essential for proper functioning of all living cells [1]. All organisms in many proteins and other types of organic molecules use iron because it can readily exist in two ionic forms, iron (II) and iron (III) [2]. In humans, iron is a vital component of proteins which functions as oxygen carrier in haemoglobin. The ability of iron atom to bind oxygen, as O_2, makes it valuable. Iron deficiency can occur due to lack of iron in human diet and can lead to anaemia. Iron deficiency is common in young children and women [1,3-6]. Excessive amounts are toxic therefore its monitoring is very important.

The bioavailability of iron from diet depends from the sources, which can be food, water and medication (added as supplement). Iron is an active participant in aquatic redox processes namely, in oxidation of organic matter, water bearing rocks and soil, surface water (at pH<6), at oxic-anodic boundaries [7]. It occurs in natural water at extremely low concentration and this is due to its strong tendency to hydrolyze in aqueous solutions and form relatively insoluble hydrous iron oxide [1]. Iron is a problem in ground water because its level ranges from 0 to 50 mg/ℓ whereas World Health Organization (WHO) recommended less than 0,3 mg/ℓ. The level higher than 0,3 mg/ℓ, have unpleasant taste which is apparent in drinking water. An iron limit in drinking water is based on aesthetic parameter rather than toxicity [8].

3.2 ANALYTICAL METHOD

Because iron occurs at extremely low concentration in natural waters, there is a need for more sensitive analytical method. Several authors using different methods and techniques have studied its determination in different oxidation states.

Chromatographic methods used for the separation of iron (III) are high performance liquid chromatography (HPLC) [9-11], thin layer chromatography (TLC) [12] and column chromatography [13]. Atomic absorption spectrometry (AAS) has been used in combination with SIA. Rubi *et al.* [14] used pre-concentration step of iron on micro column packed with chelating resin (Chelex 100) whereas de Compos Costa *et al.* [15] used extraction process. The above, methods have lower reproducibility, involves number of steps and the elution of sorbed iron is difficult [14,16].

Few fluorimetric methods have been applied for Fe (III) determination and most of those reported are based on fluorescent quenching [17-20]. This is due to the fact that iron does not usually form fluorescent chelates [17]. Cha *et al* proposed the fluorimetric method for Fe (III) determination in the presence of surfactants [20]. Electro analytical techniques, e.g. voltammetry [21-23], potentiometry [24,25], and polarography [26,27] are employed for determination of Fe (III). Most of these methods involve simultaneous determination and extraction method. The methods worked well but are time consuming and need much effort.

Spectrophotometric methods have been used to determine Fe (III) directly and indirectly. Fe (III) is measured indirectly by two steps. Firstly Fe (II) is complexed with the reagent and the absorbance is measured. In the next step Fe (III) is reduced to total iron followed by absorbance measurement. After

the two step measurements, Fe (III) concentration is calculated from the difference between measured values of Fe (II) and total iron complexes [17,28-31]. In direct method Fe (III) is reacted with the reagent then Fe (II) present is oxidized to total iron for measurement. Some reagents that have been employed for the direct determination of Fe (III) are shown in Table 3.1 [7,32-42].

Table 3.1 Reagents used for direct determination of iron (III)

Reagents	Reference
Squaric acid	[32]
2 Carboethoxy-1,3-indandione (CEIDNa)	[33]
Pyrogallol	[34]
Thioglycolic acid	[35]
Tiron	[36-39]
Thiocyanate	[7,40-42]

Lynch [43] and Senior *et al*. [44] described the method that uses two reagents in the same manifold for iron (III) and iron (II) determination simultaneously. Iron (III) and iron (II) were determined using thiocyanate together with 1,10-phenanthroline [43] and acetohydroxamic together with 1,10-phenanthroline [44]. But both methods are complicated by number of chemical operations involved. The methods are limited when the ratio of iron in two oxidation states is almost the same as it makes it difficult to calculate the second oxidation state [22].

Thiocyanate and Tiron methods have been used widely [7,36-42], improved by incorporating ion exchange resin and immobilizing the reagent [36,41] and using differential kinetic methods [36]. Differential kinetic methods provided poor selectivity when applied to real samples whereas immobilizations of reagents waste time. All Tiron and thiocyanate methods have been reported to

be sensitive with good detection limit and wide linear range but the results are based on different samples and techniques used.

The aim of this work was to compare the existing Tiron [42] and thiocyanate methods [40]. The methods have been compared for Fe (III) determination in different water samples with respect to convenience, simplicity and general application. SIA offers many advantages such as automatic control of reaction condition and manipulation of sample and reagents. This technique in conjunction with spectrophotometry was used.

3.3 EXPERIMENTAL

3.3.1 *Apparatus*

SIA manifold (Figure 3.1) was constructed from the following components:

A holding coil made of 500 cm × 0,89 mm id PTFE tubing. Teflon tubing served as connectors between tubes and the system. A Unicam 8625 UV-visible spectrophotometer (Cambridge, UK) equipped with cell holder and flow through cell (10mm) from Hellma (Mulheim/Baden, Germany) was used as a detector. The wavelength was set at 480 nm and 667 nm for the thiocyanate and the Tiron method respectively. A Valco 10 port selection valve (Houston, Texas) was used to select ports. Peristaltic pump (Gilson, Velliers-le-bel, France) pumping at the flow rate of 15 mℓ/min. Computer equipped with FlowTEK software for data acquisition and system control. The data obtained was automatically converted to the final response versus time profile (Figure 3.2) and maximum peak height measurement was used for evaluation. For replicates, obtained peak heights were averaged to give mean peak height.

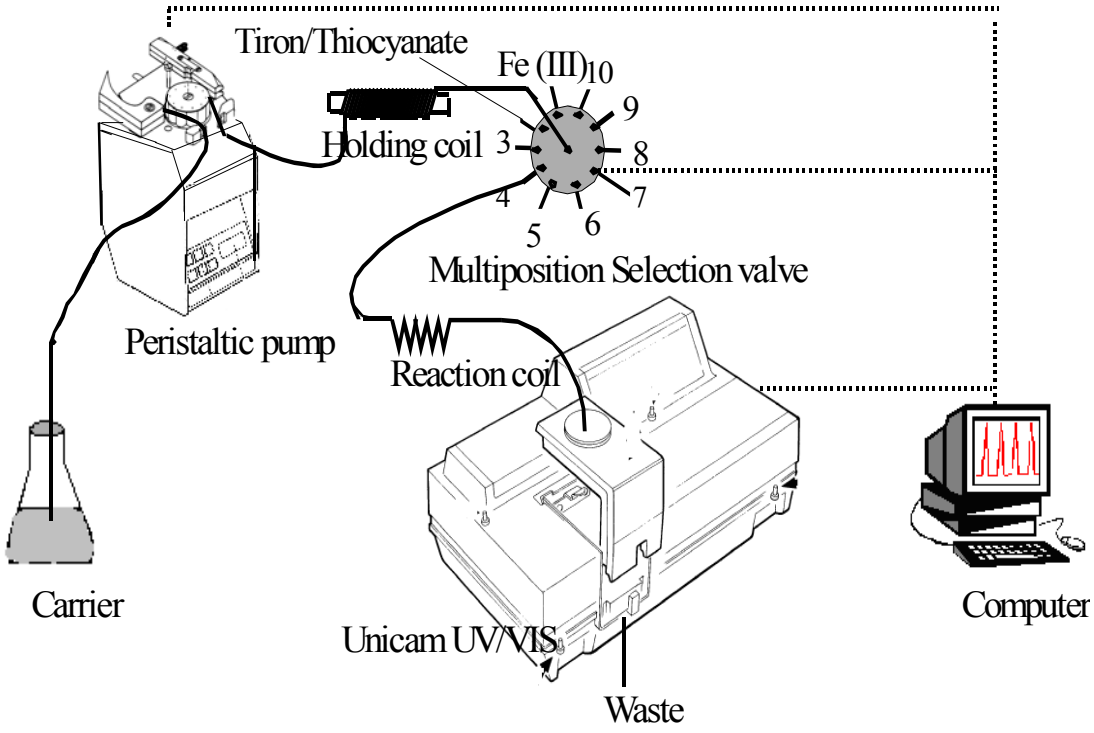

Figure 3.1 SIA manifold used for the experiment. Only thiocyanate is aspirated from port 1 in thiocyanate method and only Tiron in the Tiron method.

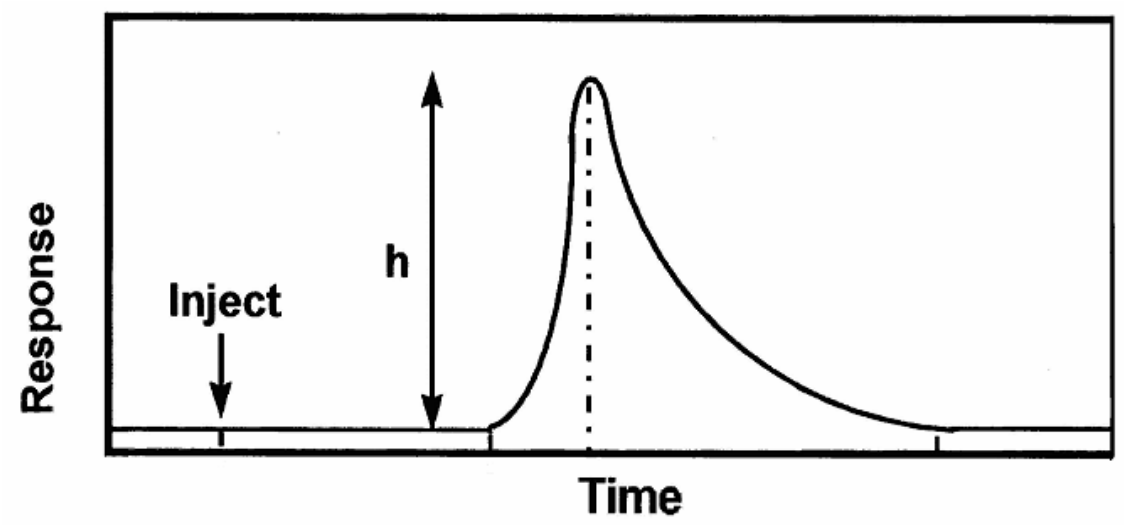

Figure 3.2 SIA signal used for data evaluation. (h - Peak height)

3.3.2 *Reagents and solutions*

All chemicals were of analytical reagent grade unless otherwise specified. The de-ionized water from Modulab systems (Continental water systems, San Antonio, Texas) was used throughout.

Stock solutions:

Iron (III) solution (1000 mg/ℓ)

7,3 g of Fe $(NO_3)_3.9H_2O$ (Aldrich) was dissolved in 0,02 mol/ℓ nitric acid for thiocyanate method and in 0,03 mol/ℓ perchloric acid for Tiron method.

Tiron solution (0,1 mol/ℓ)

3,32 g of Tiron (Merck) were dissolved in 100mℓ of water.

Thiocyanate solution (0,1 mol/ℓ)

0,76 g of ammonium thiocyanate (Protea) was dissolved in 100 mℓ of water.

Standards solutions:

Tiron and thiocyanate working standards solutions were prepared by dilution of stock solutions in water. Fe (III) working standards solutions were prepared by dilution of stock solutions in perchloric ($HCLO_4$) and nitric (HNO_3) acid for the Tiron and thiocyanate method respectively.

Other solutions:

Nitric acid (0,02 mol/ℓ) was prepared from 0,1 mol/ℓ HNO_3 (ACE) prepared in water.

Perchloric acid (0,03 mol/ℓ) was prepared from 0,1 mol/ℓ $HCLO_4$ (Merck) prepared in water.

Sample treatment

Samples were stored in polyethylene bottles until the analysis. Three different types of water (tap, mine and ground) used were filtered and were diluted to the desired volumes with nitric acid and perchloric acid solutions for thiocyanate and Tiron method respectively.

3.3.3 *Experimental Procedure*

Table 3.2 shows the flow procedure for the determination of iron (III) in water samples for both methods.

Thiocyanate method

SIA system was cleaned and filled with 0,02 mol/ℓ nitric acid as carrier solution. Standard solution of iron (III) (port 1) was aspirated into the holding coil, followed by thiocyanate reagent (port 2). The stacked zones were propelled forward through flow cell detector creating a detectable complex.

Tiron method

SIA system was cleaned and filled with 0,03 mol/ℓ perchloric acid as carrier solution. Standard solution of iron (III) (port 1) was aspirated into the holding coil, followed by Tiron reagent (port 2). The stacked zones were propelled forward through flow cell detector creating a detectable complex.

Table 3.3 SIA flow procedure used for both experiments.

Time (s)		Pump	Valve
Thiocyanate	Tiron		
0	0	Off	Home-select the sample solution
2	2	Reverse draw up the sample solution	
10	5	Off	
11	6		Advance-select reagent solution
12	7	Reverse draw up the reagent solution	
16	10	Off	
17	11		Advance-select the detector line
18	12	Forward	
33	33	Off	
40	40		Home-returns to position 1

3.4 RESULTS AND DISCUSION

Fe (III) reacts with Tiron and thiocyanate reagent to form blue and red coloured complex respectively. The colour is detected by UV-visible spectrophotometer and the signal is recorded in the form of peak. Marshall *et al.* [45] noted that good precision can be obtained if peak height is measured instead of peak width and area which give poorer precision.

Optimization of instrumental parameters is necessary to obtain good response from the system. In order to optimize the system the influence of parameters such as flow rate, inner diameter and length of reaction coil, sample and reagent volume, reagents concentrations and pH on peak height and accuracy was investigated. Parameters were optimized by altering each variable while keeping the other constant. The plots of optimized parameters are presented as mean peak height versus optimized parameters.

3.4.1 *Optimization*

A. Thiocyanate method

Physical parameters

Flow rate: Flow rate is proportional to pump speed therefore changing the pump speed of the system changes the flow rate of the reagents hence the volumes. The volume (mℓ) pumped into the system (for 60 seconds) was measured by pumping water from one end of pump tube and collecting from the other end in measuring cylinder. The volume of liquid collected at that time gives the flow rate at mℓ/min. The process was repeated for different pump speed in revolutions per minute (rpm) giving different flow rates. Table 3.3 shows different pump speed and corresponding flow rates.

Table 3.3 Pump speed with corresponding flow rates

Pump speed (rpm)	Flow rate (mℓ/min)
5	0,7
10	1,5
15	2
20	3
25	3,5
30	4,3
35	5
40	6
45	6,4

Flow rate at which the sample/reagent zones are dispersed to the flow cell has an effect on the magnitude of the signal. The influence of flow rate on mean peak height was varied from 0,6 to 3,5 mℓ/min. In order to keep reagent and sample volumes constant while changing the flow rate the aspiration times were altered accordingly. Flow rates beyond 3,5 mℓ/min were not tested because at very high flow rates back pressure is experienced and reaction time will be insufficient for colour development.

Results are shown in Table A3.1 and Figure 3.2. Results indicate that the mean peak height increased by double with increasing flow rate up to 3,5 ml/min but no significant difference was observed between 3,5 and 3,0 mℓ/min. Although higher sampling time was obtained at 3,5 mℓ/min, poorer precision was also observed. Then 3,0 mℓ/min was chosen to further the experiment as a compromise between sampling time and precision.

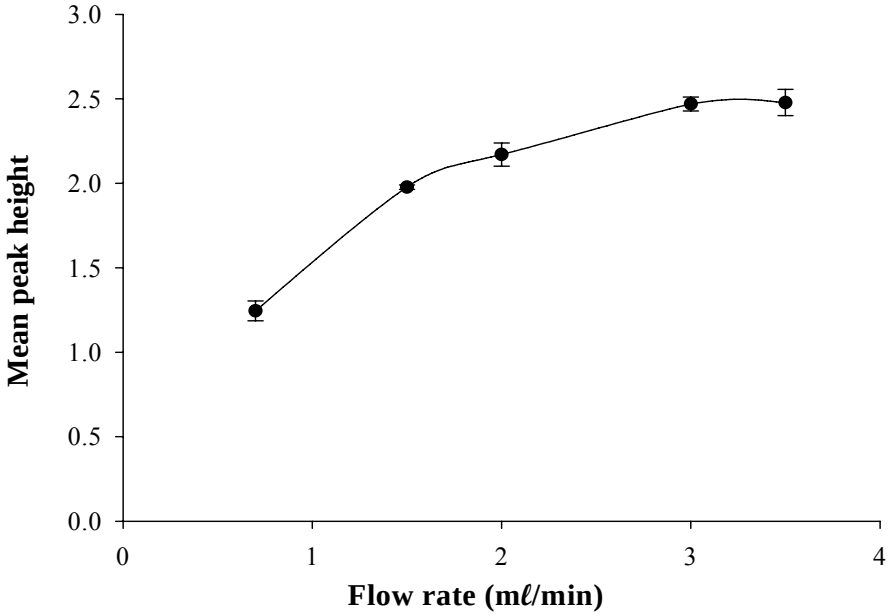

Figure 3.2 Effect of flow rate on peak height

Conditions: Fe (III), 20 mg/ℓ; Thiocyanate, 0,2 mol/ℓ; Nitric acid concentration, 0,02 mol/ℓ

Coil length: Reaction coil length affects the dispersion of zones and influences sensitivity of the method. Long coils lead to loss of sensitivity as the reaction reaches completion before reaching the detection point and reduces the sampling time. Therefore sensitivity is expected to decrease as the coil length increases. Reaction coil length was optimized ranging from 45 to 110 cm, Figure 3.3 and Table A3.2 shows the results.

The formation of Fe (III)-thiocyanate complex is very fast so the reaction coil need be as short as possible. The sensitivity increased with increasing coil length from 45 cm to 50 cm, further increase in coil length led to the reduction in sensitivity due to increase in dispersion of the complex. Lower peak heights were observed for 45 cm coil probably due to incomplete mixing of reagent and sample zones since the coil was very short. 50 cm with inner diameter (i.d.) of 0,76 mm was chosen as optimum.

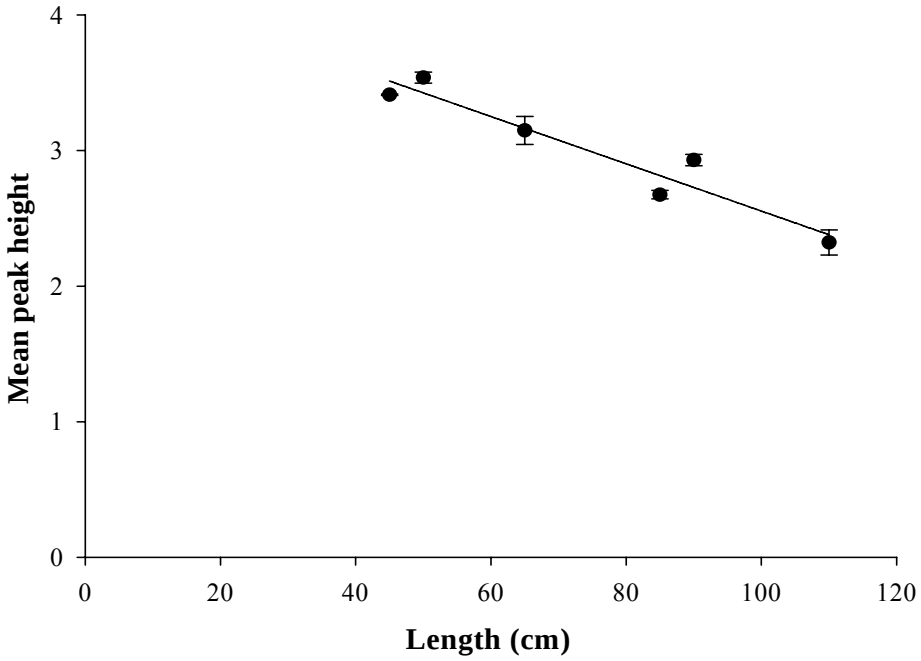

Figure 3.3 Effect of reaction coil length on peak height

Conditions: Fe (III), 20 mg/ℓ; Thiocyanate, 0,2 mol/ℓ; Nitric acid concentration, 0,02 mol/ℓ

Volume: Sample and reagent volumes have effect on the degree of zones penetration (overlap) hence the sensitivity. Using optimized flow rate of 3,0 mℓ/min and 50 cm reaction coil the effect of sample and reagent volume was evaluated between 50 to 400 µℓ. Aspiration time was altered according to give the desired volumes. 400 µℓ of sample and 200 µℓ of reagent were the optimum found, Figure 3.4 and Table A3.3 and A3.4 shows the results. Reagent volume was optimized before sample therefore 400 µℓ of sample was the highest volume tested to avoid incomplete reaction with the reagent.

Theory indicates that reagent volume should be greater than sample volume to ensure complete reaction as the zones travel through the reaction coil [46] but it is not the case in this work. Higher sample volume compared to the reagent volume gave higher peak height probably due to highly concentrated reagent used as compared to low concentrated Fe (III). To ensure complete reaction

between sample and reagent, equal volumes were used in subsequent experiments and still the objective of reducing reagent was accomplished.

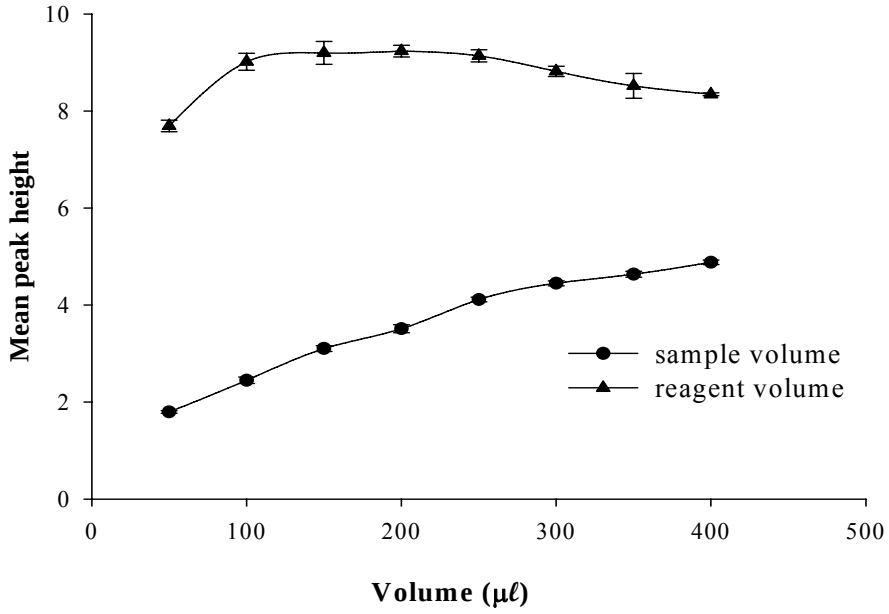

Figure 3.4 Effect of iron (III) and thiocyanate reagent volume on peak height

Conditions: Fe (III), 20 mg/ℓ; Thiocyanate, 0,2 mol/ℓ; Nitric acid concentration, 0, 02 mol/ℓ

Chemical variables

Nitric Acid concentration: The formation of iron (III) thiocyanate complex is dependent on acidic medium. Acidification is important to prevent iron hydrolysis. To ensure stability of the complex throughout, dilute acidic solution was used as a carrier instead of water. Mostly hydrochloric acid (HCl), nitric acid (HNO_3), sulphuric acid (H_2SO_4) or perchloric acid is used however nitric acid was chosen because it minimizes the fading of the colored [Fe (III)-SCN]$^{2+}$ complex [47]. Examining the effect of HNO_3 concentration as carrier the response of the complex was measured ranging the concentration from 0,05-0,4 mol/ℓ, Figure 3.5 and Table A3.5. Results show

slow increase in mean peak height with increasing concentration up to 0,2 mol/ℓ and slow decrease beyond. Large error was observed in the results. Then 0,2 mol/ℓ was used for the experiment as it shows highest response..

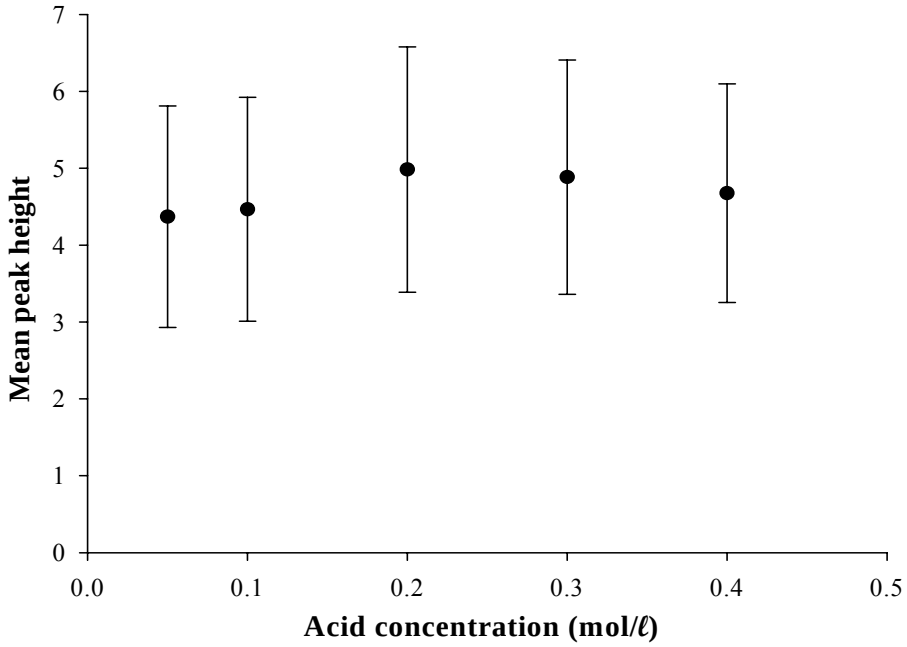

Figure 3.5 The effect of acid concentration on peak height

Conditions: Fe (III), 20 mg/ℓ; Thiocyanate, 0, 2 mol/ℓ

Reagent concentration: The influence of thiocyanate concentration was examined over the concentration range of 0,06 - 0,20 mol/ℓ, Figure 3.6 and Table A3.6. Fluctuation of results was observed from 0,06-0,14 mol/ℓ with slow tendency of decrease and increase with small difference. The concentration of 0,18 mol/ℓ showed the highest response. Mean peak height decreased with the concentration of thiocyanate reagent beyond 0,18 mol/ℓ.

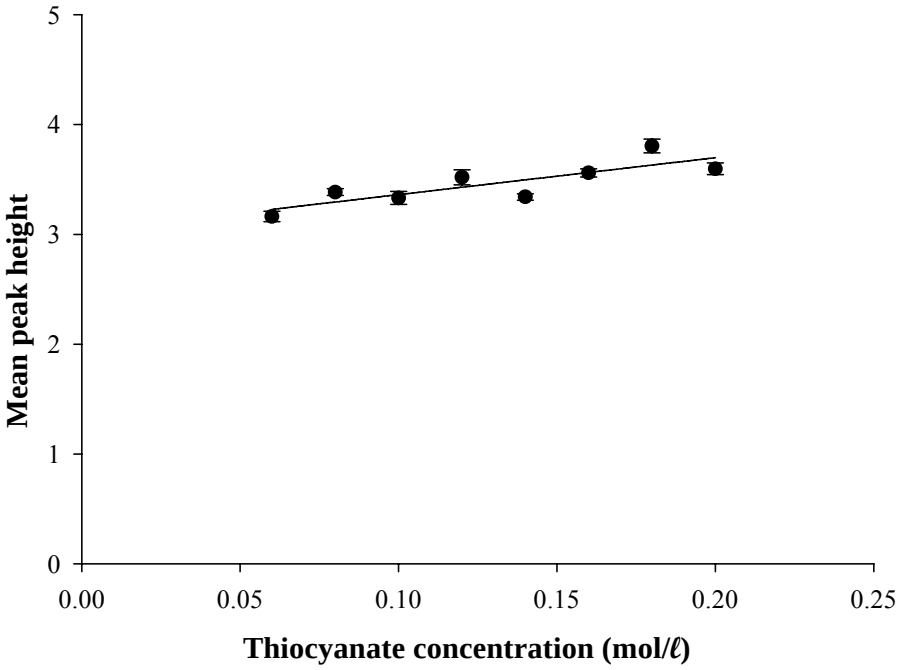

Figure 3.6 Effect of thiocyanate concentration on peak height

Conditions: Fe (III), 20 mg/ℓ; Nitric acid concentration, 0, 2 mol/ℓ

B. Tiron method

Physical variables

Flow rate: The sensitivity of SIA depends mainly on the dispersion of the flow system and the penetration of sample and reagent zone. The influence of flow rates of carrier and the reagents was varied from 1,5 to 6 mℓ/min and results are in Table A3.7. Since the reaction is fast, higher flow rates are expected to give maximum response. Figure 3.7 shows results where mean peak height increased with increasing flow rate and gave constant response from 5mℓ/min.

Higher flow rate minimized the dispersion and increased sensitivity and sampling rate. For too high flow rates complete complex formation and reproducibility of the method could be limited because very high flow rates are associated with back-pressure. Since the aspiration extend sampling cycle,

it is desirable to use highest flow rate that gives an acceptable precision in volumes aspirated. Maximum of 4,3 mℓ/min was chosen to further the experiment.

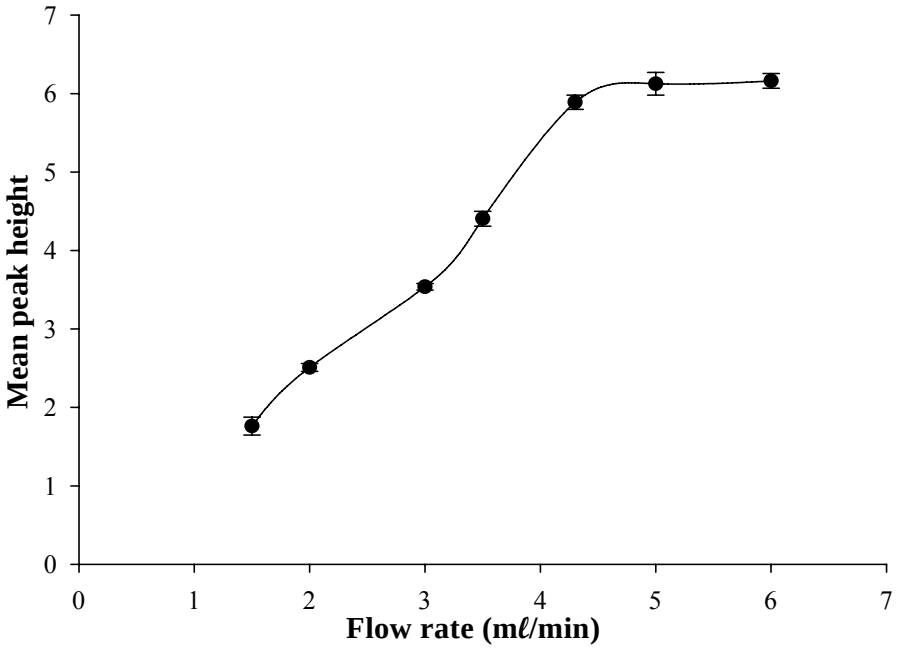

Figure 3.7 Effect of flow rate on peak height

Conditions: Fe (III), 20 mg/ℓ; Tiron, 0,02 mol/ℓ; Perchloric acid ($HCLO_4$) concentration, 0,03 mol/ℓ

Coil length: Reaction coil length was optimized ranging from 35 to 90 cm, Table A3.8 and Figure 3.8. Fe (III)-Tiron reaction is very fast and as expected higher peak heights were obtained with short coil length of 45 cm, due to less dispersion experienced by the complex. Longer reaction coils increased the residence time of the complex, increasing the dispersion and hence decrease in peak heights. Straight tube was used because it promotes axial dispersion in SIA system, hence increase in sensitivity. For inner diameter the optimization was varied from 0,68 to 0,86 mm id. 0,64 mm id provided maximum peak.

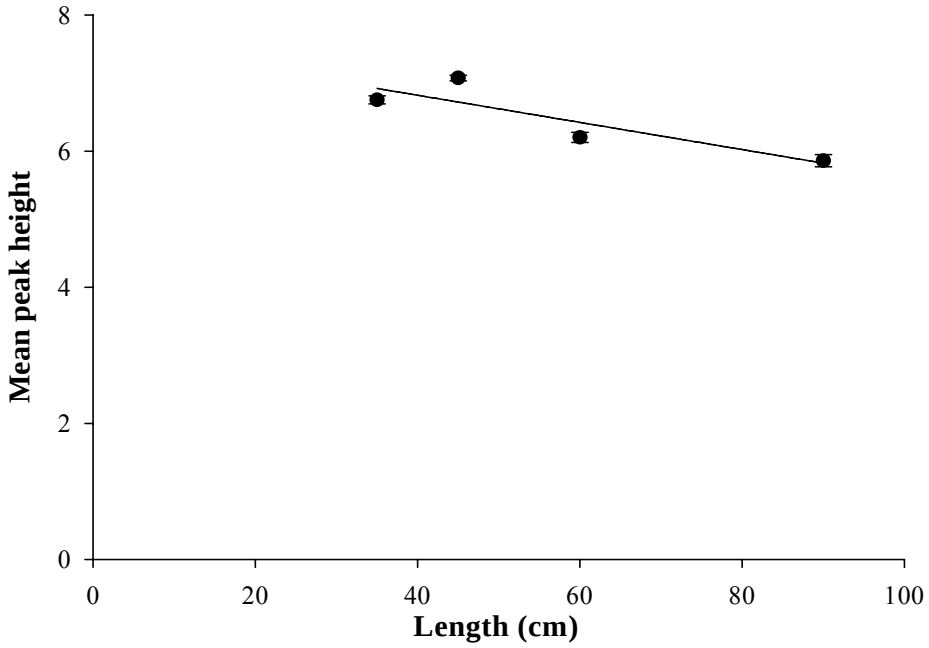

Figure 3.8 Effect of reaction coil length on peak height

Conditions: Fe (III), 20 mg/ℓ; Tiron, 0,02 mol/ℓ, $HCLO_4$ concentration, 0,03 mol/ℓ

Volume: The effect of sample and reagent volume was evaluated between 50 to 200 µℓ. Aspiration time was altered according to give the desired volumes. Simultaneous increase in sample and reagent volume is not an effective way to increase peak height and sensitivity of measurement [46]. Therefore, one of the volumes was optimized while keeping the other constant. Increase in both sample and reagent volume (not simultaneously) influences increase in peak height. With higher volumes (above150 µℓ) signals decreased owing to low overlap of zones and increased dispersion. The Fe (III)-Tiron complex gave optimum response for the volume of 150 µℓ for both sample and reagent. Figure 3.9 and Table A3.9 and A3.10 shows the results.

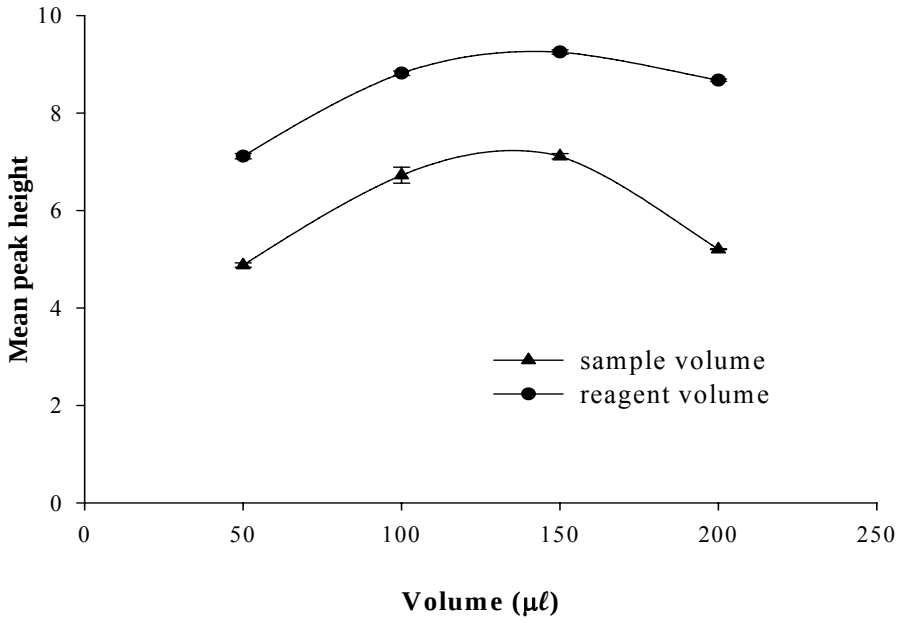

Figure 3.9 Effect of iron (III) and Tiron reagent volume on peak height

Conditions: Fe (III), 20 mg/ℓ; Tiron, 0,02 mol/ℓ, $HCLO_4$ concentration, 0,03 mol/ℓ

Chemical variables

Acid concentration: The effect of acid concentration on the complex formation was investigated. As compared to other acids perchloric acid suited the method because it darkens the colour of the complex [47] making it easy to detect. The acid concentration was varied from 0,01-0,09 mol/ℓ as shown in Table A3.11 and Figure 3.10. In strong acidic media (pH <5,6) Fe (III) react with Tiron in molar ration of 1:1 forming blue coloured complex. 0,01 mol/ℓ and 0,03 mol/ℓ has highest peak, but 0,03 mol/ℓ was chosen as optimum to ensure more acidic solution.

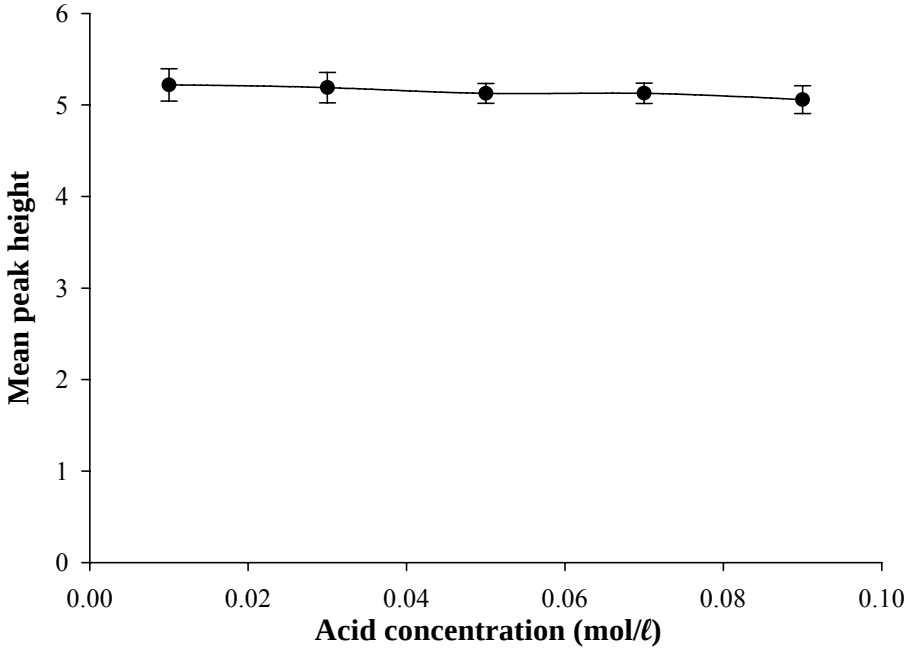

Figure 3.10 Effect of perchloric acid on peak height

Conditions: Fe (III), 20 mg/ℓ; Tiron, 0,02 mol/ℓ

Reagent concentration: The reagent concentration was tested from 0,001-0,003 mol/ℓ, the signal increased with the increase in concentration up to 0,0025 mol/ℓ and beyond that the concentration increased but the peak shape was broadened. Therefore, 0,0025 mol/ℓ was chosen as the optimum. Results are shown in Figure 3.11 and Table A3.12.

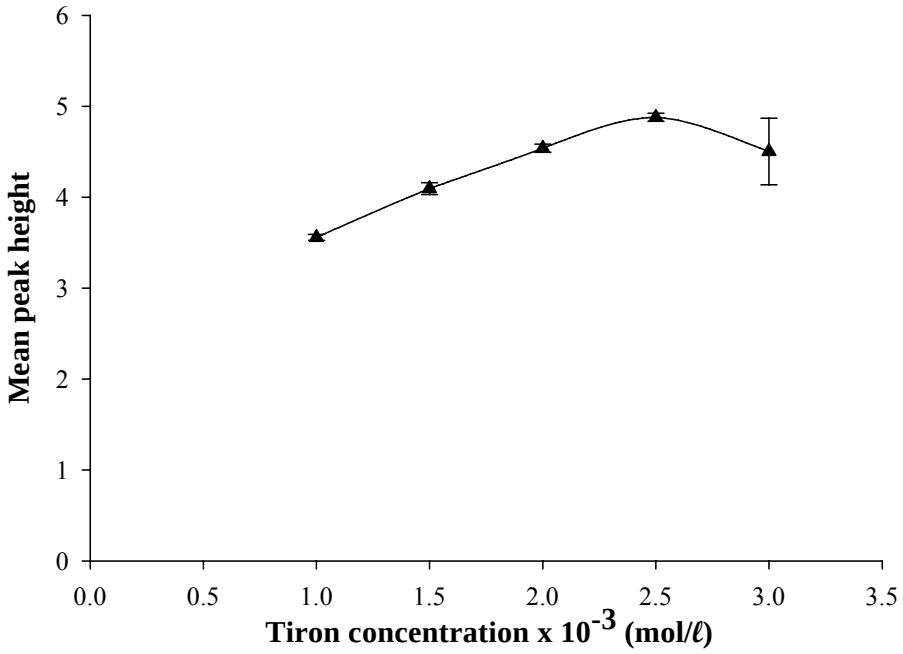

Figure 3.11 Effect of Tiron concentration on peak height

Conditions: Fe (III), 20 mg/ℓ; Acid concentration, 0,03 mol/ℓ

3.4.2 *Method evaluation*

Linearity and detection limit (DL)

Using SIA apparatus in Figure 3.1 calibration graphs (Figure 3.12-thiocyanate and 3.13-Tiron) were obtained under the optimized conditions shown in Table 3.4. Calibration was performed in the range of 1 to 50 mg/ℓ for the Tiron method and 2 to 50 mg/ℓ for the thiocyanate method.

Calibration curve for the thiocyanate method:

Y =0,1730 [Fe (III)] +0,6046 (r^2 = 0,997),

and for the Tiron method:

Y =0,1747 [Fe (III)] + 0,5262 (r^2 = 0,9979)

Y represents peak height. Detection limit of the method refers to lowest concentration that can be measured using the proposed method. Detection limit was calculated based on equation 3.1 [48]:

$$DL = \frac{(3\delta + K)(K - C)}{m} \qquad (3.1)$$

Where δ is the relative standard deviation of the blank, K is the average signal of the blank, m the slope and c the intercept of the calibration graph. DL obtained is 0,2 and 0,1 mg/ℓ for thiocyanate and Tiron method respectively.

Table 3.4 Optimum conditions used for evaluation

Parameter	Optimum value	
	Tiron	Thiocyanate
Flow rate (mℓ/min)	4,3	3,0
Reaction coil length (cm)	45	50
Acid concentration (mol/ℓ)	0,03	0,2
Sample volume (µℓ)	150	200
Reagent volume (µℓ)	150	200
Reagent concentration (mol/ℓ)	0,0025	0,18

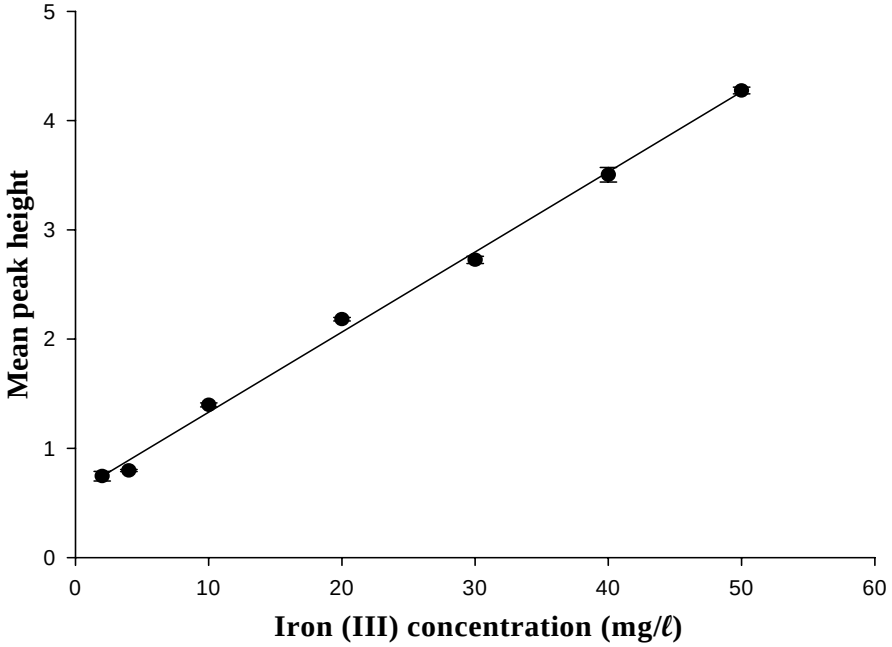

Figure 3.12 Linear curve of standard solutions of iron (III) under optimum experimental conditions using thiocyanate method.

Conditions: Thiocyanate, 0,18 mol/ℓ; Nitric acid concentration, 0,2 mol/ℓ

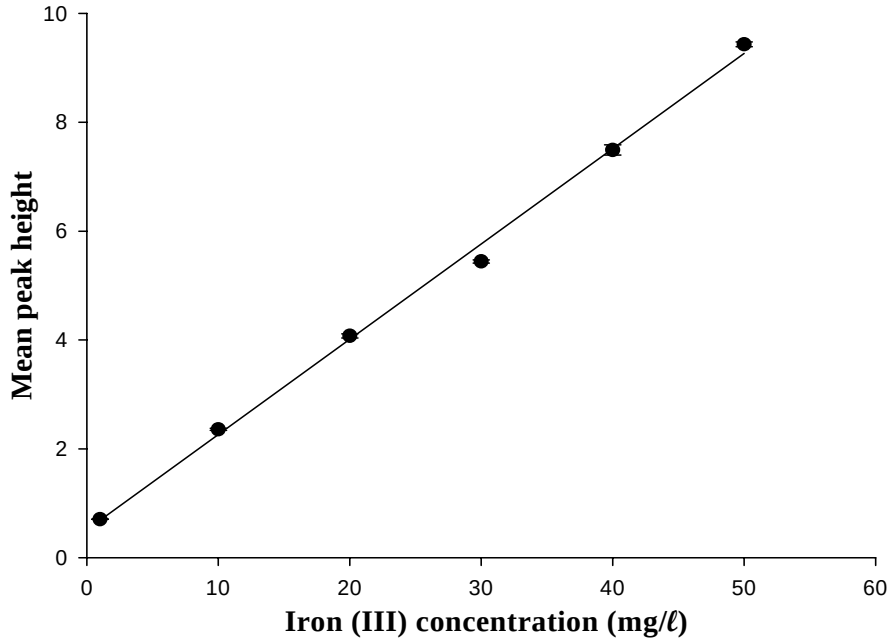

Figure 3.13 Linear curve of standard solutions of iron (III) under optimum experimental conditions using Tiron method.

Conditions: Tiron, 2,5 $\times 10^{-3}$; Perchloric acid concentration, 0,03 mol/ℓ

Accuracy and precision

The above-optimized method was applied for the determination of Fe (III) in different water samples. Comparing the results of the method used and the reference method of atomic absorption spectrophotometer (AAS), the accuracy of the method was determined. The results are summarized on Table 3.5 with good agreement. In order to test the reliability of the method, real samples were spiked with known concentration of standard Fe (III) solutions and recovery of the analyte was determined. The samples were then analysed and the concentration was calculated using calibration curve equation. The calculated value was used together with the known concentration of the sample and standards added to determine the recovery. The formula used was:

$$\%\,\mathrm{Recovery} = \frac{[spiked\ sample]}{[unspiked\ \ sample] + Added\ known\ \ concentration} x100 \qquad (3.2)$$

The recoveries were in the range 98 – 102 % for thiocyanate method and 92 – 105 % for Tiron method. The precision was studied by using Fe (III) standard solution of 10 mg/ℓ with 10 determinations under optimized conditions. Using the following equation:

$$\%\,RSD = \frac{S}{\overline{X}} x100 \qquad (3.3)$$

Where S is standard deviation and $\overline{X}$ is mean peak height. Relative standard deviation of 1,4 % for thiocyanate method and 1,83 % for Tiron method was obtained.

Table 3.5 Results obtained from the analysis of different types of water

Samples	SIA METHOD (mol/ℓ)		AAS (mol/ℓ)
	Thiocyanate	Tiron	
Tap water	0,20 ± 0,02	0,20 ± 0,01	0,37 ± 0,01
Mine water	0,45 ± 0,01	0,44 ± 0,01	0,48 ± 0,02
Ground water 1	4,80 ± 0,02	4,49 ± 0,02	4,98 ± 0,01
Ground water 2	25,00 ± 0,02	23,00 ± 0,01	23,00 ± 0,02

Interference study

The effect of possible interferences were checked, (Table 3.6) especially with those anions and cations that tend to form strong and stable complexes with Fe (III) and reagents (thiocyanate & Tiron) respectively. No interference was found in the presence of copper in the concentration range of 0,05-1 mg/ℓ for both methods. Other possible interferences, such as cobalt (II) and mercury (II) are very unlikely since they are found in most natural waters at lower concentration [12].

None of the anions have shown any interference. Keeping the complexes in acidic medium and using high concentration of reagents controlled the possible interference of the anions. Fluoride forms a complex with iron (III). To eliminate interference of fluoride, the pH of complexes was kept at pH 3 because the complex of Fe (III) and fluoride is complete at pH 2 and $Fe(OH)_3$ precipitates may occur at pH>5 [12]. High concentration of thiocyanate reduces the errors of chloride, phosphate and other ions forming complex with ferric ions in acidic medium [13]. The interference of Al (III) was determined as this ion is most likely to be encountered in water samples and likely to form strong complex with Tiron. The existence of this ion did not interfere with the determination in samples because the complex is colorless and weak.

Table 3.6 Percentage (%) signal of interferences for iron (III) determination

Ions	Recovery (%)
Cl^-	99,3
NO_3^-	97
F^-	97
Cu^{2+}	103
Mg^{2+}	95
Co^{2+}	104
Al^{3+}	103

3.5 CONCLUSIONS

The used sequential injection methods for Fe (III) determination are simple involving single reagent, are sensitive and fast with sampling frequency of 72 samples per hour. No sample pre-treatment which prolong the analysis time was required. Speciation was not conducted in this work, as simplicity was one of the aims of the work. The thiocyanate method has better precision but high detection limit as compared to the Tiron method.

Calibration curves for both methods range between 1-50 mg/ℓ. Both methods are useful in acidic medium and employ inexpensive and readily available chemicals. Theory reports that thiocyanate is harmful to aquatic organisms; however the use of SIA minimizes generated waste with the average of less than 3mℓ per determination. The two methods are comparable to the reference method giving acceptable results. It is concluded that both reagents are convenient for iron (III) determination.

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CHAPTER 4

SPECTROPHOTOMETRIC DETERMINATION OF FORMALDEHYDE (HCHO) IN WASTEWATER

4.1 INTRODUCTION

Formaldehyde (HCHO) is the most stable, chemically reactive carbonyl compound originating from exhaust fumes, vegetation emissions and photochemical reaction of alkanes and alkenes. Formaldehyde is also manufactured by oxidation of dehydrogenation of methyl alcohol and environmentally hazardous substances [1,2]. It is important because of its widespread use. Formaldehyde is toxic and recognized as one of the main air pollutants [3].

Formaldehyde is a colorless, pungent-smelling gas and is an important chemical, primarily used in manufacture of urea, phenol, and melamine resins and for variety of special industrial chemicals. The well-known use of formaldehyde is as preservative in medical laboratories and as an embalming agent in mortuaries. Formaldehyde is widely used in resin that binds wood products, pulp and papers. It is also used in glass wool and rock wool insulator, in plastics and coating, and textile finishing [4-8]. Formaldehyde is found in cosmetic products as one of the ingredients and it preserves cosmetic raw materials [9,10].

Formaldehyde is one of the compounds found in both rural and urban ambient air and its reaction with hydrochloric acid in moist air form a bis (chloromethyl) ether which is carcinogenic to animals and possibly to human beings [6,9,11]. It affects the acid generating capacity of atmospheric water because it inhibits oxidation of S (IV) to sulphuric acid, and because it is a precursor to formic acid [12]. It has been known for its irritant properties such

as dermatitis, asthma, irritation of eyes, nose, and throat, respiratory tracts and pulmonary edema [13,14]. Because of high toxicity of formaldehyde, the allowed exposure limit in all work places (except agriculture) is 0,75mg/l according to regulatory standards [6]. Commonly, the formaldehyde that is found in drinking water is the results of the discharge of industrial wastes and oxidative water treatments [15]. During manufacture and use, formaldehyde finds its way into water streams and leaves liquid waste that human life may be exposed to and causes danger. Due to the large amount that is exposed to human life, it's monitoring and control of exposure in both industries and environment is of great concern.

4.2 ANALYTICAL METHOD

Different methods for assessing formaldehyde contamination in different technological fields (e.g food chemistry, pharmaceutical, etc.) have been reported [16-22]. Electroanalytical determinations include amperometry, polarography, voltametry and potentiometry [16-22]. In these methods it is necessary to remove traces of oxygen and to cater for interference by enzymes. This limits these methods because analysis time is increased. [20]

The chromatographic methods for the determination of formaldehyde are based on pre- and post- column chemical derivatization. The most used methods are based on derivatization of formaldehyde with 2,4-dinitrophenylhydrazine (DNPH) [23-27], and other reagents such as Nash reagent [28] and ethyl-3-oxobutanoate with ammonia [29] followed by separation with liquid chromatography coupled to some detectors. Other methods such as capillary electrophoresis [30,31], gas chromatography (GC) [32,33] and TLC [34,35] have been used. However, the methods require long measuring time per sample due to pre column reactions and cannot be used for continuous analysis [36]. The derivatization reactions may be affected by

other components in samples [37]. Enzymatic methods for the determination of formaldehyde in combination with other techniques have been used. The method based on oxidation of formaldehyde with oxidised nicotinamide adenine dinucleotide (NAD^+) and catalysed by formaldehyde dehydrogenase to form formic acid and reduced dinucleotide (NADH) [38-40] and alcohol oxidase (AOC) [20] are used for determination of formaldehyde. The problem with enzymes is that they are expensive, not stable and may precipitate out of solution resulting in reduced response [36].

Many fluorescence methods have been reported by several workers to determine formaldehyde. Hantzch reaction, involve the cyclization of amine, aldehyde and β-diketone to form dihydropyridine derivative [41]. Other methods, involve the reaction with 5,5-dimethylcyclohexane-1,3-dione [42], Floral P [43], hadralazine [44], 3,4 diaminoanisole [45] and the use of surfactant [46]. The problem with fluorimetric method is the interference of some compounds present in the samples and long analysis time.

Spectrophotometric methods are most widely used for their inexpensive instrument, simple procedure and rapid analysis. The National Institute for occupational safety and health (N.I.O.S.H) recommended method [47,48] uses chromotropic acid and sulphuric acid for the determination of formaldehyde. However, this method suffers from interferences, long heating time and has the problem of using strong, hazardous and corrosive acid. Fagnani *et al* [3] improved the method by replacement of sulfuric acid (H_2SO_4) with hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) whereas Gigante *et al* [49] replaced H_2SO_4 by H_3PO_4 and H_2O_2 but still, the method requires long heating time and lacks sensitivity.

Pararosaniline methods (with and without the use of mercury reagent) [50-54] have been used. Walters [54] used complicated instrument and some provide

higher detection limit with slow color development. The less often used methods are 5,5'-dithiobis(2-nitrobenzoic acid) DTNB [55], 3-methyl-2-benzothiazone hydrazone (MBTH) [33], redox reaction of crystal violet and potassium bromate [56], brilliant cresyl blue [57], malachite green-sulfite [58] and brilliant green-sulfite [59]. The latter has been used for determination of traces of sulfite [60]. Safavi and Ensafi [59] employed a kinetic spectrophotometric method coupled to flow injection analysis (FIA). They exploited the inhibition of the brilliant green-sulfite (BG) reaction by formaldehyde. This method is suitable for low concentration measurements and offers acceptable detection limit.

In this work, sequential injection analyzer (SIA) coupled to kinetic spectrophotometric method has been used for the determination of formaldehyde. SIA shows some advantages in terms of less reagent consumption, rapidity, reproducibility and simplicity. Coupling SIA with kinetic spectrophotometric method makes the method more sensitive and selective.

4.3 EXPERIMENTAL

4.3.1 Apparatus

The SIA manifold (Figure 4.1) was constructed from the following components:

PTFE tubing and Teflon tubing was serving as connectors between tubes and the system. A Unicam 8625 UV-visible spectrophotometer (Cambridge, UK) equipped with cell holder and flow through cell from Hellma (Mulheim/Baden, Germany) was used as detector at wavelength of 612 nm. A Valco 10 port selection valve (Houston, Texas) was used to select ports. Peristaltic pump (Gilson, Velliers-le-bel, France) pumping at the flow rate of 15 ml/min. Computer equipped with LabVIEW software for data acquisition

and system control. The data obtained was automatically converted to the final response versus time profile (Figure 3.2) and maximum peak height measurement was used for evaluation. For replicates, obtained peak heights were averaged to give mean peak height.

For the reference method, spectrophotometric measurement was carried out at 510 nm using the above spectrophotometer and cuvettes.

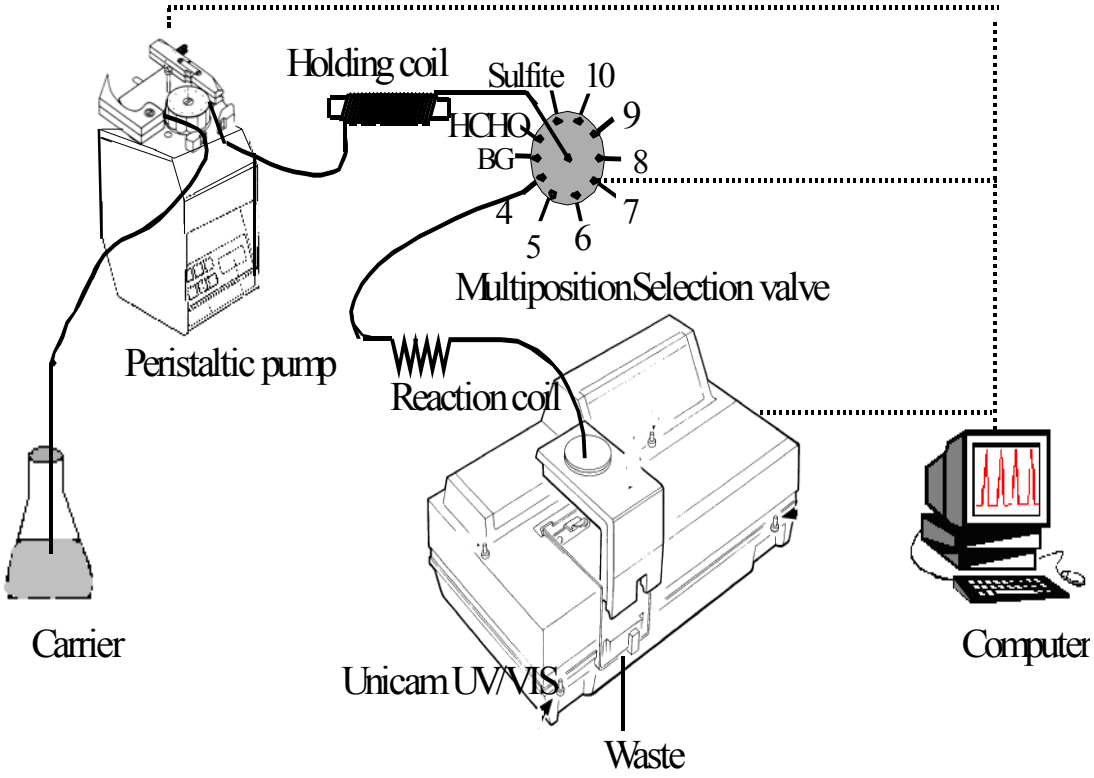

Figure 4.1 SIA manifold used for the experiment. BG-brilliant green and HCHO-formaldehyde

4.3.2 Reagents and solutions

All chemicals were of analytical reagent grade unless otherwise specified. The de-ionized water from Modulab systems (Continental water systems, San Antonio, Texas) was used throughout.

Stock solutions:

Formaldehyde solution (1000 mg/ℓ)

2,5 ml of 37 % formaldehyde (BDH) was diluted to 1ℓ with water and the

solution was standardised by sulfite method [48].

Brilliant green solution ($1{,}04\times10^{-4}$ mol/ℓ)

0,005 g of Brilliant green (BG) (Aldrich) was dissolved in water and the solution was diluted to 100 mℓ with water.

Sodium sulfite solution (1000 mg/ℓ)

0,015 g of anhydrous sodium sulfite [Na_2SO_3] (Merck) was dissolved in 100 mℓ of phosphate buffer solution.

Standards solutions:

All working standards solutions were prepared by the diluting of stock solutions with water.

Buffer solution:

Phosphate buffer solution was prepared by dissolving 0,5 g potassium phosphate dibasic [K_2HPO_4] (PAL) and potassium phosphate monobasic [KH_2PO_4] (PAL) in 1000 mℓ of water.

Other solutions:

Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were used to adjust the pH.

Sample treatment and preparation

The samples were kept in polyethylene bottles until the analysis. During analysis the samples were filtered and diluted. The BG-sulfite reaction method was applied.

4.3.3 Experimental procedure

A carrier solution (water) was pumped into the channel. Sulfite (port 1), formaldehyde (port 2), and brilliant green (port 3) solutions were aspirated sequentially into a holding coil the stacked zones were forwarded into the

flow cell detector. The flow was stopped and the formed complex was arrested in the flow cell, and the reaction response was recorded during the stopped time. The pump was restarted and the solution was washed out. Table 4.1 show the sequence used.

Table 4.1 SIA procedure used for the experiment

Time (s)	Pump	Valve
0	Off	Starting position 1(reagent 1)
2	Reverse-draws regent 1	
5,5	Off	
6		Step to position 2 (sample)
7	Reverse-draws sample	
14	Off	
15		Step to position 3 (reagent 2)
16	Reverse-draws regent 2	
26,5	Off	
27		Step to position 6 (detector)
28	Forward until product reaches the detector	
60	Off	
120	Forward	
145	Off	
150		Return to position 1

4.4 RESULTS AND DISCUSSION

BG dye decolorizes in the presence of sulfite, and formaldehyde inhibits the reaction. Reaction response of uninhibited and inhibited was recorded spectrophotometically at 612 nm in the form of peak. Peak height measurement of the transient signal was used for quantification. The

difference between each peak height of uninhibited and inhibited reaction was calculated and the average of the difference was reported as mean of difference in peak height (DMPH). Water was used in place of formaldehyde for uninhibited reaction.

Optimization of instrumental parameters is necessary to obtain good response from the system. Parameters such as flow rate, inner diameter and length of reaction coil, sample and reagent volume, reagents concentrations and pH were optimized by altering each variable while keeping the other constant. The plots of optimized parameters are presented as mean of difference in peak height versus optimized parameters.

4.4.1 Optimization

Physical parameters

Flow rate: The volume (mℓ) pumped into the system (flow rate in mℓ/min) was determined as in chapter 3 and Table 3.3, which shows different pump speed and corresponding flow rates, was used for flow rate optimization. The effect of flow rate was ranged form 1,5 to 3,5 mℓ/min (Figure 4.2 and Table A4.1). Flow rate influence the mixing of sample and reagents. Changing from 1,5 to 2 mℓ/min, an increase in mean peak height (difference) was observed and the decrease thereafter. Too low flow rate lead to poor reproducibility and incomplete mixing was experienced. At flow rate of 2 mℓ/min complete reaction between three zones was obtained giving maximum peak height. At higher flow rate the signal decreased dramatically due to insufficient reaction of HCHO, BG and sulfite. At higher flow rate peak heights for inhibited and uninhibited reaction are very close indicating that the response is only due to diluted BG-reagent (by HCHO for inhibited and water for uninhibited) and this results to decrease in difference.

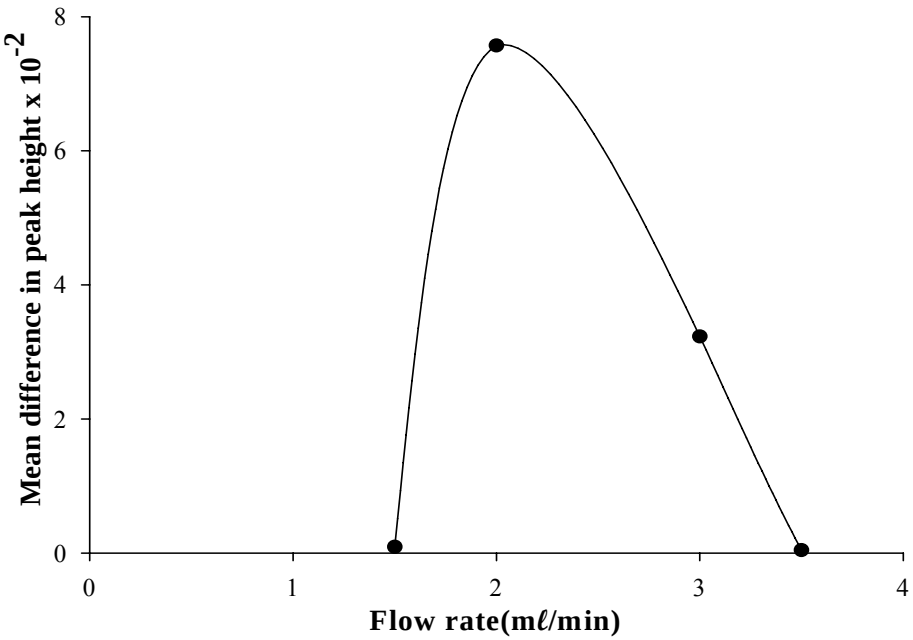

Figure 4.2 Effect of flow rate on peak height.

Conditions: Formaldehyde, 2,5 mg/ℓ; BG, 1,04 x 10^{-4} mol/ℓ; sulfite, 6 mg/ℓ; pH 7.

Coil length: Reaction coil length and its inner diameter influence the dispersion which affect zones penetration hence the sensitivity. The effect of reaction coil length was studied in the range of 90 to 310 cm and corresponding mean peak is shown in Table A4.2. Figure 4.3 shows mean peak height increasing from 90 cm to 200 cm of reaction coil length and decreasing beyond. Coils less than 200 cm were too short for efficient mixing and the reaction was not complete. Longer reaction coil of 200 cm resulted in increase in sensitivity as a result of promoting better mixing of sample and reagents. Very long reaction coil (longer than 200 cm) decreased peak height probably due to the fact that reaction between zones reaches completion before reaching the detector where the sensitivity is suppose to be increased by stopping the flow. 200 cm was chosen for the next optimization.

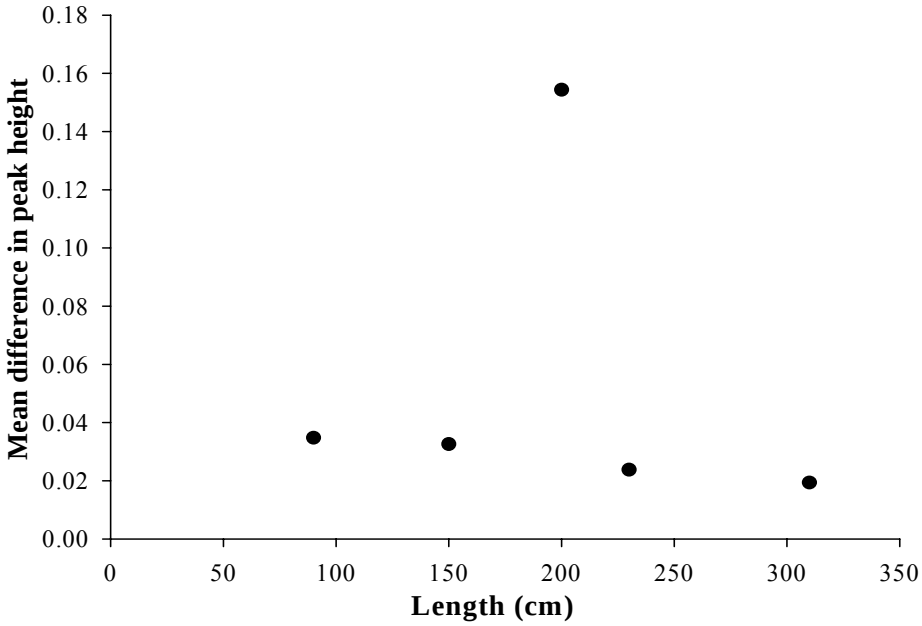

Figure 4.3 Effect of reaction coil length on peak height.

Conditions: Formaldehyde, 2,5 mg/ℓ; BG, 1, 04 x 10^{-4}; sulfite, 6 mg/ℓ; pH 7.

Volume: The influence of sample and reagent volume was investigated by aspirating volumes in the range of 175 µℓ up to 700 µℓ of formaldehyde, 35 µℓ to 700 µℓ of sulfite and 105 µℓ to 700 µℓ BG reagent. Results for investigation are shown in Figure 4.4 and Table A4.3, A4.4 and A4.5. Sulfite was aspirated first in the holding coil followed by formaldehyde then reagent was introduced last into the holding coil.

As expected an increase in BG reagent increased peak height for both inhibited and uninhibited reactions but the difference in their peak height decreased. At higher volumes BG reagent response is independent of the other reagents resulting in the peak height values for both inhibited and uninhibited reactions being almost equal or close. Sulfite reagent reduces BG reagent colour from green to colourless. Using BG reagent optimized volume, the increase in sulfite should decrease the peak height as more of sulfite will be reacting with fixed volume of BG reagent. Increased volume decreased peak

heights for inhibited and uninhibited reactions and their differences were also decreased.

Formaldehyde was used to inhibit BG-sulfite reaction. Water was used in place of HCHO for uninhibited reaction in order to have equal zones for both reactions. Increasing HCHO volume up to 350 µℓ increases the peak height and the difference in peak height because of an increase in inhibition effect. Above 350 µℓ peak heights continued to increases but difference in peak height decreases. This is due to less interaction between BG and sulfite reagents.

HCHO/water acts as a spacer between the two therefore as the HCHO/water volume continue to increases the respond obtained from both inhibited and uninhibited reaction result only from BG reagent. Although the response was not exactly the same due to dilution of BG reagent by water, their differences were very small. The optimum sensitivity was observed at 350 µℓ for formaldehyde, 175 µℓ for sulfite and 525 µℓ for BG (Figure 4.4).

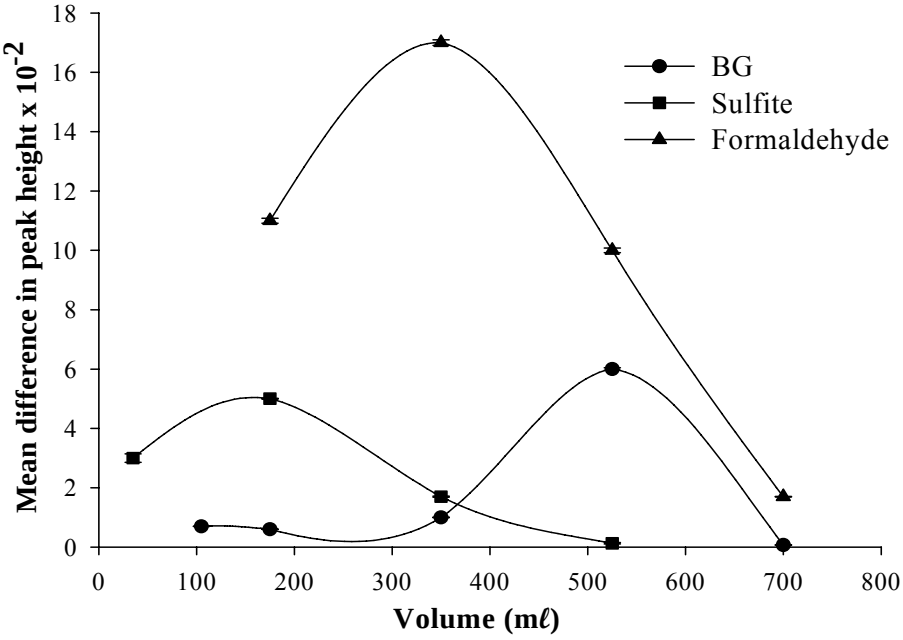

Figure 4.4 Effect of formaldehyde, BG and sulfite volumes on peak height.

Conditions: Formaldehyde, 2,5 mg/ℓ; BG, 1,04 x 10^{-4}; sulfite, 6 mg/ℓ; pH 7.

Reaction time: The sensitivity for the determination of formaldehyde depends on reaction time. The effect of time before and after stopping was investigated. Longer reaction time could produce larger signal and higher sensitivity could be obtained. Time before stopping the flow was optimized in the way that the complex reaches the visualization point of detector before equilibrium point to allow the reaction to continue during stopped flow and data collection. 60 s had higher response.

Higher sensitivity could be obtained by longer reaction time because the reaction continues without dilution with carrier solution. Reaction time was optimized from 20 to 100 s and the sensitivity increased with increasing time. Although there was significant difference between 60s, 80s and 100s a compromise between sensitivity and sampling rate was made and 60 s (better precision) was chosen as optimum. Results are shown in Figure 4.5

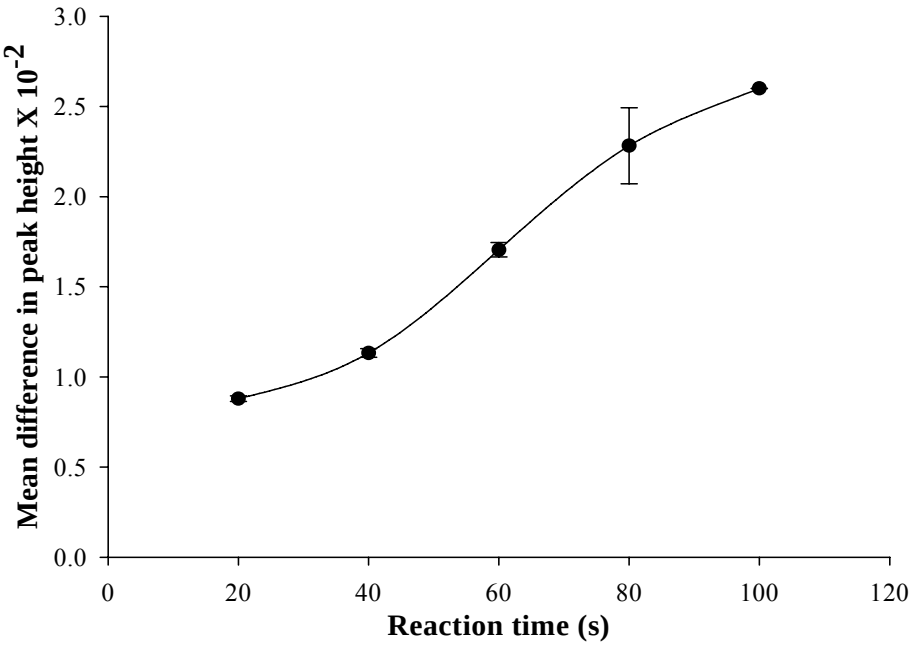

Figure 4.5 Effect of reaction time on peak height.

Conditions: Formaldehyde, 2,5 mg/ℓ; BG, 1,04 x 10^{-4}; sulfite, 6 mg/ℓ; pH 7.

Chemical variables

pH: Stability of the reaction is always a very important factor for system optimization. The effect of pH on peak height was studied ranging from 4 to 9. The change in mean peak height increased with increasing pH up to pH =8, and decreased beyond pH 8 (Figure 4.6 and Table A4.6). At higher and lower pH values the concentration of HSO_3^- decreases by its conversion to SO_3^{2-} and H_2SO_3 respectively and reagent is not stable [3]. pH 8 gave maximum response.

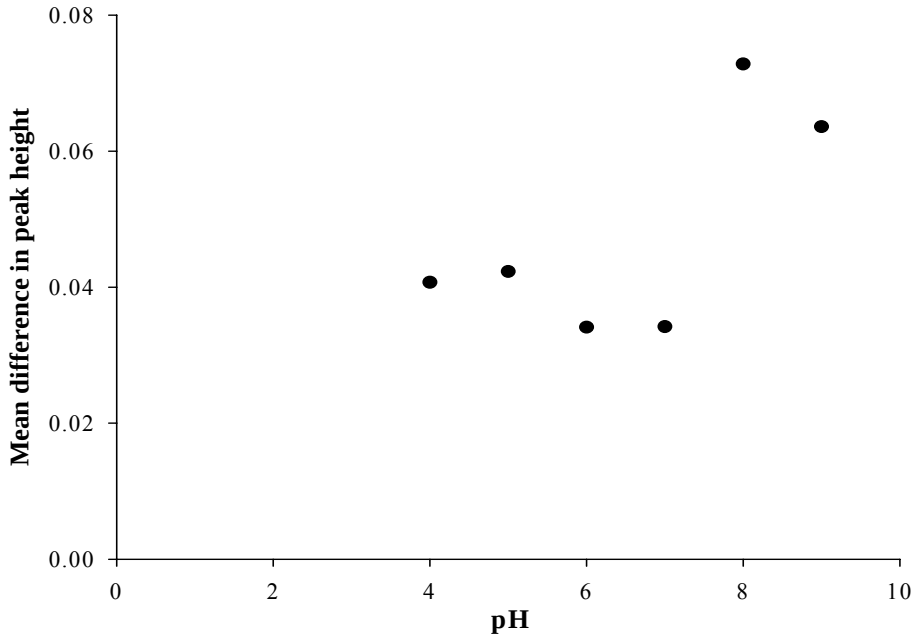

Figure 4.6 Effect of pH on peak height.

Conditions: Formaldehyde, 2,5 mg/ℓ; BG, 1,04 x 10^{-4}; sulfite, 6 mg/ℓ.

Reagents concentration: The effect of sulfite concentration was studied with pH 7 ranging from 2 to 20 mg/l (Table A4.7). Figure 4.7 shows the increase in change of mean peak heights when sulfite concentration is increased.

However from 8 mg/ℓ and higher concentrations sulfite continue to bleach BG reagent to extend that the effect of water and HCHO becomes negligible and the signals obtained are very close to give small difference in mean peak heights as observed in Figure 4.7. 8 mg/ℓ was selected for the subsequent optimization.

The concentration of brilliant green was another chemical variable optimized. The effect was studied between 5 $\times 10^{-6}$ and 4×10^{-5} mg/ℓ. Figure 4.8 and Table A4.8 shows the results where the difference in mean peak heights increased with increasing concentration. Higher concentrations were not tested because of high molar absorptivity of BG and because it can cause reagent adsorption on PTFE tubes. The adsorption of BG reagent will increase the analysis time because extra time will be required to clean the tubes. Therefore 4×10^{-5} mg/ℓ was chosen as optimum.

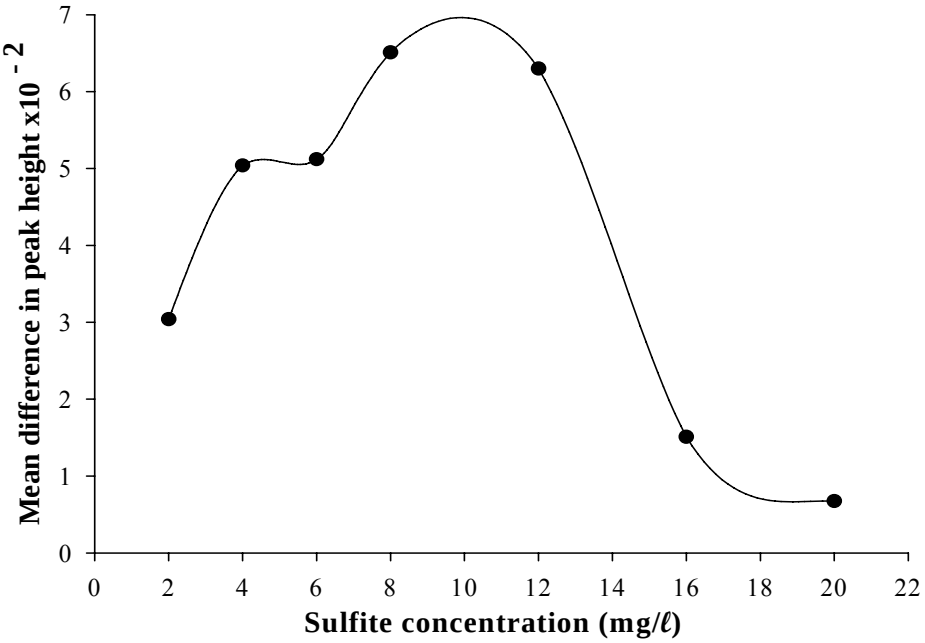

Figure 4.7 Effect of sulfite concentration on peak height.

Conditions: Formaldehyde, 2,5 mg/ℓ; BG, 1,04 x 10^{-4}; pH 8.

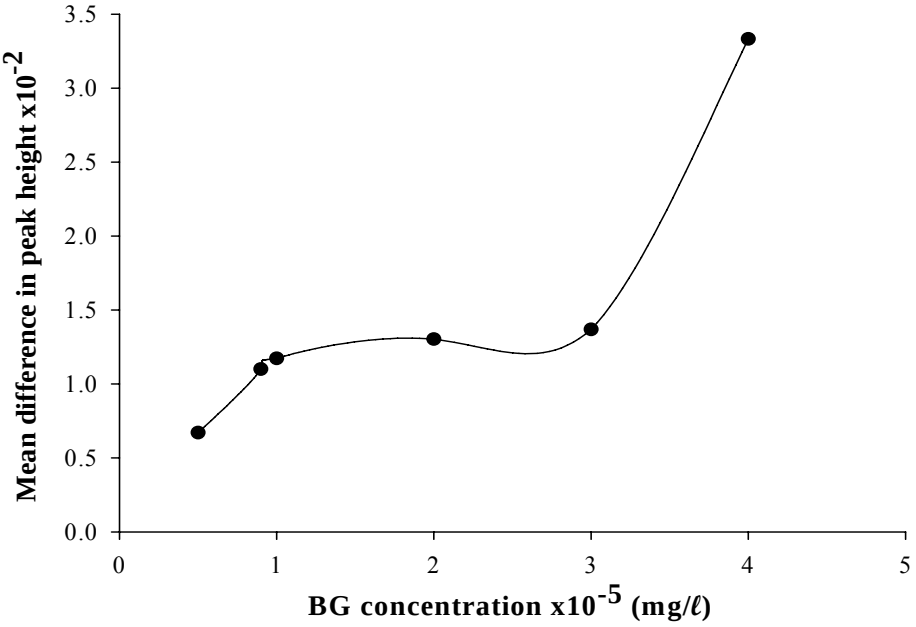

Figure 4.8 Effect of BG concentrations on peak height.

Conditions: Formaldehyde, 2,5 mg/ℓ; Sulfite, 8mg/ℓ; pH 8.

4.4.2 *Method evaluation*

Linearity and detection limit (DL)

Using the above optimized SIA system (Table 4.2) for formaldehyde determination, the series of standards solution and the blank were run five times each to obtain linear expression. Calibration graph (Figure 4.9) was linear over the range of 0 to 3 mg/ℓ.

Linear equation is given by:

$$Y = 0{,}1047\ [HCHO] + 2{,}7781\ (r^2 = 0{,}9917)$$

Where Y = peak height and [HCHO] is the concentration of formaldehyde in mg/ℓ. Detection limit was determined using equation 3.1. DL obtained was 0,06 mg/ℓ.

Table 4.2 Optimum conditions used for evaluation

Parameter	Optimum value
Flow rate (mℓ/min)	2
Reaction coil length (cm)	200
Reaction time (s)	60
BG-reagent volume (µℓ)	525
Sulfite reagent volume (µℓ)	175
Sample volume (µℓ)	350
BG-reagent concentration (mg/ℓ)	4×10^{-5}
Sulfite reagent concentration (mg/ℓ)	80
pH	8

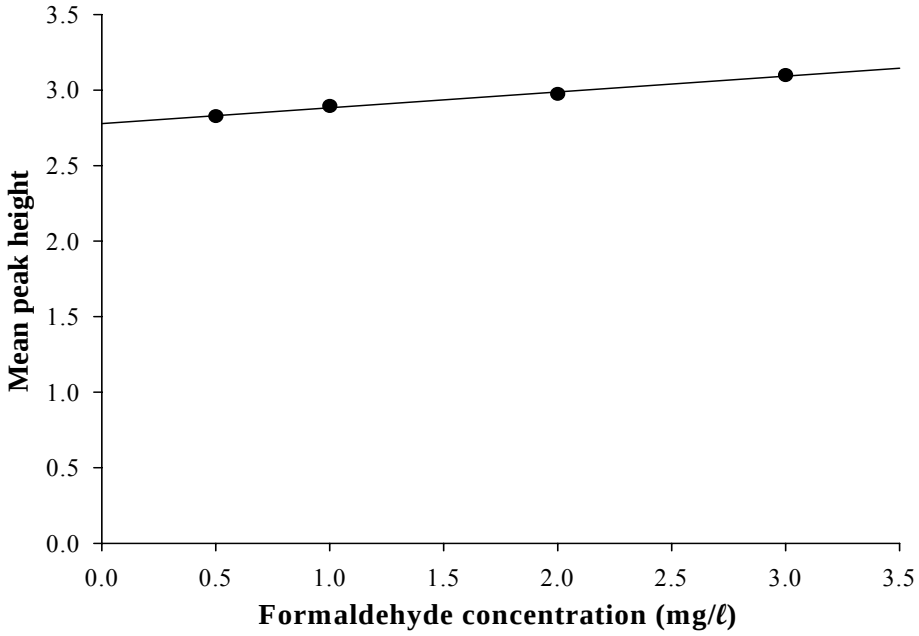

Figure 4.9 Linear curve for standard solutions of formaldehyde under optimum experimental conditions

Accuracy and precision

The method was applied to the determination of formaldehyde in 2 types of wastewater from different places. The results obtained by the SIA were

compared with the Chromotropic acid method as a reference method by applying t-test at the 95 % confidence level (Table 4.3). The critical value of *t* for 5 degree of freedom and the 95% confidence level is 2,45 suggesting that there is no significant difference between the results obtained by the two methods. The method shows good reproducibility with relative standard deviation of 1,5 % for 0,5 mg/ℓ of formaldehyde (n = 5).

Table 4.3 Results obtained for analyses of wastewater

Samples	SIA (mg/ℓ)	Reference (mg/ℓ)	*t*-Calculated
SAMPLE 1	0,527 ± 0,034	0,429 ± 0,006	0,739
SAMPLE 2	0,793 ± 0,055	0,729 ± 0,002	0,289

Interference study

Anions and cations present in the samples were checked for interfering effect for the determination of 0,5 mg/ℓ (Table 4.). No interference from ammonium nitrate, ammonium sulfate, sodium nitrate, sodium carbonate, sodium chloride, sodium sulfate, propionaldehyde and benzaldehyde were found at concentration of 500 mg/ℓ which was a maximum limit tested. Ethanol, methanol and acetone interfered at concentration above 50 mg/ℓ whereas acetaldehyde and sulfide interfered above 25 mg/ℓ and 2 mg/ℓ respectively.

Table 4.4 Tolerance limits of foreign species on determination of 0,5 mg/ℓ formaldehyde

Species	Tolerance limit (mg/ℓ)
NH_4^+, SO_4^{2-}, NO_2^- CO_3^{2-}, Cl^-, Na^+, propionaldehyde and Benzaldehyde	500
Acetaldehyde	25
Sodium sulfide	2
Methanol, ethanol, acetone	50

4.5 CONCLUSIONS

Combination of the SIA with the kinetic method is more advanced compared to FIA due to its discontinuous nature which allows the flow to be stopped and restarted by computer control of the pump during the analysis. And this verifies the SIA system long-term stability and versatility. With kinetic analysis high selectivity has been achieved for the determination of formaldehyde by reducing the interferences of major components in wastewater thus making SIA kinetic method suitable for analysis of environmental samples.

The applicability of the method to real samples has been demonstrated by analyzing HCHO samples and compared with a reference method. The lack of a statistically significant difference between the results from the two methods verifies the accuracy of results obtained by the SIA method. As compared to the brilliant green-sulfite reaction method using FIA, brilliant green and sulfite reagent consumption with SIA technique has reduced. This is one of the sensitive and selective methods, which allow the determination of formaldehyde at lowest concentration with very low interference.

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CHAPTER 5

SPECTROPHOTOMETRIC DETERMINATION OF ASCORBIC ACID IN PHARMACEUTICALS, SWEET AND ORANGE JUICE

5.1 INTRODUCTION

Ascorbic acid also known as vitamin C is a water soluble vitamin, less stable among the vitamins and is highly sensitive to the light, heat and the air that causes its oxidation. When exposed to the oxidant, it is reversibly converted to dehydroascorbic acid (DHAA) [1]. Ascorbic acid is a dienol form of γ-lactone of 2-desoxy-2-keto-L-gulonic acid. Enol group [-C (OH)=C(OH)-] is responsible for the molecule's acidity and its reducing properties [2,3]. It is stable in solid form, but according to the reaction in solutions it is oxidized by dissolved oxygen. [4].

Ascorbic acid occurs naturally in fruits and vegetables. In some sweets it is purposely added to attract consumers and act as antioxidant to prolong shelf life of the commercial products and it is also added in pharmaceutical preparations to add a supplementary source of vitamin C in human diets [5]. During storage especially in the ambient temperature and in the presence of heavy metals it breaks down [6].

The primary function of ascorbic acid is to assist in the production of collagen in connective tissues, neurotransmitters, steroids hormones, carnitine, and conversion of cholesterol to bile acid and enhances iron bioavailability. Ascorbic acid prevents the degenerative diseases such as cataracts, certain cancer and cardiovascular diseases. It is a great antioxidant and protects the body from foreign substances.

Ascorbic acid is very important for normal tissues growth and repair such as healing of wounds and burns. Too much intake of ascorbic acid can cause gastrointestinal problems whereas lack of ascorbic acid in the body causes scurvy with the symptoms of slow wound healing, soft and spongy bleeding gums and loose teeth. Edema is another condition caused by inadequate amount of vitamin C and its symptoms are lack of energy, painful joints and bronchial infection and colds [7-9].

5.2 ANALYTICAL METHOD

Various techniques have been used for ascorbic acid determination such as titrimetry [10-14], electroanalytical techniques [15-22], flourimetry [23,24], separation techniques [25-28], enzymatic method [1,6,29-31] and spectrophotometry [34-42].

Titration methods used oxidizing agents 2,6 dichlorophenolindophenol [10], N-bromosiccinimide in the presence of potassium iodide/starch indicator [11,12], ferricyanide [13] and thallium III [14]. The titration methods were limited by the difficulty in visualization of the end point in colored samples and in the presence of other reductors.

Potentiometric titration [15-17] method was employed to eliminate problems of colored samples. Other electroanalytical methods amperometry [18-20], voltametry [21] and polarography [22] were used for the determination of ascorbic acid. However, these methods suffer from the interferences present in the samples containing redox compounds, they require long analysis time and are not reproducible [21,22]. Ion selective electrode of potentiometry needs bubble oxygen, which needs sufficient current to be produced.

Perez-Ruiz *et al*. [23] and Wu *et al*. [24] and other authors reported the use flourometric method with different reagent to yield fluorescing product. The method is time consuming with low sensitivity, complicated and may not be perfect because aerobic oxidation of ascorbate to dehydroascorbate is non-stoichiometric [26,27]

Separation techniques have been used for ascorbic acid determination combined with different detectors. Gas chromatography (GC) [25,26], high performance liquid chromatography (HPLC) [19,27] and capillary zone electrophoresis [28] methods have drawbacks such as a use of expensive instruments, the need of operator's attention and long analysis time of converting analyte to its derivatives before subjecting to analysis

Enzymatic methods [1,29-31] in combination with other techniques, has been useful for the analysis but they are costly, too specialized for ordinary laboratory use, and slow in analyzing large number of samples.

Spectrophotometric methods for ascorbic acid determination are widely used owing to their fast applicability to routine analysis and sensitivity. Most of the methods are based on oxidation-reduction process and ability to produce colored complexes. The use of reactions catalyzed by Cu (II) [32,33] and Fe (III) [3,5,34-42], have been widely used to oxidize ascorbic acid to dehydroascorbic acid.

The use of Cu (II) catalysis was complicated by the use of buffers, which interact to different extents with Cu (II) ions to form complexes [42] and the mechanism of reaction using high concentration of reactants [43]. Reduction of Fe (III) to Fe (II) is the most sensitive approach to the determination of ascorbic acid [35]. The generated Fe (II) can complex with different reagents,

ferrozine [34,35], 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (Br-PADAP) [36], 2,2'dipyridyl [37], *p*-carboxyphenylfluorone [38] and 1,10 phenanthroline [3,39-42] that has being mostly used. Phenanthroline reagent forms stable complex with Fe (II). It is slightly soluble in water but soluble in acidic solution and this favors the use of Fe (III) because at higher pH iron oxide precipitates forms.

Much work has been done based on this reagent method with few improvements. Prodromos *et al*. [39] employed an aqueous micellar medium formed by cationic surfactant cetyl-pyridinium bromide with long analysis time of 30 min. in water bath and the cooling before the measurements. Flow analytical method has been applied to this method.

Pereira and Fatibello-Filho [40] inserted a column containing Fe $(OH)_3$ immobilized in polyester resins in FIA system. However this method is limited by long analysis time due to the preparation and immobilization of Fe $(OH)_3$. Memon and coworkers [41] used single line FIA manifold and Luque-Perez *et al*. [3] used photochemical method using irradiation to enhance the redox reaction. The latter method provided higher sensitivity.

SIA is another flow injection method that has been used on the same concept. Sultan and Desai [2] investigated the kinetics and mechanism of chemical reaction involved. A redox reaction is used in this work using SIA. Ascorbic acid reduces Fe (III) to Fe (II) and the produced Fe (II) reacts with 1,10 phenanthroline to form ferroin complex. The complex was measured spectrophotometically in combination with photochemical method. The photochemical method uses visible light to enhance the reaction between ascorbic acid and Fe (III).

5.3 EXPERIMENTAL

5.3.1 Apparatus

SIA manifold (Figure 6.1) was constructed from the following components. PTFE and Teflon tubes served as connectors between tubes and the system. A Unicam 8625 UV-visible spectrophotometer (Cambridge, UK) equipped with cell holder and flow through cell from Hellma (Mulheim/Baden, Germany) was used as detector at wavelength of 510 nm. A Valco 10 port selection valve (Houston, Texas) was used to select ports. Peristaltic pump (Gilson, Velliers-le-bel, France) pumping at the flow rate of 15 ml/min. Computer equipped with LabVIEW software for data acquisition and system control. For reference method, the titration method using burette was used. Ultra violet visible lamp of 400 Watts (W) was used.

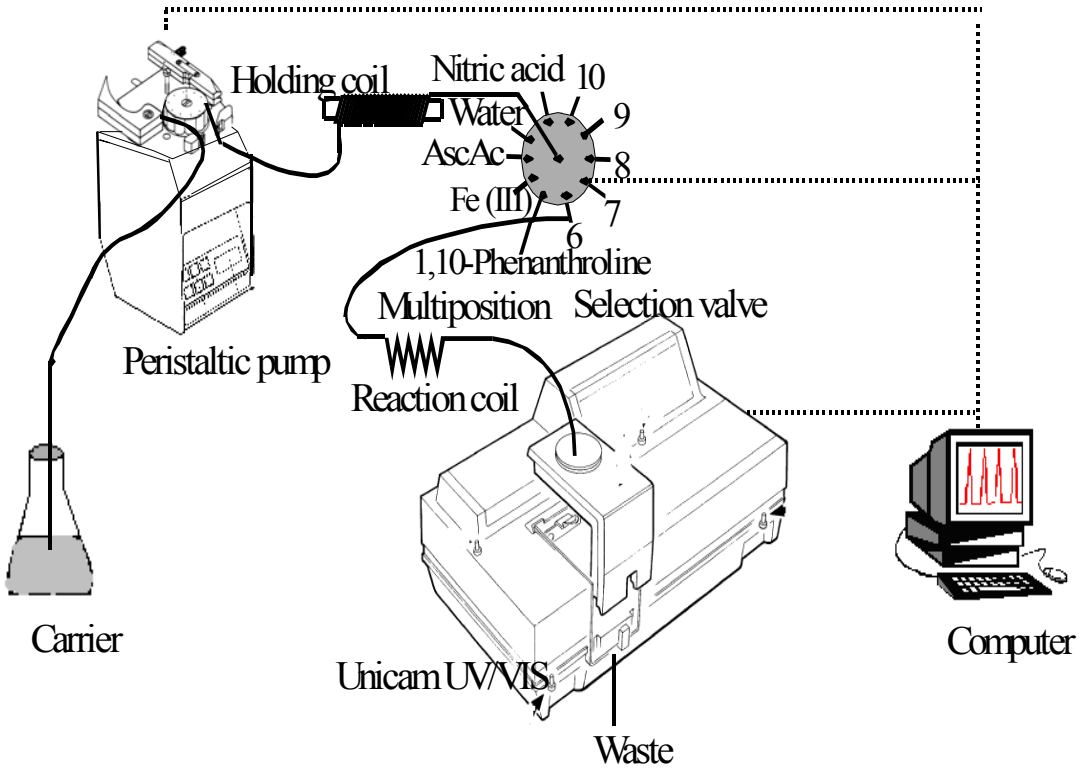

Figure 5.1 SIA manifold used for the experiment

5.3.2 *Reagents and solutions*

All chemicals were of analytical reagent grade unless otherwise specified. The de-ionized water from Modulab systems (Continental water systems, San Antonio, Texas) was used throughout.

Stock solutions:

Ascorbic acid solution (1000 mg/ℓ)

Ascorbic acid solution was prepared daily by dissolving 0,1 g of ascorbic acid (Merck) in 100 mℓ of acetate buffer

Fe (III) solution (0,01 mol/ℓ)

The solution was prepared by dissolving 0,27 g of Fe (III) chloride (B.Owen Jones) in 100 mℓ of 0,01 mol/ℓ H_2SO_4

Phenanthroline (phen) solution (0,03 mol/ ℓ)

1,10 phenathroline was prepared by dissolving 0,59 g of 1,10 phenanthroline (Aldrich) in 100 mℓ of 0,01 mol/ℓ H_2SO_4

Standards solutions:

All working standard solutions were prepared by dilution of stock solutions in water.

Buffer solution:

Acetate buffer solution (pH 3,5) was prepared by mixing a 6,4 mℓ of 2 mol/ℓ sodium acetate (PAL) and 93,6 ml of 2 mol/ℓ acetic acid (Merck).

Other solutions:

H_2SO_4 was prepared by diluting 0,08 mℓ of H_2SO_4 (univAR). 1 % starch was prepared by dissolving 1 g of starch (Protea) in 10 mℓ of boiling water and added to 90 mℓ of saturated sodium chloride solution. 0,1 % (w/v)

N-Bromosuccinimide (Aldrich) solution was prepared by dissolving the powder in hot water, cooled and diluted to 100 mℓ.

Sample treatment and preparation

Sweets (Vita C) and tablets (Scorbex and Cal-c-vita) were purchased from the local supermarket and chemist respectively. They were dissolved in water and the concentration was measured per sweet and per tablet. Squeezing and filtering the juice from the fruit prepared fruit juice. The filtrates were diluted to obtain the desired concentration.

5.3.3 Experimental procedure

A carrier solution (water) was pumped into the channel. Nitric acid (Port 1) used as wash solution was aspirated first into a holding coil in order to prevent complex adsorption on the inner walls of the tubes. Water (port 2) was aspirated secondly to prevent the mixing of HNO_3 and ascorbic acid. Ascorbic acid (port 3) and Fe (III) standard solution (port 4) were aspirated sequentially; the flow was stopped in the holding coil under irradiation for the reduction of Fe^{3+} to Fe^{2+} to take place. 1,10 phenanthroline (port 5) was aspirated into the holding coil to allow the reaction with generated Fe^{2+} as the product is propelled forward forming a ferroin complex. The complex was propelled towards the detector for signal reading. The whole procedure is shown in Table 5.1

Table 5.1 Operation procedure of SIA system.

Time(s)	Pump	Valve
0	Off	Starting position 1(wash solution)
2	Reverse-draws HNO_3	
27	Off	
28		Step to position 2 (blank)
29	Reverse-draws blank	
44	Off	
45		Step to position 3 (reagent 1)
46	Reverse-draws regent 1	
61	Off	
62		Step to position 4 (sample)
63	Reverse-draws sample	
78	Off	
108		Step to position 5 (reagent 2)
109	Reverse-draws reagent 2	
129	Off	
130		Step to position 6 (detector)
131	Forward	
194	Off	
200		Return to position 1

5.4 RESULTS AND DISCUSSION

Fe (III) does not form stable complex with phenanthroline (phen) reagent, it is therefore reduced to Fe (II) which, forms stable red-orange ferroin $[Fe\ (phen)_3]^{2+}$ complex which is monitored at 510 nm.

Oxidation of ascorbic acid by iron (III) occurs according to the reaction,

$C_6H_8O_8 + 2Fe^{3+} \rightarrow C_6H_6O_8 + 2H^{2+} + 2\ Fe^{2+}$

$Fe^{2+} + 3phen \rightarrow Fe^{2+}(phen)_3$

In the solution $[Fe\ (phen)_3]^{2+}$ complex if favoured at 1:3 mole ratio of Fe^{2+}:phen [44]

Optimization of instrumental parameters is necessary to obtain good response from the system. Parameters such as flow rate, inner diameter and length of reaction coil, sample and reagent volume, reagents concentration and pH were optimized by altering each variable while keeping the other constant. The signal is recorded in the form of peak. Obtained peak heights were averaged to give mean peak heights. The plots of mean peak heights versus optimized parameters are shown in this chapter.

5.4.1 Optimization

Physical parameters

Flow rate: The flow rate in mℓ/min was determined as in chapter 3. Table 3.3, which show different pump speed and corresponding flow rates, was used for flow rate optimization. The influence of flow rate on sensitivity was ranged from 1,5 mℓ/min to 3,5 ml/min the results are given in Table A5.1.

Flow rate influences the mixing of sample and reagents hence the sensitivity of the system. Since reaction between ascorbic acid and Fe (III) is slower, higher flow rates leads to decrease the sensitivity. As shown in Figure 5.2 mean peak heights decreased dramatically at flow rates higher than 2 mℓ/min owing to less interaction time of reagents which results to incomplete reaction and short residence time of the complex. 2 mℓ/min was chosen as optimum.

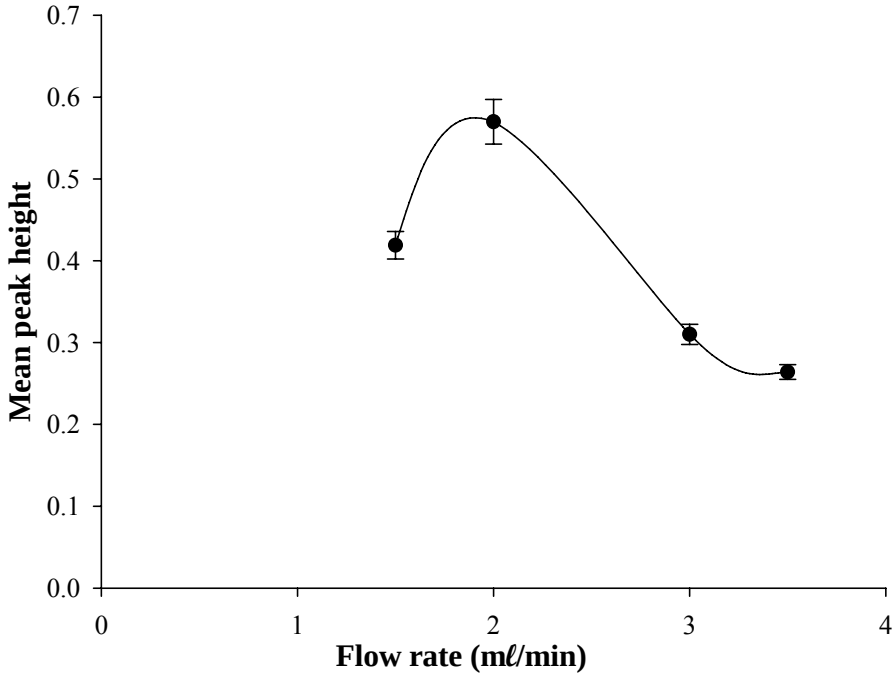

Figure 5.2 Effect of flow rate on peak height

Conditions: Phenanthroline, 1,0 x 10^{-3} mol/ℓ; Ascorbic acid, 20 mg/ℓ;
Fe (III), 5 x 10^{-3} mol/ℓ

Coil length: Reaction coil length and its inner diameter have strong influence on the sensitivity of sample-reagent product. The greater the length and diameter, the higher the dispersion resulting in lower sensitivity. It is important that the reaction coil be short with small inner diameter to give minimum dispersion. The effect of reaction coil length and diameter was studied in the range of 50 to 80 cm and 0,51 to 0,89 mm respectively. The results are given in Table A5.2.

Since the reduction process took place in holding coil, higher response from the complex was obtained from short reaction coil owing to fast reaction between Fe (II) and 1,10 phenanthroline. Mean peak height decreased with increase in reaction coil length because by the time the product zone reaches the reaction coil it has undergone quite a degree of dispersion and lost its

sensitivity. With very small diameter, poor precision was obtained, which might be due back pressure related to pump speed used. 50 cm presented maximum mean peak height as shown in Figure 5.3 and 0,64 mm i.d was used as optimum diameter.

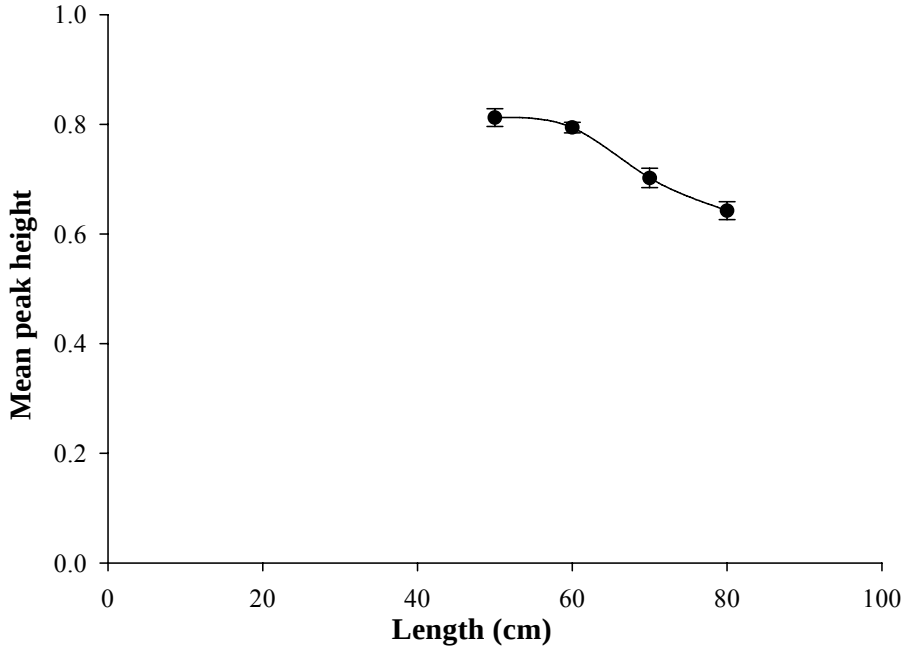

Figure 5.3 Effect of reaction coil length on peak height

Conditions: Phenanthroline, 1,0 x 10^{-3} mol/ℓ; Ascorbic acid, 20 mg/ℓ;
Fe (III), 5 x 10^{-3} mol/ℓ

Order of aspiration: The sensitivity due to order of aspiration was studied. Although three zones were used for introduction, the mechanism resembled two-zone penetration. Ascorbic acid was aspirated first followed by Fe (III) into holding coil, two zones were allowed reacted for a certain time under irradiation to reduce Fe (III) to Fe (II). Phenanthroline was introduced lastly. When ascorbic acid was sandwiched between Fe (III) and Phenanthroline, smaller peak were observed due to incomplete reaction and less interaction between the reduced iron and Phenanthroline. When phenanthroline was

introduced before Fe (III) and ascorbic acid the sensitivity was decreased to the dilution of phenanthroline during the stopping period. Iron was sandwiched between ascorbic acid and phenanthroline to allow its complete conversion and complete reaction resulting in higher sensitivity.

Volume: The influence of sample and reagents volume were investigated by aspirating volumes in the range from 250 µl-1250 µl ascorbic acid, 250 µl – 1000 µl Fe (III) and 250 µl – 1250 µl of 1,10 Phenanthroline reagent. Results are given in Table A5.3, A5.4 and A5.5.

Sensitivity increased with increase in Fe (III) volume up to 750 µl as the interaction time interval increased. With less volume the sampling time was increased but peak heights were very low. The maximum response was 750 µl. Increase in ascorbic acid volume improved the reduction process however at higher volume above 750 µl it increased the gap between generated Fe (II) and phenanthroline leading to lower response. Using fixed 750 µl of ascorbic acid and Fe (III), phenanthroline reagent volume was optimized. 1000 µl of phenanthroline increased sensitivity of measurement. This result agrees with Gubeli *et al* [45] work that sufficient reagent excess promote sensitivity of the method. The response for the volumes is shown in Figure 5.4

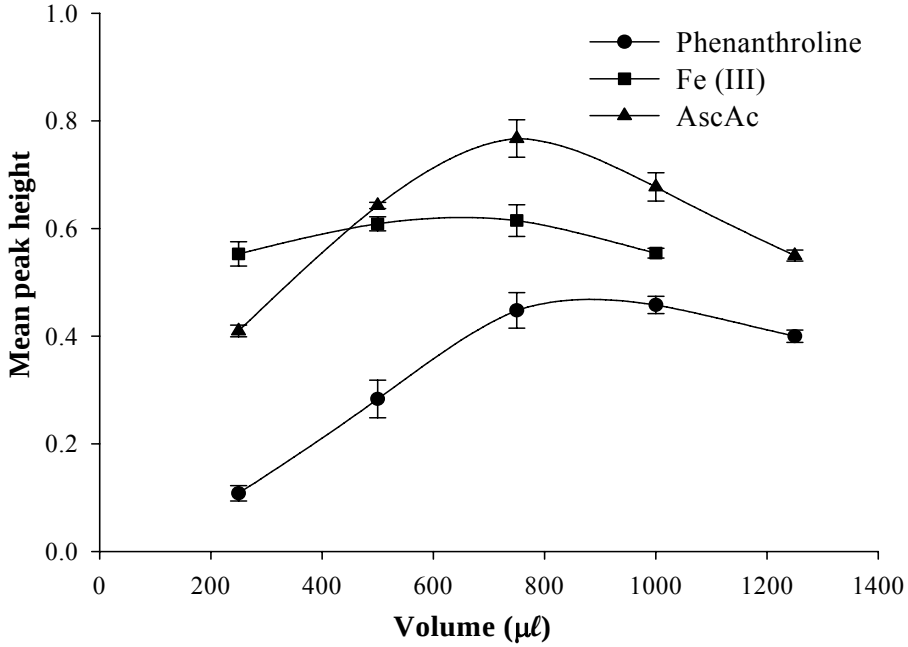

Figure 5.4 Effect of Ascorbic acid (AscAc), Fe (III) and phenanthroline volumes on the peak height

Conditions: Phenanthroline, 1,0 x 10^{-3} mol/ℓ; Ascorbic acid, 20 mg/ℓ; Fe (III), 5 x 10^{-3} mol/ℓ

Reaction time: A visible light lamp of the power 400W being the only one available was used. The lamp was used to enhance the reaction and improve sensitivity. The time before and after stopping the flow in the holding coil was studied. The reaction was stopped in the holding coil to allow the reaction of Fe (III) and ascorbic acid and conversion Fe (III) to take place before addition of reagent and to improve the mixing as the product is propelled to the detector. For a very short stopping time (before stopping), Fe (III) and ascorbic acid does not reach the radiation position hence the decrease in the sensitivity. The sensitivity increased with increasing time and later decreased. The decrease in sensitivity was due to incomplete conversion since the reagents passes the radiation point. Table A5.6 shows the results for optimization of reaction time (during irradiation).

Reaction time as expected increased with the increase in peak heights (Figure 5.5). However, longer reaction times were not studied in order to reduce sampling time. 30 s was taken as a compromise.

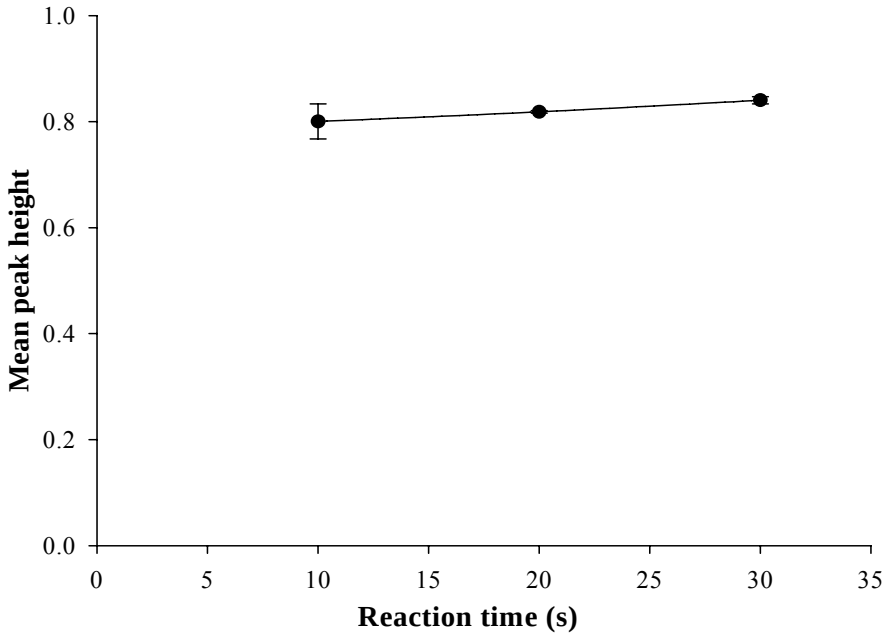

Figure 5.5 Effect of reaction time on peak height

Conditions: Phenanthroline, 1,0 x 10^{-3} mol/ℓ; Ascorbic acid, 20 mg/ℓ;
Fe (III), 5 x 10^{-3} mol/ℓ

Chemical variables

pH: The reduction of Fe (III) by ascorbic acid is dependent on pH. The complex formed by the reaction pH was studied in the range of pH 3-5 (Table A5.7). pH below 3 and above 5 was not studied because Fe (III) tends to undergo hydrolysis forming Fe (III)-oxide, and ascorbic acid is less stable in neutral media. As a result pH was studied from pH 3-5. Fluctuation of results is observed after pH 3,5 (Figure 5.6). In order to favor the stability of Fe (III), pH 3,5 was chosen as maximum.

Reagents concentration: Fe (III) concentration was optimized ranging between $0,8\times10^{-4}$ –10×10^{-4} mol/L and the results are given in Table A5.8. The peak height increased with increase in concentration until $7,5x10^{-4}$ mol/L. Thereafter almost constant response was observed showing the independence of response on Fe (III). The change in response is shown in Figure 5.7. Higher concentration of Fe (III) cause incomplete reduction.

Another variable, 1,10 phenanthroline concentration was studied within the range of $0,5x10^{-3}$ to $3x10^{-3}$ mol/L. The results for optimization are given in Table A5.9. The sensitivity of the method increases with the increase in concentration as shown in Figure 6.8. However highly concentrated reagents were not tested because of low solubility of the reagent and broad peaks that started to appear from $3x10^{-3}$ mol/L. Then 3×10^{-3} mol/L was chosen as optimum

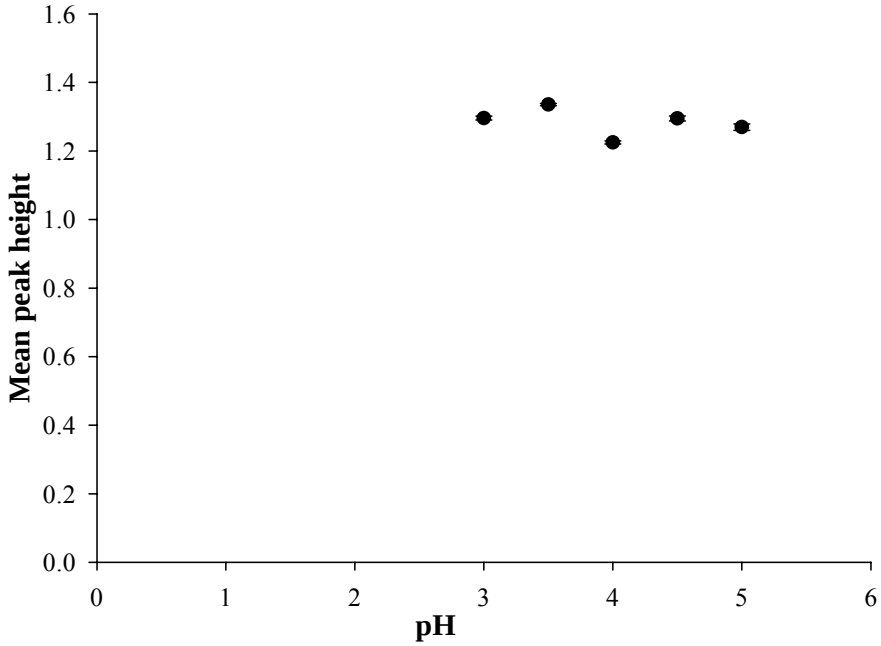

Figure 5.6 Effect of pH on the peak height

Conditions: Phenanthroline, 3 x 10^{-3} mol/ℓ; Ascorbic acid, 20 mg/ℓ; Fe (III), 7,5 x 10^{-4} mol/ℓ

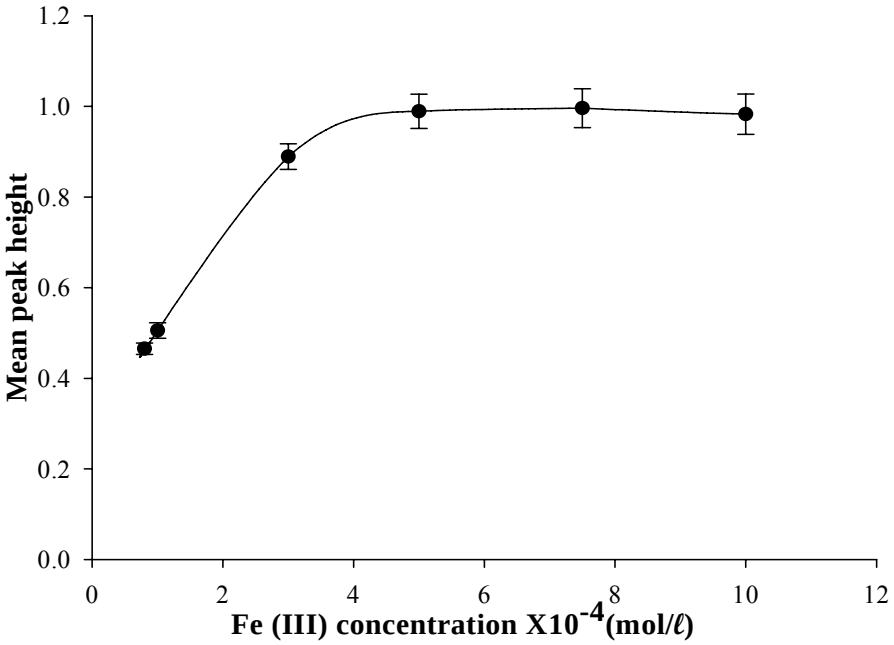

Figure 5.7 Effect of Fe (III) concentrations on the peak height

Conditions: Phenanthroline, 1,0 x 10^{-3} mol/ℓ; Ascorbic acid, 20 mg/ℓ

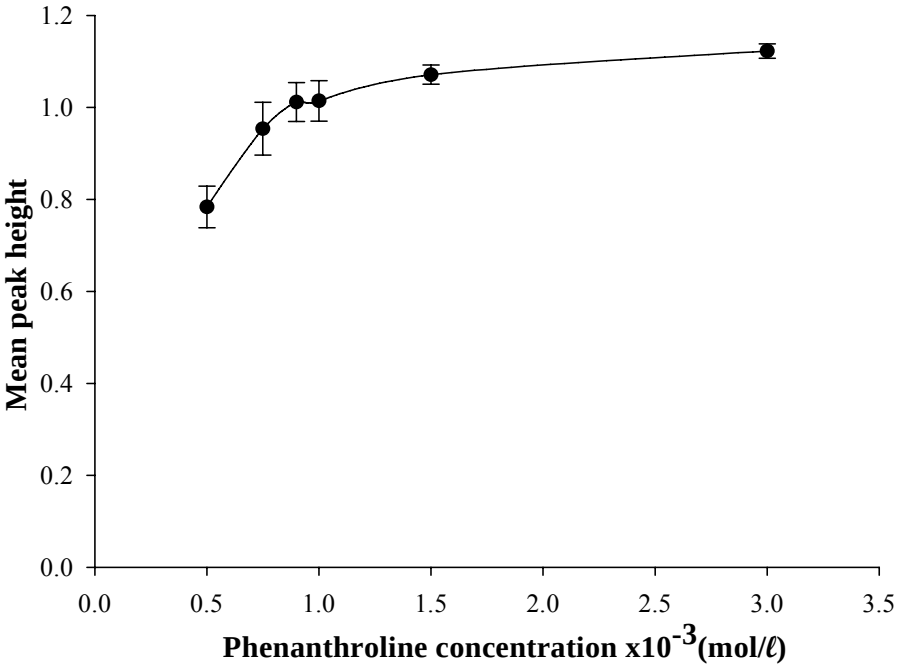

Figure 5.8 Effect of phenanthroline concentration on the peak height

Conditions: Ascorbic acid, 20 mg/ℓ; Fe (III), 7,5 x 10^{-4} mol/ℓ

5.4.2 Method evaluation

Linearity and detection limit (DL)

The calibration graph (Figure 5.9) was obtained under the optimized conditions in Table 5.2 using SIA apparatus in Figure 5.1. Linearity ranged from 5 to 30 mg/ℓ with correlation coefficient of r^2 = 0,9927 for 10 determinations. The curve is described by linear equation:

$$Y = 0{,}1184[X] + 0{,}5865$$

where [X] is the concentration of ascorbic acid and Y is peak height. Using equation 3.1, detection limit of 0,06 mg/ℓ was obtained.

Table 5.2 Optimum conditions used for evaluation

Parameter	Optimum value
Flow rate (mℓ/min)	2
Reaction coil length (cm)	50
Fe (III) reagent volume (µℓ)	750
Phenanthroline reagent volume (µℓ)	1000
Sample volume (µℓ)	750
pH	3,5
Fe (III) reagent concentration (mol/ℓ)	$7{,}5 \times 10^{-4}$
Phenanthroline reagent concentration (mol/ℓ)	3×10^{-3}
Reaction time (s)	30

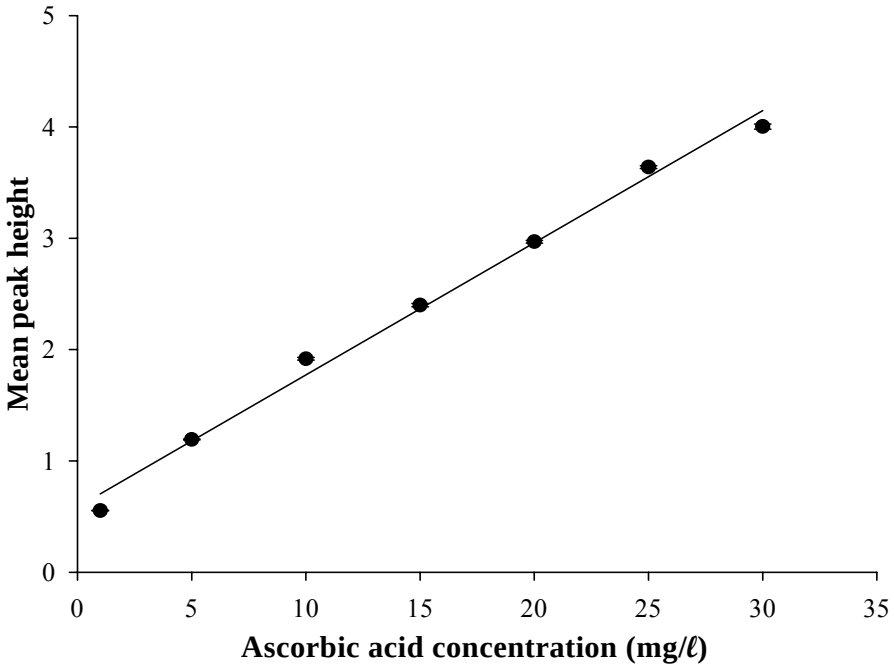

Figure 5.9 Linear curve for standard solutions of ascorbic acid, under optimum experimental conditions.

Accuracy and precision

The above, optimized method was applied for the determination of ascorbic acid in sweets, tablets and fruit juice. The accuracy of the method was checked by comparing the results of the method used with the claimed values of the samples and N-bromosuccinimide method (reference method). The results are summarized on Table 5.3 with good agreement. Real samples were spiked with known concentration of standard ascorbic acid solutions and recovery calculated using equation 5 was between 99,6 and 114 %. The precision was studied by using ascorbic acid standard solution of 15 mg/l with 10 determinations under optimized conditions. Relative standard deviation of 1,3 % was obtained.

Table 5.3 Results obtained from five determinations of ascorbic acid in tablets, sweet and orange juice

Samples	Claimed	SIA	Reference method
1. Scorbex	500 mg/tablet	504 mg/tablet	500 mg/tablet
2. Cal-c-vita	1000 mg/tablet	1008 mg/tablet	1003 mg/tablet
3. Vita-c	60 mg/sweet	69 mg/sweet	64 mg/sweet
4. Orange juice	40 mg/ml	39 mg/ml	40 mg/ml

Interference study

The effect of common species in sweets, fruit juice and tablets was studied and the results are presented in Table 5.4. The tolerance limit was defined as the concentration that causes the error of ±5 % of the absorbance obtained for 10 mg/l ascorbic acid. No interferences were observed up to 100 folds excess of glucose, fructose and calcium. Vitamin B6 and tartaric acid interfered when their concentrations exceeded that of the ascorbic acid. Citric acid and malic acid interfered at all concentrations. This might be due to the fact that they all act as reducing agents.

Table 5.4 Percentage (%) signal of foreign species for the determination of ascorbic acid (Ratio of ascorbic acid: interfering species))

Foreign species	1/1	1/10	1/50	1/100
Citric acid	92	88	61	53
Malic acid	90	90	86	84
Tartaric acid	95	93	90	85
Glucose/Fructose	100	100	101	105
Vitamin B_6	99	89	87	55
Ca^{2+}	99	97	96	95

5.5 CONCLUSIONS

Photochemical method for ascorbic acid determination has been determined by FI technique [3]. FI methods are consuming lot of reagents and require number of channels. The SI method with photochemical reaction method enhances the sensitivity of the determination and reduces the analysis time of the determination by reducing Fe (III) directly in the SIA system. The adaptation of photochemical method to SI technique provides a very fast and simple way to determine low concentrations of analytes without time consuming mixing step in mixing chamber.

Colored samples did not affect the method since they were highly diluted before the analysis. Citric acid and malic acid are usually present in the samples. Because of their reducing properties they tend to interferes with ascorbic acid determination. Therefore it is advisable to eliminate

photochemical method for sample with high ascorbic acid concentration since the iron (II) and the 1,10-phenanthroline-reaction method is sensitive. The lack of statistically significant difference between the results from the two methods verifies the accuracy of the results obtained by 1,10 phenanthroline reagent methods.

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CHAPTER 6

FINAL CONCLUSIONS

6.1 INTRODUCTION

Quality control is very important in process environment, hence the need for routine analysis. Flow systems have made progress in replacing the manual operations in process environment, introducing automated, reliable and flexible systems. This dissertation focused on environmental, food and pharmaceutical analysis because there are established regulations on maximum limits of their contents and their quality control is very important. Sequential injection analysis (SIA) principles fulfill the economic aspects of the modern analysis and it was chosen for the application. It offers fast and simple analysis that can be automated and manipulated and offers economic analysis that minimizes the consumption of expensive reagents hence reduction of waste production.

Most importantly SIA system is flexible to employ stopped flow method at any time and at any point. Stopped flow method allows increased sample contact time resulting in optimized sensitivity. Combination of SIA with stopped flow method is more advanced compared to FIA due to SIA discontinuous nature which enables the flow to be stopped and restarted by computer control of the pump during the analysis. SIA incorporates various detection methods into simple, versatile and automated measurement system. The applied methods used UV-VIS spectrophotometer as a detection mode in all analysis presented here.

To prove this advantages SIA technique has been combined with spectrophotometric technique to monitor Fe (III) in ground, tap and mine

water samples, HCHO in waste water and ascorbic acid in tablets, sweets and orange juice. Conclusions drawn from each case are given below.

6.2 DETERMINATION OF IRON (III)

The research started with the simple, single reagent system employing non-stop flow method (except when changing from one port to another) comparing two reagent methods. It is observed that the analysis time for both methods was shorter offering the sampling rate of 72 samples in an hour. The sensitivity of the methods was found to be different with 0,1 and 0,2 mg/ℓ for Tiron and thiocyanate respectively and improved as compared to other Tiron and thiocyanate methods. Good results were obtained from both methods. The Tiron method offered better sensitivity and detection limit whereas the thiocyanate method offered better precision.

Both methods showed a wide linear range as compared to other Fe (III) determination methods and compared well with reported reference methods. In terms of general application and reliability both methods work very well in acidic medium, are convenient to use with cheap and common reagents but thiocyanate is not environment friendly because it is harmful to aquatic organisms. However the use of SIA minimised the waste that need to be disposed with average of less than 3 ml per determination. SIA simplified the method because no pre-treatment was required before analysis.

6.3 DETERMINATION OF FORMALDEHYDE

The use of stopped flow method provided a convenient means of increasing the sensitivity of the methods then achieving lower detection limits of 0,06 mg/ℓ proving the feasibility of SIA kinetic method to determine analytes at trace levels. Complex manifold has been reported to be the problem of flow

injection analyzer (FIA) when more than one reagent is used where more channels are required to accommodate additional reagents. Using SIA in work with three reagents no physical configuration or addition of channels was necessary and no special technique was required to perform kinetic determinations. Reaction time was increased without increasing a reaction coil and the dispersion and good sampling rate of 24 samples per hour was obtained. Without increasing the dispersion waste production was also decreased.

6.4 DETERMINATION OF ASCORBIC ACID

In this chapter the two reagents (ascorbic acid and Fe (III)) were stopped in the holding coil to allow the reduction of Fe (III) to Fe (II) by ascorbic acid under UV-light irradiation. Irradiation allowed the reduction of Fe (III) before reaching the reaction coil where the complete reduction and reaction with phenanthroline reagent takes place.

The reduction process was successful in improving the sensitivity of the method giving the detection limit of 0,06 mg/ℓ demonstrating the feasibility of using this approach. The flexibility of SIA employing stop flow photochemical method allowed reduction process to be accomplished via flow programming avoiding manual addition process and hence a decrease in analysis time. Slight interferences were observed with this method from substances present at the similar concentrations as ascorbic acid but they can be reduced by using non-photochemical SIA method.

In conclusion, application of SIA to this work has been successful in spite of the two disadvantages associated with it. Undoubtedly its application to various fields with complex matrices will continue to be most interesting area of research.

APPENDIX A

OPTIMISATION DATA

Table A3.1 Optimization of flow rate

Flow rate (mℓ/min)	Mean peak height	% RSD
0,7	1,2446	4,67
1,5	1,9766	0,63
2	2,1704	3,17
3	2,4695	1,67
3,5	2,4778	3,14

Table A3.2 Optimization of reaction coil length

Coil length (cm)	Mean peak height	% RSD
45	3,4570	1,22
50	3,3574	3,20
65	3,1570	2,91
85	2,6741	1,17
90	2,9299	1,43
110	2,3212	3,98

Table A3.3 Optimization of sample volume

Sample volume (µℓ)	Mean peak height	% RSD
50	1,7956	1,43
100	2,4475	2,69
150	3,1035	1,93
200	3,5112	2,31
250	4,1152	1,20
300	4,4488	1,15
350	4,6365	1,31
400	4,8816	0,96

Table A3.4 Optimization of reagent volume

Reagent volume (µℓ)	Mean peak height	% RSD
50	7,6919	1,56
100	9,0166	1,96
150	9,1973	2,57
200	9,2344	1,32
250	9,1387	1,36
300	8,8179	1,20
350	8,5178	3,00
400	8,3452	0,37

Table A3.5 Optimization of acid concentration

Acid concentration (mol/ℓ)	Mean peak height	% RSD
0,05	4,3700	32,96
0,1	4,4660	32,59
0,2	4,9836	32,01
0,3	4,8848	31,20
0,4	4,6755	30,41

Table A3.6 Optimization of reagent concentration

Reagent concentration (mol/ℓ)	Mean peak height	% RSD
0,06	3,1621	1,51
0,08	3,3838	0,90
0,1	3,3313	1,78
0,12	3,5193	1,95
0,14	3,3401	0,88
0,16	3,5593	1,04
0,18	3,8040	1,64
0,2	3,5969	1,47

Table A3.7 Optimization of flow rate

Flow rate (mℓ/min)	Mean peak height	% RSD
1,5	1,7592	6,61
2	2,5088	1,99
3	3,5371	1,20
3,5	4,4039	2,14
4,3	5,8896	1,54
5	6,4443	3,38
6	7,0239	2,45

Table A3.8 Optimization of reaction coil length

Coil length (cm)	Mean peak height	% RSD
35	6,7527	0,88
45	7,0754	0,59
60	6,2114	0,95
90	5,8597	1,51

Table A3.9 Optimization of sample volume

Sample volume (µℓ)	Mean peak height	% RSD
50	4,8788	0,91
100	6,7257	2,45
150	7,1142	0,74
200	5,1691	2,96

Table A3.10 Optimization of reagent volume

Reagent volume (µℓ)	Mean peak height	% RSD
50	7,1142	0,74
100	8,8175	0,54
150	9,2517	0,49
200	8,4988	0,83

Table A3.11 Optimization of acid concentration

Acid concentration (mol/ℓ)	Mean peak height	% RSD
0,01	5,2195	3,39
0,03	5,1899	3,19
0,05	5,1267	2,10
0,07	5,1673	3,16
0,09	5,0581	3,03

Table A3.12 Optimization of reagent concentration

Reagent concentration (mol/ℓ)	Mean peak height	% RSD
1×10^{-3}	3,5580	0,91
$1,5\times10^{-3}$	4,0946	1,60
2×10^{-3}	4,5801	0,98
$2,5\times10^{-3}$	4,8786	0,91
3×10^{-3}	4,3361	3,16

Table A4.1 Optimization of flow rate

Flow rate (mℓ/min)	(MDPH)	% RSD
1,5	$0,09 \times 10^{-2}$	0,53
2	$7,57 \times 10^{-2}$	14,23
3	$3,22 \times 10^{-2}$	0,43
3,5	$0,04 \times 10^{-2}$	0,93

Table A4.2 Optimization of coil length

Coil length (cm)	MDPH	%RSD
90	0,0348	0,38
150	0,0326	1,69
200	0,1543	0,67
230	0,0238	3,25
310	0,0194	7,51

Table A4.3 Optimization of BG-reagent volume

BG-reagent volume (µℓ)	MDPH	% RSD
105	$0,76 \times 10^{-2}$	0,53
175	$0,64 \times 10^{-2}$	0,38
350	$1,32 \times 10^{-2}$	0,97
525	$6,54 \times 10^{-2}$	2,85
700	$0,08 \times 10^{-2}$	0,10

Table A4.4 Optimization of sulfite volume

Sulfite volume (µℓ)	MDPH	% RSD
35	$3,03 \times 10^{-2}$	6,29
175	$5,31 \times 10^{-2}$	0,60
350	$1,79 \times 10^{-2}$	1,07
525	$0,13 \times 10^{-2}$	1,32

Table A4.5 Optimization of sample volume

Sample volume (µℓ)	MDPH	% RSD
175	$11{,}58 \times 10^{-2}$	2,35
350	$17{,}51 \times 10^{-2}$	1,17
525	$10{,}44 \times 10^{-2}$	3,59
700	$1{,}72 \times 10^{-2}$	2,90

Table A4.6 Optimization of pH

pH	MDPH	% RSD
4	0,0407	0,87
5	0,0432	0,02
6	0,0341	0,06
7	0,0342	0,29
8	0,0729	3,64
9	0,0636	3,26

Table A4.7 Optimization of sulfite concentration

Sulfite concentration (mg/ℓ)	MDPH	% RSD
2	$3{,}04 \times 10^{-2}$	1,91
4	$5{,}04 \times 10^{-2}$	1,03
6	$5{,}12 \times 10^{-2}$	0,58
8	$6{,}51 \times 10^{-2}$	0,22
12	$6{,}30 \times 10^{-2}$	0,84
16	$1{,}51 \times 10^{-2}$	1,26
20	$0{,}68 \times 10^{-2}$	0,11

Table A4.8 Optimization of BG-reagent concentration

BG-concentration (mg/ℓ)	MDPH	%RSD
5×10^{-6}	$0{,}67 \times 10^{-2}$	14,45
9×10^{-6}	$1{,}10 \times 10^{-2}$	3,75
1×10^{-5}	$1{,}17 \times 10^{-2}$	6,01
2×10^{-5}	$1{,}30 \times 10^{-2}$	1,07
3×10^{-5}	$1{,}36 \times 10^{-2}$	0,19
4×10^{-5}	$3{,}33 \times 10^{-2}$	0,44

Table A5.1 Optimization of flow rate

Flow rate (ml/min)	Mean peak height	% RSD
1,5	0,4189	4,01
2	0,5698	4,78
3	0,3100	3,98
3,5	0,2642	3,43

Table A5.2 Optimization of reaction coil length

Coil length (cm)	Mean peak height	% RSD
50	0,8123	2,01
60	0,7941	1,20
70	0,7022	2,51
80	0,6426	2,55

Table A5.3 Optimization of Fe (III) reagent volume

Fe (III) volume (µℓ)	Mean peak height	% RSD
250	0,5527	4,09
500	0,6088	2,17
750	0,6147	4,82
1000	0,5542	1,66

Table A5.4 Optimization of phenanthroline reagent volume

Phenanthroline volume (µℓ)	Mean peak height	% RSD
250	0,1079	13,42
500	0,2832	12,32
750	0,4477	7,37
1000	0,4580	3,50
1250	0,3997	2,88

Table A5.5 Optimization of sample volume

Sample volume (µℓ)	Mean peak height	% RSD
250	0,4097	2,66
500	0,6427	0,89
750	0,7671	4,53
1000	0,6773	3,87
1250	0,5498	1,85

Table A5.6 Optimization of reaction time

Reaction time (s)	Mean peak height	% RSD
10	0,8004	4,15
20	0,8185	0,29
30	0,8404	0,81

Table A5.7 Optimization of pH

pH	Mean peak height	% RSD
3	1,2960	0,43
3,5	1,3354	0,26
4	1,2249	0,38
4,5	1,2949	0,55
5	1,2695	0,73

Table A5.8 Optimization of Fe (III) reagent concentration

Fe (III) concentration (mol/ℓ)	Mean peak height	% RSD
8×10^{-5}	0,4653	2,71
1×10^{-4}	0,5054	3,40
3×10^{-4}	0,8891	3,19
5×10^{-4}	0,9892	3,82
$7,5 \times 10^{-4}$	0,9962	4,30
1×10^{-3}	0,9830	4,53

Table A5.9 Optimization of reagent concentration

Phenanthroline concentration (mol/ℓ)	Mean peak height	% RSD
5×10^{-4}	0,7837	5,78
$7,5 \times 10^{-4}$	0,9537	6,03
9×10^{-4}	1,0116	4,18
1×10^{-3}	1,0412	4,33
$1,5 \times 10^{-3}$	1,0712	1,95
3×10^{-3}	1,1224	1,39