Application of Flow Injection in the Assay of Selected Substances in Food and Clinical Analysis

by

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Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in the Faculty of Natural, Agricultural and Information Sciences

University of Pretoria

Pretoria

October 1999

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SYNOPSIS

The concept of flow injection (FIA), introduced as a simple and convenient principle, has established itself as a well defined analytical technique. With achievement and improvement of FIA as a general analytical laboratory technique, on-site monitoring and process analysis becomes a reality. It introduces a great improvement in the objectivity of the analysis, accuracy of the analytical information and greatly enhances the rate of the analytical process.

The aim of this research was to determine the content of selected substances of biological importance from food industry (chloride, zinc, α-amylase), and also from the pharmaceutical industry (paracetamol). The importance of adopting FIA systems for these purposes is to increase the objectivity and the speed of their determination, and also to give the possibility of on-line assay.

Because only reliable methods can be automated, it was necessary to choose between the methods available for the assay of chloride, zinc, α-amylase and paracetamol. The next step was to optimize the FIA system, and after it to evaluate the FIA/spectrophotometric systems.

The results obtained for all substances proved a high reliability. The sample rate was high. Practically no sample interaction was recorded. These characteristics obtained for the proposed methods made them suitable to be used on-line in food and pharmaceutical industry, respectively.

Toepassing van Vloei-inspuitanalise in die Bepaling van Geselekteerde Stowwe in Voedsel en Kliniese Analise

deur

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SAMEVATTING

Die konsep van vloei-inspuitanalise (FIA) wat as 'n eenvoudige en geskikte beginsel bekend gestel is, is tans as 'n goed gedefinieerde analitiese tegniek gevestig. Met die daarstelling en verbetering van FIA as 'n algemene analitiese tegniek, het aan-lyn monitering en prosesanalise 'n realiteit geword. Dit het 'n groot verbetering in analise objektiwiteit en akkuraatheid van analitiese inligting teweeg gebring en die tempo van die analitiese proses grootliks verbeter.

Die doel van hierdie projek was om geselekteerde stowwe wat van biologiese belang in die voedselindustrie (chloried, sink, α-amylase) en farmaseutiese bedryf (paracetamol) is, te bepaal. Dit was belangrik om FIA sisteme vir hierdie bedrywe te ontwikkel, nie net om vinniger resultate te verkry nie, maar ook vir aan-lynproseskontrole.

Omdat slegs betroubare metodes geoutomatiseer kon word, was dit nodig om geskikte metodes vir die bepaling van chloried, sink, α-amylase en paracetamol te selekteer. FIA sisteme is vir die spesifieke bepalings geoptimiseer en ge-evalueer.

Die resultate vir die verskillende voorgestelde sisteme het goed met standaard metodes vergelyk. 'n Hoë monsternemingstempo is verkry sonder enige noemenswaardige monsteroordrag. Die vloeisisteme is betroubaar en behoort besonder geskik te wees vir aan-lynmonitering in die voedsel en farmaseutiese bedryf.

Acknowledgments

I give praise and honor to the Holy Trinity.

First and foremost I would like to express my sincere gratitude to one of the best scientist in the world, an authority in his field, a man who has patiently and throughly guided me through this academic journey. Professor Jacobus F van Staden, this is another notch in your belt, I believe there is still more to follow.

To Dr Raluca Stefan, you are a star, an oasis of kindness and knowledge. You made me believe in myself, your constant enthusiasm, patience and valuable aid got me this far. You are such a wonderful person. God bless you.

To my mothers, Anna, Mary and Lucy I followed your wish. A T Mulaudzi, your financial support has been great.

To my four brothers and four sisters, you have each contributed in your special way, and I cherish our bond.

Daphney and the Mishiyi family, I appreciate your support and understanding by filling up the gaps when I was away. You gave the best love. Hulisani, chemistry took two years from your tender age, I will surely pay them back, son.

To Professor J J Prinsloo for believing in me.

Bra Hopi and Makonde for being there all the time.

Bobby and Mack, you were instrumental from A to Z. Thank you. Kgomotso, it was through your encouragement, concern, and unwavering support that I have obtained this academic milestone.

To University of Pretoria, National Research Foundation and Irish government bursary, for their financial support towards their study and for making me realize my dreams.

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CHAPTER 1

FLOW INJECTION ANALYSIS - AN IMPORTANT TOOL FOR ANALYTICAL CHEMISTRY

1.1 Introduction

Flow injection analysis (FIA) is an unsegmented-flow technique that involves direct injection of a sample into a carrier stream, and controlled, reproducible partial dispersion thereof. Neither physical nor chemical equilibrium is ever reached and the operation timing is highly reproducible [1].

The principle of FIA is very simple: a liquid sample, in the form of a well defined zone, is injected into a non-segmented carrier stream that transports the zone towards a detector where an absorbance, electrode potential or other physical parameter is continuously recorded with the passage of the dispersed sample zone through the flow cell [2]. In contrast to any other technique utilizing continuous flow, FIA is based on (1) sample injection, (2) the controlled dispersion of a well defined sample zone, and (3) the exact timing of all events within each analytical cycle. This approach yields a highly reproducible readout even when the mixing is incomplete, the chemistry does not reach equilibrium, and the signal is transient.

In its simplest form, the FIA analyser (Figure 1a) consists of a propulsation unit (such as a peristaltic pump), an injection device (such as a valve or several magnetic valves), a mixing coil, and a flow-through detector. A typical recorder output has the form of a peak whose height is related to the concentration of the assayed species (Figure 1b). Microliter sample volumes and rarely more than one millilitre of reagent are consumed per assay, the result of which is available within seconds after sample injection. The miniaturization, economy of reagent and time, simplicity of experimental setup and applicability of the method to a wide range of detectors and analytical techniques was leading to a widespread use of FIA within the last number of years.

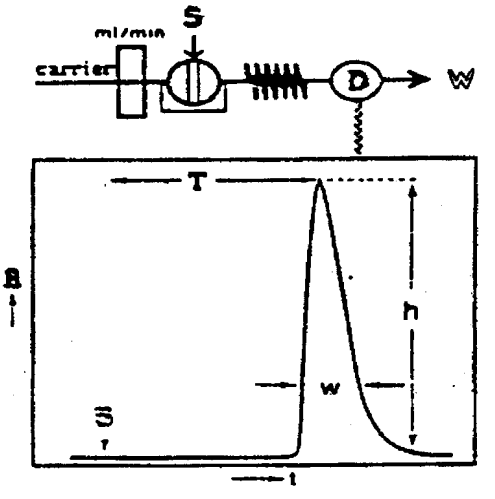

Figure 1.1 Schematic diagram of a single line flow injection analyser (a) and a typical recorder output (b). S, sample injection; T, residence time; H, peak height; W, waste; FC, flow cell [2].

The basic characteristics of FIA are the absence of are bubbles and the rapid injection of an aqueous sample into a continuously moving unsegmented carrier stream of water or reagent solution.

FIA has established itself nowadays as the method of choice in various fields as an analytical tool which is suitable for automation and increasing sample output in most analytical laboratories. FIA is well documented in more than 5900 papers. The majority of FIA publications were devoted to water, followed by environmental, food (e.g.,milk), pharmaceutical, clinical, and industrial areas [3].

1.2 Continuous flow - Flow injection. Profile comparison.

The advantages of FIA over continuous flow analysis, are: high sample throughput (1 to 400 samples per hour), low carry over; no air bubble and consequently no need to control the air bubble size and air bubble pattern, no debubbler, except for detector warm up, the system is ready for instant operation, immediately the sample is injected, read out time is 3 to 30 s, reduced pulsation because of absence of air bubbles, manifolds are easily assembled and/or exchanged; no wash cycle, robust and a high degree of flexibility in terms of sensitivity and automation.

The beneficial effect of air segmentation in conventional continuous flow analytical systems has been so obvious that the necessity of introducing air bubbles was never really doubted, although the drawbacks of its presence in the flow stream are well known: because of the compressibility of air, the stream tends to pulsate, streams have to be debubbled before they enter the flowcell, size of air bubbles has to be controlled for faster sampling rates and wetting agent is required, pressure drop and flow velocities vary in the presence of air for different tubing material, and there is a contribution to the magnitude of the lag phase reducing sampling rates.

Table 1.1 Comparison continuous flow - flow injection

	Continuous flow	**Flow injection**
capacity	up to 80 samples per hour	up to 400 samples per hour and possibility of executing "STAT procedure
start up time	max. 30 min	max 20 s
reproducibility	1 ... 2 %	1 ... 2 %
concentration read out (dwell time)	240 s or longer	max 60 s
carry over	lag phase 20 s	lag phase approaching zero, because of absence of the air bubble
reaction time	2 to 30 min	3 to 60 s
sample volume required for actual analysis	0.2 to 2 ml/min in 14 steps because the volume is dictated by the d.i. of the pump tubing	any volume between 1 µl and 25 ml because the volume is dictated by the volume of the calibrated loops. At this stage of development more sample is needed than CF
sample splitting	maximum of 24	max of 5 due to the limited available sample valves
mixing	through turbulent flow caused by wall friction by air bubble, mixing coils	through controlled diffusion processes
sample route	sample flows via the peristaltic pump to analytical manifold	sample directly into the analytical manifold
dilution	possible 1 to 50 in 1 stept and is limited by flow rate, inner diameter of sample pump tubing dialyser length	1 to 150 in 1 step is achieved through the increase of the inner diameter of stream carrier tubing, sample volume dialysis and flow rate.
dialysis	more efficient	less efficient in some circumstances due to higher flow rate
liquid extraction	possible	possible
reaction heating	more efficient	less efficient due to higher flow rate
ion-exchange	more efficient	less efficient due to higher flow rate
on-line dilution	standard with on-line dilutor	standard with on-line dilutor
distillation	more efficient	less efficient due to higher flow rate
reagent additions	more possibilities	less possibilities
titrimetry	not possible	possible
concentration ranges from one sample intake	one concentration	multiple concentration ranges from one sample possible by splitting the sample reagent stream into different pass length
wash cycle	essential	not required
detectors	all kinds	all kinds
data aquisition	recorder	computer

Although it might appear that the role of the bubbles is to divide the stream into a number of slugs, this cause wall frictions with resultant turbulent, rather than laminar flow, which is responsible for carry-over even at low pumping velocities. In contrast, FIA is based on laminar flow.

If one had to compare the parameters, and methods involved in automation, by comparing continuous flow and flow injection an improvement can be easily seen when FIA is utilized.(Table 1.1)

1.3 The aim of the study

FIA was applied in all major fields of analytical chemistry: environmental analysis, food analysis, and clinical analysis. Only reliable methods were selected for FIA. The automation of these methods will increase the rate of the sample analysis as well as the objectivity of the analysis itself.

For environmental and clinical chemistry the sample is unique; the proposed method must be reliable and objective. The quality of food is very important for the health of peoples. In the processing of foodstuffs, quality control is important. This requires fast and reliable analytical systems which need automation of the food analysis.

The method must be selected in accordance with the complexity of the matrix of the sample. To select the best method, theoretical knowledge concerning FIA and detectors are necessary. To be able to use FIA effectively it is necessary to understand the concept of dispersion [2].

It is not possible to use all the colour reactions in FIA; it is necessary to also take into account the rate of these reactions. Low rate reactions are not recommended for flow systems. Sample rate can be improved by utilization of electrochemical detectors in FIA, where the analytes are measured directly from the solution involved.

Enzymatic reactions are used successfully in FIA. They are fast and reliable when a flow-through cell is used, and furthermore when the enzymatic solution is mixed with the sample stream. This method was applied for the assay of the activity of α-amylase [4]. The importance of developing such a method for the assay of α-amylase is given by the role of it in foodstuffs (quality of bread, sugar, starch, beer, saccharification of starch to glucose, production of ethanol) and clinical analysis (occlusion of the pancreas, parotitis).

The concentration of chloride in milk is one of the indicators defining the quality of milk. A high chloride concentration indicates mastitic milk. Mastitis is recognise as one of the major disease problems concerning the dairy industry. A reliable FIA/spectrophotometric method is proposed for the assay of chloride in milk [5].

Zinc was established as an essential element which occurs abundantly in nature in different forms, it is involved in many biochemical processes and contributes significantly to human health. Zinc is essential for the functioning of over 70 enzymes that control protein synthesis and the growth and repair of cells. Zinc forms an integral part in physiological processes such as taste, appetite control and wound healing. An FIA/spectrometric system is proposed for the assay zinc in different types of beer, energy drinks, Coca Cola, steel wool, and fertilizers [6].

The determination of paracetamol in pharmaceutical formulations is essential because a supra-doze of the active substance can be responsible for the death of a patient. A method based on the oxidation of paracetamol followed by the reaction with phenol, gives very good results when it is used in FIA.

To find the best FIA system for the determination of α-amylase, chloride, zinc and paracetamol, a study of FIA modes, as well as of the FIA instrumentation was necessary.

The advantages of the proposed methods are simplicity in operation, elimination of separation steps, and rapidity of analysis.

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CHAPTER 2

THEORETICAL BACKGROUND

2.1 Introduction

It is recognized that FIA is a well known technique for the rapid serial analysis of a variety of sample solutions, but it would, however be a pity if FIA was regarded only as a means of rapid, economical handling of samples with the aim of performing the analysis of a large series of samples. There are some concepts, like the controlled dispersion of the sample zone, that must be considered.

The concept of homogeneous mixing of sample and reagent solutions has been replaced by the concept of controlled dispersion, the understanding of which allowed the development of a variety of entirely new so-called gradient FIA techniques [1].

The control of dispersion is playing the most important role for the quality of the peaks obtained in FIA, and affect the quality of analytical information. Therefore, attention was mainly given to establish the factors that affect the dispersion as well as some theoretical models concerning it.

Previously special attention was given to the convection and diffusion in a micro-flow injection system [2]. There is not much literature on numerical modeling of the dispersion of a dye injected into a carrier stream flowing through micro-manifolds. Although, a lot of work was done in the field of computational fluid dynamics (CFD), the models proposed in the field of FIA is restricted to straight macro-tubes [3,4]. The numerically models for micro-devices are not yet well established. The CFD is the only valuable technique to model accurately the fluid dynamics, presently available. Akker *et al.* [2] proposed a numerical model that provides advantages compared to the equations used in FIA to predict the dispersion. An appropriate numerically model for the micro-flow injection systems will give the opportunity to construct a more precise and reliable one. That will represent an important step in micro-technology.

2.2 Partial dispersion as a foundation of FIA

Dispersion is the dilution undergone by a sample volume injected into a flowing stream. The concept of homogeneous mixing of sample and reagent solutions has been replaced by the concept of controlled dispersion, the understanding of which allowed the development of a variety of entirely new so-called gradient FIA techniques.

A dispersed sample zone consists of a continuum of profiles in both the axial and radial directions. Depending on the characteristics of the detector used, these concentration profiles are reflected in the shape of the response curve. One may imagine that these concentrations are composed of an infinity of individual elements of fluid and it is useful to relate the original concentration of the sample solution before injection (C_0) to the concentration of the sample material in that particular element of fluid (C) that corresponds to a section of the response on which the analytical readout

is being based. Thus dispersion (D) is defined as:

$$D = \frac{C_0}{C} \tag{1}$$

or in the most frequent case, when the peak height is used as a readout,

$$D = \frac{C_0}{C_{max}} = \frac{H_0}{H_{max}} \tag{2}$$

which means that for D = 2 the sample solution has been diluted with carrier stream in a ratio 1:1 (C_{max} is the concentration of the analyte within that element of fluid that yields the peak maximum (H_{max}); H_0 is the correspondent peak of C_0).

The practical use of the concept of dispersion is now well established as it is recognized that the flow injection system must be constructed according to the type of assay to be performed. Thus limited dispersion (D = 1 to 3) is used when a FIA system only serves as a means of transport and the sample material should not become mixed with the carrier stream (e.g., sensors, atomic absorption, conductivity). Medium dispersion (D = 3 to 10) has a wide range of applications as it allows chemical reactions to occur after the mixing of reagent, present in the carrier stream, with the sample components as part of the dispersed sample zone. Presently, large dispersion (D > 10) is used only when the sample material to be analyzed is too concentrated to be accommodated within the detector range [1].

2.3 Factors contributing to dispersion

The transport of matter along the tubes of a flow system is essentially created by laminar flow and is not the result of turbulent flow.[5] There are two mechanisms that contributes to dispersion of the injected sample: convective transport and diffusional transport.

2.3.1 Convective transport

Convective transport occurs under laminar flow conditions, and produces a parabolic profile where the molecules at the tube walls have zero linear velocity, and those at the centre of the tube twice the average velocity. (Figure 2.1)

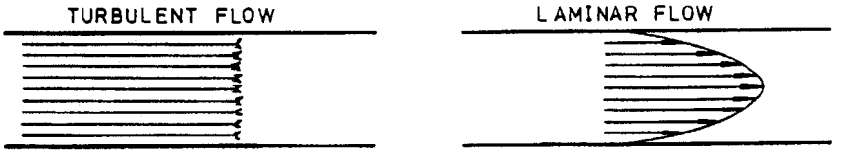

Figure 2.1 Convection transport

2.3.2 Diffusional transport

The parabolic profile produces concentration gradients that cause the sample molecules to diffuse in multiple directions, axial and radial, both at the front and at the back (Figure 2.2).

Axial diffusion is due to the horizontal concentration gradients at the leading and trailing edges of the injected sample zone. Its contribution is not significantly to the overall dispersion.

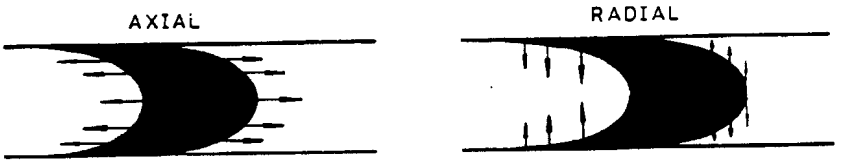

Figure 2.2 Diffusional transport

Radial diffusion results from concentration differences perpendicular to the direction of flow, and it contributes significantly to the overall dispersion. It balances concentrations in such a way that the molecules located at the tube walls tend to move to the centre, whereas those from the centre travel outwards. Indeed, this motion slows down convective transport, thus hindering progressive dilution of the zone in the carrier stream.

2.4 Quantitative evaluation of dispersion

Various attempts have been made to derive a general expression C = f(t) accounting for the signal profile and relating its characteristics (travel, residence, and baseline-to-baseline times, and peak height or area) to the experimental parameters of a FIA system (flow rate, reactor length, etc.).

A discussion follows below on the models that have been developed for laminar flow conditions, to define the theoretical principles of FIA and derive mathematical expressions, of the form C=f(t), accounting for the physical behavior of an injected plug.

2.4.1 Taylor's model

Taylor's model was the first attempt dealing with the derivatization of a mathematical description for the typical response of FIA methodology. It accounts for two different situations, namely:

(1) when concentration changes arising from convective transport along a tube, the response will be faster, and as a result the contribution of molecular diffusion is negligible.

(2) when the transport is slower relative to the period during which diffusional concentration changes to the radial direction, the response decreases to a fraction of the initial value.

Taylor's model is only valid for low flow rates and very long reactors, and as a result this compensate for radial concentration changes and enhancing the diffusion phenomena. This model justifies the Gaussian distribution defined by C = f(t) in a form which depends on the chosen parameters which are:

$$C = \frac{m}{r^2}\frac{1}{4\pi Dt}\exp\left[-\frac{(x-L)^2}{4Dt}\right] \qquad (3)$$

$$C = \frac{C_0 V_i}{q\sigma(2\pi)^{1/2}}\exp\left[\frac{(t-\bar{t}_r)^2}{2\sigma^2}\right] \qquad (4)$$

where C is the concentration of the sample at a given time, m is the injected solute mass ($m = C_0V_i$); σ is the standard deviation (Gaussian distribution); x is the axial distance from the injection point; t is the time after injection, C_0 is the initial concentration of the injected sample and V_i is the volume of the injected sample.

This model can be applied only if: $V_i << V_r$ (the injected volume is negligible compared to the volume of the reactor and only valid for low flow rates and very long reactors. Thus, this model is not applicable under normal FIA conditions [6,7].

2.4.2 Tanks - in series model

Růžička and Hansen were the first to apply their model to FIA [8]. The method bears strong resemblance to liquid chromatography in terms of theoretical plates. This method is said to be an improvement of the Taylor's model and is based on the assumption that the fluid flow passes sequentially through a large number of mini chambers where there is instantaneous mixing. The mathematical equation is:

$$C = \frac{1}{(\bar{t}_r)_N}\left[\frac{t}{(\bar{t}_r)_N}\right]^{(N-1)}\frac{1}{(N-1)!}\exp\left[-\frac{t}{(\bar{t}_r)_N}\right] \qquad (5)$$

$(\bar{t}_r)_N$ is the mean time of an element of fluid in a given tank. The method fails for small N values. The model is offering an accurate dispersion for single bead reactors as given by Reijn *et al.* [9].

2.4.3 Mixing chamber model

This model was devised by Pardue and Fields [10] and is a derivative of the Tanks - in - series model. It relies on the following steps:

(1) Chemical reactions should be fast;

(2) Sample reaches the mixing chamber undiluted;

(3) Immediate mixing of analyte and reagent inside the chamber;

(4) There is further dilution between the mixing chamber and the detector.

This model is comprising three stages:

Stage one: - characterized by instantaneous mixing of reaction and dilution which reduces the reagent concentration. The decrease in the reagent concentration due to chemical reaction and dilution is given by:

$$-\frac{dC_{bg}}{dt} = \frac{qC_{as}^{0}}{nV_g} + \frac{qC_{bg}}{V_g} \qquad (6)$$

and

$$C_{bg} = \left(\frac{c_{as}^{0}}{n} + C_{bg}^{0}\right)\exp\left(-\frac{qt}{V_g}\right) - \frac{c_{as}^{0}}{n} \qquad (7)$$

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

Stage two ($t_i < t < t_2$) The rest of the sample enters into the chamber and its initial concentration inside the chamber increases:

$$\frac{dC_{ag}}{dt} = \frac{q}{V_g} C_{as}^0 - \frac{qC_{ag}}{V_g} \tag{8}$$

and

$$C_{ag} = C_{as}^0 \{1 - \exp[-\frac{q}{V_s}(t - t_1)]\} \tag{9}$$

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

Stage three. The reagent enters the sample-filled chamber and the sample concentration in the chamber decreases due to the mass flow and chemical reaction:

$$\frac{C_{ag}}{dt} = \frac{q}{V_g} C_{ag} + n\frac{q}{V_g} C_b^0 \tag{10}$$

and

$$C_{ag} = C_{ag,\max} \exp\left[-\frac{q}{V_g}(t - t_2)\right] - nC_b^0 \{1 - \exp\left[-\frac{q}{V_g}(t - t_2)\right]\} \tag{11}$$

it is valid between t_2 and t_3 (end point), where C_b^0 is the undiluted reagent concentration in the

flowing stream; C_{ag} is the sample concentration in the chamber; q is the flow rate (mℓ/min), and V_g is the volume of the chamber.

The concentration of the sample in the mixing chamber increases to reach a maximum value at t_2 after which the concentration decreases again. The rate of the decrease of the sample concentration in the chamber depends on the value of initial sample concentration relative to the undiluted reagent concentration. The same expression is valid when the carrier stream contains no reagent and the injected sample is only diluted in the chamber.

2.4.4 Generale model (Concentration/time profile)

The general model is taking into account both convective and diffusional modes of transport.[11] It described completely the overall physical dispersion phenomena. The expression found for this model is:

$$\frac{\partial C}{\partial t} = D_m\left(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial r^2} + \frac{1}{r}\frac{\partial C}{\partial r}\right) - u_{\max}\left(1 - \frac{r^2}{a^2}\right)\frac{\partial C}{\partial x} \qquad (12)$$

where C is the concentration at the point (x, r, t), D_m the molecular diffusion coefficient of a sample plug due to transport along a tube, x the distance along the tube axis, r the radial from the tube centre, t the point in time, a the tube radius and u_{max} the maximum linear flow velocity at the tube centre.

Two mechanisms from the convection - diffusion equation contribute to sample zone deformation in laminar flow conditions. The primary convective transport deforms the whole slug by a velocity

distribution. This deformation causes, however, a secondary molecular diffusional transport phenomenon acting against the deformation as a combination of axial and radial diffusion. At the front side of the tube deformed slug there will be a considerable concentration gradient towards the tube wall, whereas at the rear side there will be a gradient towards the axis.

2.4.5 Practical definition of dispersion

Dispersion is defined from the parameters characterizing the transient signal, since the shape and position of this signal are directly related to dispersion of the sample at the detector. An FIA peak is characterized, at least qualitatively, by

(1) its position, as defined by the travel time, t_a,

(2) its bandwidth, characterized by the baseline-to-baseline time, Δt,

(3) the co-ordinates of the band maximum (T, C_{max}).

2.4.6 Růžička's dispersion coefficient

Růžička [1] introduced this dilution coefficient in 1982. It represents the ratio between the concentrations before (C_0) and after (C) the dispersion process has taken place (see equations (1) and (2)). Another way proposed for the expression of dispersion is as a ratio between the peak heights obtained for the undiluted signal (h^0) and for the recorded peak signal (h_{max}):

$$D = \frac{h^0}{h_{max}} \tag{13}$$

2.4.7 Van der Slice's expression

Van der Slice *et al.* [12] found a numerical solution for the general diffusion - convection equation by providing two simple expressions as relating to travel time and the base line - to base line time and emphasized the relation between geometric and hydrodynamic characteristics of the FIA system.

$$t_0 = \frac{109R^2D^{0.025}}{f}\left(\frac{h}{q}\right)^{1.025} \tag{14}$$

$$\Delta t = \frac{35.4R^2 f\left(\frac{l}{q}\right)^{0.64}}{D^{0.36}} \tag{15}$$

where t_0 is the travel time, Δt is the base line - to - base line time, q is flow rate, f is the accommodation factor, R is the tube radius, D is the molecular diffusion coefficient, h and l are various tube lengths.

The above equations provide time related information on the FIA signal, but has no correspondence to the C_{max} or D.

The accommodation factor (f) is critical in both equations. It is an adjustable parameter for fitting the theoretical expressions to the experimental observations [13].

2.5 Influence of various factors on dispersion

The dispersion in FIA represents the sum of the partial dispersions from the four main parts of the system [14]

$$D = D_{injection} + D_{transport} + D_{detector} + D_{connectors} \qquad (16)$$

where $D_{injection}$ represents the dispersion due to the sample volume, and geometric aspects of the injection system; $D_{transport}$ is the contribution of the reactor geometry and flow rate; $D_{detector}$ is the contribution of the flow cell geometry (shape and dimension); $D_{connectors}$ is the contribution of the connectors to the dispersion.

The most important contribution to the value of dispersion is given by $D_{transport}$

2.5.1 Sample volume

Figure 2.3 is showing that FIA signals obtained with an elementary FIA system into which increasing volumes ($V_1 < V_2 < V_3 < V_4$) of a dye have been introduced, and recorded by starting from the same position on the chart so as to obtain a series of superimposed curves. Careful scrutiny of this figure reveals that,

(1) the travel time does not depend on the injected sample volume;

(2) the residence time, and hence t', increases with the injected volume ($T_1 < T_2 < T_3 < T_4$);

(3) the baseline-to baseline time also increases with the injected volume ($\Delta t_1 < \Delta t_2 < \Delta t_3 < \Delta t_4$);

(4) the dispersion coefficient decreases with increasing sample volume.

According to Růžička, D and V_i are inversely proportional to each other, i.e.

$$D = \frac{k}{V_i} \tag{17}$$

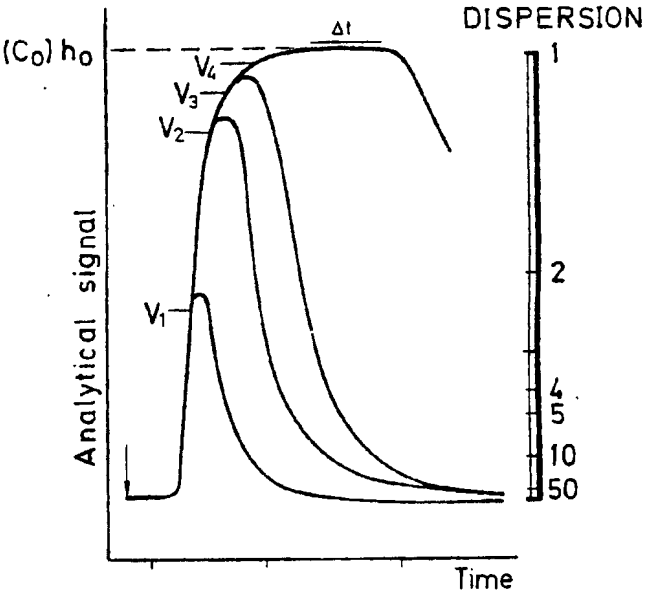

Figure 2.3 Influence of the injected sample volume on the dispersion coefficient. When the injection volume is very large compared to that of the detector, there is a time span, Δt, during which the sample undergoes no dispersion on passing through the detector.

At very high sample volumes, the peak forms a plateau at the top that differs markedly from the typical FIA peak. The dispersion coefficient is decreasing by increasing the injected volumes and also when the residence and baseline-to-baseline times are increased. When the sample volume is very large, the central portion of the sample plug remains undiluted.

2.5.2 Hydrodynamic factors

2.5.2.1 Flow rate

Van der Slice's expressions provide a relationship between the flow rate (q), travel (t_a) and baseline - to baseline (Δt) times as:

$$t_a = \frac{k}{q^{0.125}} \tag{18}$$

$$\Delta t = \frac{k'}{q^{0.64}} \tag{19}$$

It follows that the dispersion is decreasing when the flow rate increases. The proposed equations are confirmed by experiments (Figure 2.4).

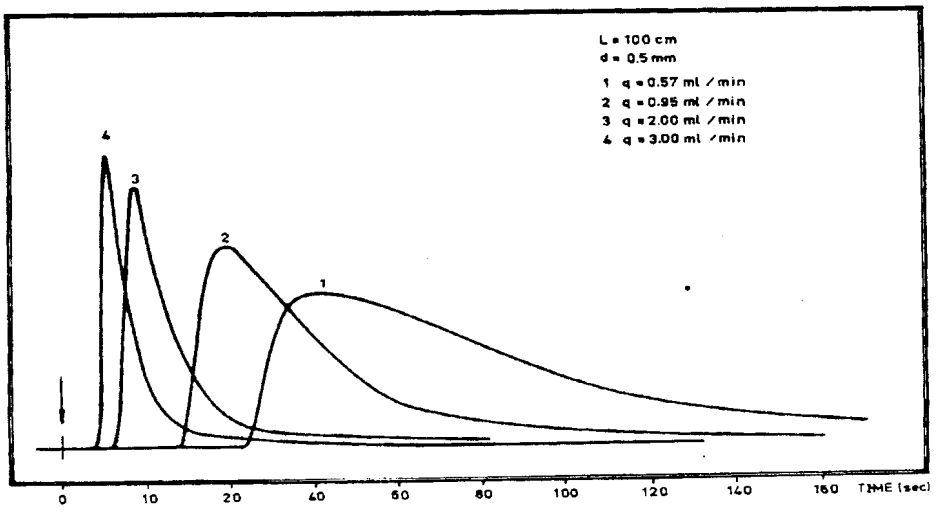

Figure 2.4 Superimposed signals obtained with the FIA system for various flow rates ($q_1 < q_2 < q_3 < q_4$)at constant tube length and tube diameter.

2.5.3 Influence of traveled distance

The dispersion starts and residence times are increased when the traveled length between the injection and detection points increases. This results in broader peaks (Figure 2.5).

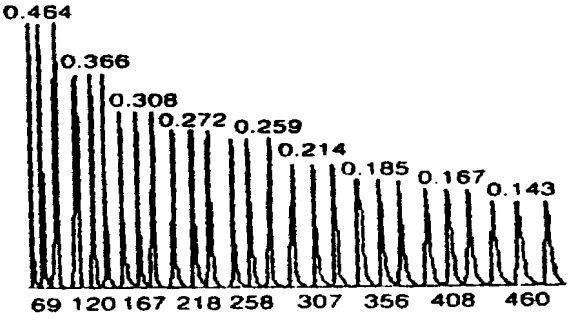

Figure 2.5 The influence of distance from injection valve to detector flow cell (cm) on the dispersion coefficient.

2.5.4 Geometric factors

2.5.4.1 Straight tubes

The lengths and diameters play a critical role in these simplest forms of reactors for FIA. Increasing the length and diameter will have an effect as the result of an increaser in dispersion - as shown by the equations given by Van der Slice:

$$t_a = kL^{1.025} \tag{20}$$

$$\Delta t = k' L^{0.64} \tag{21}$$

$$D = kL^{1/2} \tag{22}$$

2.5.4.2 Coils

Coiled tubes formed a radial type of flow due to the centrifugal force.[15] At low flow rates, the centrifugal force is not very high and the velocity is parabolic, whereas at very high flow rates; the centrifugal force is high and this forces radial diffusion. The general trend of coiled tubes is that they decrease the dispersion experienced by a sample plug normally in a straight tube of similar length.

2.5.4.3 Knotted reactors

Knotting a tube drastically reduces dispersion since there is a great similarity between coil and knotted. Knotted reactors are in essence very tight coils [16].

2.5.4.4 Packed reactors

Occasionally, FIA tubes are packed with inert solids to enhance their performance. These packings decrease dispersion in inverse proportion to the particle size of the packing. When the ratio of the inner diameter of the tube to the particle size is in the range from 5 to 50, the effect of reduction in dispersion is pronounced. If the packing is too light, high pressures arise which are not ideal for FIA.

2.5.4.5 Single bead string reactors

These types of reactors is characterized by bead diameter ranging from 60 to 80 percent of the inner diameter of the tube [17]. This type of packing decreases the peak width as it increases radial dispersion while keeping sample dispersion at an acceptable level. Dispersion is normally ten times lower under those conditions and is independent on the flow rate below about 1.5 mℓ/min.

2.5.4.6 Connector shape

Connectors defect the different solutions into a merging zone. The principle is to homogenize the sample with the diluent or conditioner. Mixing efficiency is controlled by sample viscosity and the molecular diffusivities of the solutes. T, Y or arrowhead shapes plays a major role. Dispersion follows the series as given: Y > Arrowhead > T.

2.5.4.7 Temperature

Increase in temperature has a direct proportion to the increase in dispersion. This is due to the kinetic energy that increases the Brownian movement within the tube. But this is also dependent on the nature of the reaction under investigation.

2.5.4.8 Chemical kinetics

Dispersion is not solely a physical phenomena, chemical kinetics also influences dispersion. This is achieved in two ways:

(1) when a property of the reaction product is measured, then the chemical contribution results in a decrease in the practical dispersion. The higher the reaction rate constant, the smaller is dispersion;

(2) when a property of the reactants which are consumed with time is monitored, the chemical contributes increases the dispersion coefficient. The higher the reaction rate constant, the larger is dispersion.

2.6 Dispersion in a micro-flow injection system

Akker and van der Linden [2] proposed a procedure to study the dispersion of a dye in a carrier stream flowing through micro-manifolds. At the beginning of a micro-manifold a sample is introduced (as a finite volume) into a carrier stream. The sample travel through the system undergoing dispersion. Along the flow systems detection points can be set up where the sample

concentration can be recorded as a function of time. Such a recording results in a response curve.

Navier-Stokes equations are proposed for the modeling of the dynamic behavior of fluids in a micro-flow system. The fluids used were incompressible, they have a constant density, and the assumption was made in a laminar flow regime, at 20°C. The assessment of the prevailing type of flow regime can be based on the Reynolds number (R_e):

$$R_e = \frac{Ul}{\nu} \tag{23}$$

where U is the velocity of the fluid, l is the characteristic length for the flow under consideration (diameter of the channel), and ν is the kinematic viscosity.

The comparison of the different numerical and experimental response curves was done by means of statistical moment analysis. The direct comparison is not suitable in this case because there are some assumptions made in the numerical case that makes a direct comparison irrelevant:

(1) the first assumption is that the channels are exactly 200 μm by 200 μm - this, however, is not the case. Etching downwards (12 μm) also results in a displacement sideways (2 μm). This result in a channel being 200 μm wide at the top and 234 μm wide at the bottom. The effect of the etching error will result in a higher velocity in the numerical case. The curves of the numerical flow will have a shorter residence time.

(2) the second assumption is that of the injected sample volume. Due to the method of injection there will not be a well defined sample plug placed in the carrier stream. Deconvulsion of the curve before and after the structure under observation eliminates this

problem.

(3) the use of sharp bends to establish a flow system with a certain traveling distance within a small area has minimal effects on the dispersion of a sample under the conditions described.

The proposed numerical model (Navier-Stokes equations) can be used as a numerical preanalysis tool for the determination of dispersion in micro-flow injection systems.

2.7 References

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CHAPTER 3

ESSENTIAL COMPONENTS OF A FLOW INJECTION ANALYSIS SYSTEM

3.1 Introduction

A flow injection system consists of four major components (Figure 3.1):

(1) Propelling systems;

(2) Injection systems;

(3) Transport conduits and mixing reactors;

(4) Detection and data acquisition.

An ideal assembly for a typical FIA system should allow the reproducible insertion of a volume of sample into a carrier reagent stream without altering its flow rate [1-8]. The sample volume should be modified easily, and the carrier stream should flow consistently in a pulse free manner through a conduit of uniform cross section.

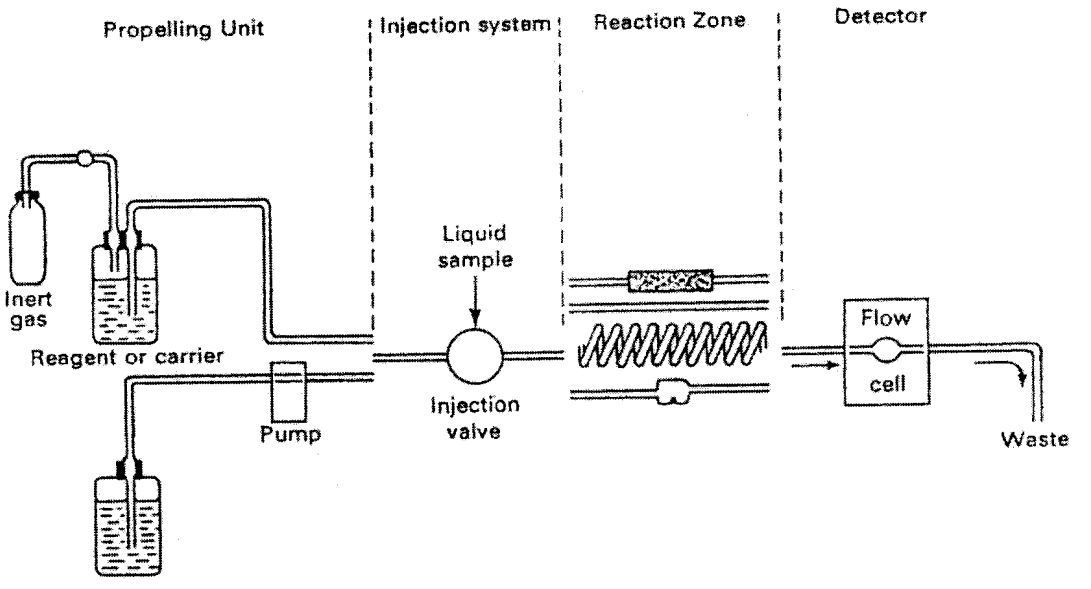

Figure 3.1 General arrangement of a FIA system

3.2 The propelling system

In transporting the stream to the detector, the propelling system must be constant and reproducible to achieve a constant dispersion and residence time. The fluids can be propelled through a FIA manifold by various mechanisms that rely on the use of a peristaltic pump, gravity and gas pressure pumps, or of an alternative type of pump for electro-osmotic flow.

3.2.1 Peristaltic pump

A peristaltic pump is the most popular propelling system (Figure 3.2). It is usually made up of channels that holds the tubes. A set of rollers simultaneously squeezes a number of flexible tubes arranged at right angles to the rollers, so that the successive flexions of the tubes forces various fluids through the tubes. The peristaltic pump does not offer a pulse free flow. The pulse can be

dampened by inserting a damping system such as a long flexible tube after the pump through which the carrier circulates before the sample is injected, or by recirculating the waste through the pump. The tubes are made from various materials, the properties of which make them suitable for use with a specified range of fluids.

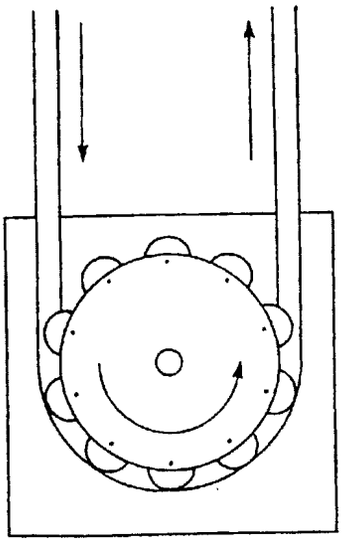

Figure 3.2 Schematic diagram of a peristaltic pump.

3.2.2 Gravity and gas pressure pumps

3.2.2.1 Gravity based propelling system

A liquid reservoir above the conduit level delivers the carrier and sets streams in motion. These pumps are applied for very simple assemblies such as a single channel manifold excluding a chemical reaction.

The pumps rely on the difference in height between the reagent and/or carrier reservoir and the

FIA flow-line. A pulse free flow is obtained and the difference in heights determines the flow rate. The most significant shortcoming of this type of propelling system is the need to maintain a constant level of the liquid(s) in the reservoir(s) in order to achieve a constant flow; this may be accomplished by using reservoirs with large cross-sectional areas. This has the advantage of an uniform and pulse free flow. The fitting of a chosen flow rate is very difficult.

3.2.2.2 Gas pressure propelling system

An inert gas is used to propel the fluids in a FIA manifold (Figure 3.3). The flow on each stream is controlled by a restrictor valve. The resulting flow is pulse free with no generated electrical interferences. The main problems with these systems are the possible bubble formation from the different solubilities of the gas in various reagents utilized. Bubbling results in flow rate oscillations and spurious responses from some types of detection frequently employed in FIA.

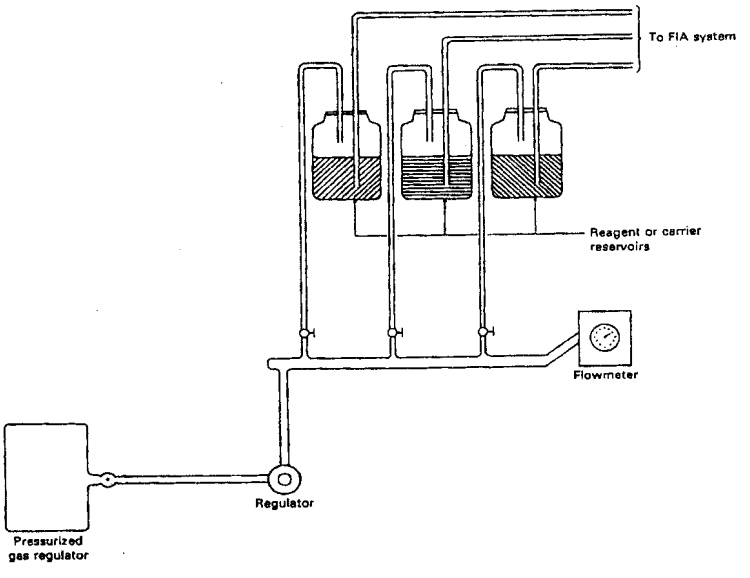

Figure 3.3 Pressure-based propelling system.

3.3 Injection system

The requirements of the injection system are that a highly reproducible sample volume is injected into a continuously flow and unsegmentated carrier stream with minimum disturbance of the carrier flow. Precision and reproducibility are quite essential for an injection system.

3.3.1 Single injection system

3.3.1.1 Syringe

This is the first injection system described by Růžička and Hansen, which relied on the use of a needless syringe (Figure 3.4) The plunger displaces a septum thereby creating a chamber accommodating the sample, which was then forced through a channel into the carrier by returning the septum to its starting position.

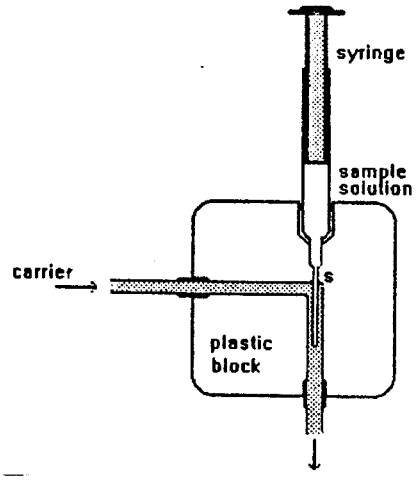

Figure 3.4 Classical way for injection the sample into the carrier stream (s is septum).

3.3.1.2 Rotary valve

The rotary valve is a typical valve used in FIA systems (Figure 3.5).

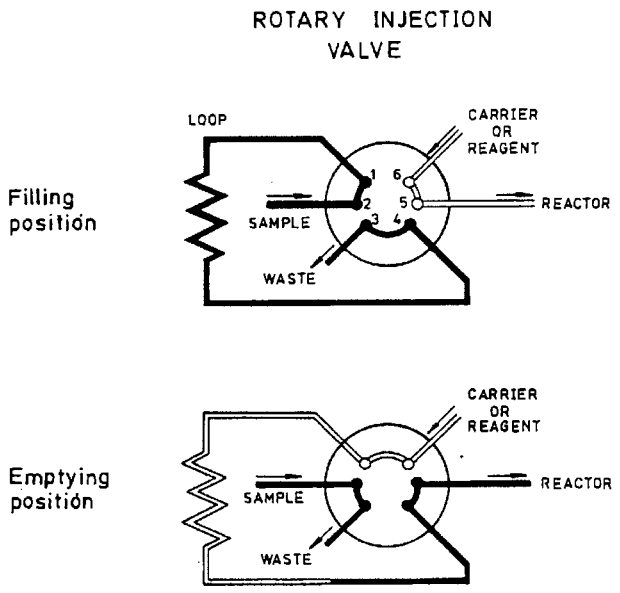

Figure 3.5 Operational scheme for a four-way rotary valve showing the sample filling and emptying positions.

This type of valve has two to ten ports which can be connected to different components of the system for single sample injection, double sample injection, dilution or pre-concentration, respectively. The one commonly employed in FIA is the valve with two ports used for load and inject operations. In load position, the sample fills the sample loop, and when switching to the inject position, the sample is flushed into the carrier to the reaction coil and detector.

3.3.1.3 Proportional injector

This injector consists of three drilled polyethylene blocks. Two blocks are fixed and one is moving. In the filling position, the two kinds of blocks coincide drawing reagents, and in the injection mode this is oppositely arranged.

3.3.1.4 Solenoid valve

The operation of such a valve is electrically controlled. The system features several operational modes, each characterized by different positions of the valves. The different valves are interconnected in an intricate pattern with respect to the overall operation.

3.4 Transport conduits and mixing reactors

This is the part that receives and passes the reagents and samples to the next level in the FIA system. It is the region where reaction and dispersion take place. They interconnect the various parts making up the working system.

3.4.1 Transport conduits

The materials used for tubing are usually Tygon, Teflon, polyethylene, and polypropylene. The internal diameter normally used is between 0.1 and 2.0 mm. The length of the tubing is dictated by the optimum experimental requirements [9,10]. A typical manifold may contain several meters of flexible tubing. It was found that to coil the tubing is convenient, because it also promote

mixing. The conduits are interconnected, and connected to other components of the system by means of connectors. These connections should be void of dead volumes, leakage, and introduction of air bubbles.

3.4.2. Reactors

The reactors are part of the FIA system where the reagents mixes and undergoes a chemical change. The resident time is spend within the reactor [11]. From the reactor the sample plug is endowed with the required characteristics for measurement by the detection unit. There are five different types of reactors which are chosen relative to the particular needs of a specific analysis to be carried out.

3.4.2.1 Open tubes

Open tubes represents the simplest reactors, straight and of fixed length. They are normally required for simple chemical reactions.

3.4.2.2 Coils

Coils are tubes which are uniformly wound helically around a support. The dispersion is influenced by their length and diameter values.

3.4.2.3 Packed reactors

This type of reactors is not very often used in FIA because of complications that arise due to their physical nature. The tubing are filled with chemically inert or active material. Chemically active packings such as ion exchange resins, oxidants, reductants and enzymes are used in FIA systems.

3.4.2.4 Single bead string reactor

The single string stream of glass is filling with inert beads 60 - 80% from the tubing but it is not filling more than 65% of the cross section of the tube. The flow meets the least resistance. Their performance has been explained by van den Berg *et al.* [12].

3.4.2.5 Mixing chambers

A mixing chamber is sometimes used when a great extent of mixing and complete sample - reagent homogenization is required. The large difference in viscosity, specific gravity, temperature or detergent content between the carrier and the sample through the mixing chamber takes place [13]. Mixing chamber systems have a lower sampling rate.

3.5 Detectors

The requirements of the detector are those normally associated with analytical instrumentation in general, namely high sensibility, rapid response, low noise, good stability, wide dynamic range, and it should make a minimal contribution to the dispersion effect. The detection limit and the

sensitivity of the detector must be taken into account the concentration level of the analyte in the sample. The sensitivity of the detector must be connected with the sensitivity that the selected FIA mode can assure.

The selectivity of the detector is a factor of major importance in its selection for a FIA system. Due the non-equilibrium status of the conditions of analyte measurement, the selectivity can be improved, but not hundred percent solved in many of the cases. Sometimes, the selection of another reagent or/and detection can solve the problem of selectivity.

The detection system selected for FIA must be reliable. The variety of detectors employed reflected the significance and widespread use in conventional analytical technique [14,15].

3.5.1 Electrochemical detectors

Electrochemical detectors are among the best incorporated into FIA systems, because no colour reaction is involved. They can be used from high concentration levels (potentiometric ones) up to low concentration levels (amperometric ones). They respond to the concentration of the analyte in the fluid, that is immediately adjacent to the sensing surface. It follows that the cell must be constructed in such a way that the concentration in the center of the zone should be sensed [16].

Electrochemical detectors are classified taking into account the geometry of the system:

(1) Annular detector - a sensitive tube is inserted into the flow line;

(2) Wire type detector - an electrode placed with its surface along the axis of flow;

(3) Cascade type detector - the flow impinges frontally (ion-selective electrodes) or spherically (dropping mercury electrode).

3.5.1.1 Amperometric

Amperometric detectors are those with a high sensitivity and also with a very low limit of detection. Modern polarographic techniques were considered as detectors systems for FIA [17]. with dropping mercury electrodes, due to the possibility of a continuously renewed surface, preferred. They assure a stable sensitivity and baseline level and were recommended for the determination of physiological active compounds.

Amperometric biosensors proved to be ideal detection systems in FIA for clinical analysis [18-20]. The reproducibility is higher, and the selectivity increases considerably due to the high selectivity of the enzymatic reaction involved.

3.5.1.2 Potentiometric

Ion-selective membrane electrodes (ISME) were chosen to be inserted in FIA systems in the potentiometric detector mode [16-18, 21-26]. Their sensitivity is not so high. They can be used generally for the assay in the 10^{-1} - 10^{-5} mol/ℓ concentration range. The non-equilibrium measuring conditions improves their selectivity.

3.5.1.3 Redox

Karlberg and Thelander proposed redox detectors for FIA systems [27]. Their sensitivity is not so good as that achieved even with ISMEs. The selectivity is a problem that was not solved for their utilization as detectors in FIA systems.

3.5.1.4 Ion-selective field effect transistor (ISFET)

An ISFET detector was proposed for a FIA system by Satoh and Aoki [28]. The sensitivity obtained for the assay of zinc can be compared as magnitude order with the ones obtained using amperometric biosensors (submillimolar level). The selectivity of this type of detection system was improved by utilization of an enzyme in a layer that covered the electrode (pH-FET).

3.5.1.5 Coulometric

The principle of these detectors is the measurement of the integral of the current - time output obtained on injection of a small volume of solution containing electroactive species into a supporting electrolyte stream with minimal mass transport.

3.6 Optical detectors

Optical detectors are intensively used in FIA systems. Morever, their reliability is more or less the same as those achieved by the utilization of electrochemical detectors. For some of them (chemiluminescence, fluorescence, ICP-MS), the sensitivity is less than that of amperometric

detectors. That fact made them more suitable for trace analysis. Their selectivity can be increased by the utilization of an enzymatic or immunoreaction.

3.6.1 Photometric

The base of this detectors is the determination of absorbance per unit length. The only limitation of its utilization is the difficulty in construction of an efficient photometric cell [29,30].

3.6.2 Atomic spectrometry

Atomic spectrometric techniques proved to be good in FIA systems due to their high sensitivity and due to their selectivity [31]. Chatterjee and Irgolic proposed the insertion of a hydride generation atomic absorption spectrometer in the conduits of a FIA system [32]. The main advantage is its reliable utilization to the assay of selenium.

3.6.3 ICP-MS

In comparison with absorption spectrometry, ICP-MS is a total technique (information for all the components of the sample can be obtain at the same time), is more sensitive and selective. Therefore, its utilization in FIA systems was extended recently [33,34].

3.6.4 UV/Vis spectrometer

The UV/Vis detectors are by far the most used in the conduits of FIA systems [35,36], mainly due

to the simplicity of the cell construction of the detection system. There are also numerous reliable reactions that can be automated. A high precision and reliability with a high speed of the analysis of analyte from a matrix is obtained.

3.6.5 Diode array

The advantage of diode array over the UV/Vis spectrometer is the possibility of scanning of a full range of wavelengths [37,38]. This property made the detection of more than one analyte possible using the same reagent, if the colours of the compounds (complexes) obtained are not the same.

3.6.6 Fluorescence

Fluorescence detectors cannot be used for the detection of all types of substances. The reaction responsible for the development of fluorescent compounds are very sensitive. These types of detectors can be used successfully in a conduits of a FIA system for trace analysis [37-39].

3.6.7 Chemiluminescence

Chemiluminescence detectors have a larger domain of utilization than fluorecence. Their sensing is based on the reaction of H_2O_2 with luminol. Rarely, another reaction is preferred. To improve the selectivity, enzymatic or immunoreactions are proposed for peroxidase development. The limit of detection can reach 10^{-18} g. This type of detectors can be successfully used in the conduits of an FIA system [40-46].

3.6.8 Optical sensors

Flow-through sensors are recommended as optical sensors in FIA [47]. The way in which the flow-through cell is constructed increased the sensitivity of the optical detection system. The reliability also has a high value when optical sensors are used as detectors for FIA.

3.6.9 Radiochemical

The automatization of radiochemical detectors is one of the most important tools of FIA due to the high contamination of the system operator during the analysis [48]. A flow-through scintillation detector cell is proposed. The reliability of the analytical information obtained using FIA - radiochemical system is good.

3.7 FIA signal

This is the signal output of a FIA system, from which any meaningful deductions and calculations with regards to quantitative analysis can be done. The basic parameters that delimits on a FIA transient signal is the peak. The height or width of the peak is evaluated with respect to baseline restoration time which determines sensitivity and sample throughput. The quality of the peak is directly related to the extent of dispersion.

3.8 Computerized control and data acquisition

Data acquisition and computer aided flow analysis was obtained from the FlowTek package

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

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

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CHAPTER 4

FLOW INJECTION ANALYSIS MODES

4.1 Introduction

FIA is a technique that has caused a great impact in analytical chemistry [1,2]. The simplest FIA mode is called elementary FIA [3]. This simplest form of FIA cannot always be applied with high precision for analytical control in clinical, agricultural, pharmaceutical, environmental and industrial processes. This led to the development of different derivative FIA modes [4]. In principle there are three distinct derivative FIA modes: gradient techniques, kinetic methods, and two phase modes. The aim of FIA modes is to derive more analytical information than is possible by simply measuring the area or height of a single peak [5]. The coupling or an introduction of a large variety of physical and chemical principles has greatly enhanced application of FIA to different analysis. The complexity of the conduits depend on the type of reaction required for a particular determination. A detailed description of the different FIA modes and their exploitation has been outlined by Valcarcel and Luque de Castro [6]. The advantages associated with the different modes are quite numerous ranging from economical implications, reduced sample and reagent volumes, improved sensitivity, acceleration of the sampling rate, widening of the linear concentration for spectrophotometric determinations, simultaneous determination and no release of dangerous fumes into the environment [7-15].

4.2 Elementary Modes.

This is the simplest and most widely applied form of FIA. It involves a single injection of a sample into a carrier stream where there may or may not be a chemical reaction. Elementary modes can be differentiated into various types distinguished by the number of channels within the system. These are single, double, triple, and those with more than three channels.

4.2.1 Closed-loop systems

In this FIA mode there is continuous recirculation of the solution with no waste outlet. This type of system may feature a regenerating unit which in some cases acts as a separator of species or reaction products [16]. This derivative of FIA has three versions :

(a) systems without a regeneration unit;

(b) systems with either a regeneration unit or removal unit;

(c) systems with both a regeneration and removal units.

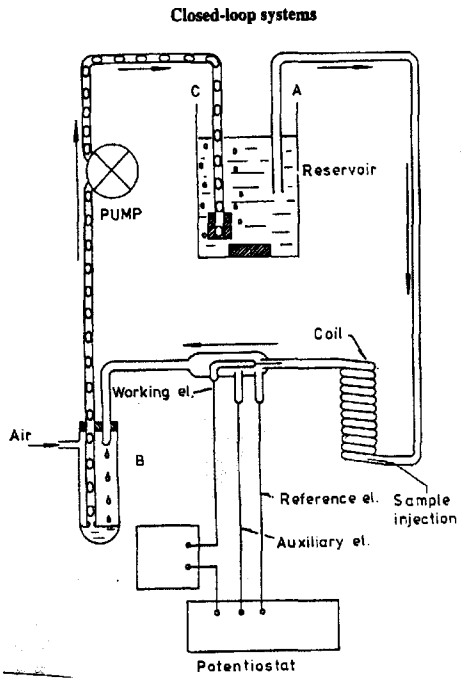

Figure 4.1 Closed-loop flow system for enzymatic determination of substrates by measurement of the level of dissolved oxygen with the aid of a three-electrode system.

4.2.2 Automated sample treatment

In most of the cases the sample undergoes a series of operations to render it suitable for either qualitative or quantitative analysis. With automatic set-ups this step can be directly linked to FIA systems as a preliminary step or wholly integrated into it. Sample treatment can be aimed at, preconcentration or dilution, removal of interferences and protection of the analytical transducer. In the case of solid samples they can be directly introduced into the FIA manifold and the component of interest isolated by leaching with an appropriate solvent. More about this and related subjects will be discussed under two-phase modes of FIA.

4.2.3 Parallel FIA

Channels are arranged parallel to each other leading to a single detector. The transport tubing bifurcates at an alternating pump following the injection system. The operation of this system is as follows, each sample is stopped, the valve turned, the sample in the other channel is detected, the next injection takes place, and the pump is again stopped and the valve turned. This mode has the advantage of fast analysis. This method was introduced by Růžička and Hansen[17] and has not as yet been fully explored with regards to chemical reactions.

4.2.4 Wash-out effect

This system employs the use of two pumps, the overall determination rate is accelerated by increasing the washing speed in the reaction coils and the flow cell. There is a reagent and wash solution pump. These pumps alternate with the wash pump operating last and consequently rinsing the conduits. The stopping of the reagent pump at certain stage reduces the amount of reagent solution that would otherwise be used.

4.2.5 Merging zones

This method was developed with the sole purpose of saving reagent and sample solutions when the system is stopped or started. The rational behind this is the injection of the sample and introducing the reagent solution in such a way that the sample zone meets the selected section of the reagent stream in a controlled manner and this is better achieved when the rest of the system is filled with the wash solution. A summary of events within this method are:

(a) injection of sample into a suitable carrier;

(b) introduction of reagent plug either into sample channel or separate channel;

(c) a point where plugs merge and mix for detection.

Merging can be achieved in three ways: by use of two pumps and a single injector, by synchronize multi-injection through a multi valve, and by intermittent pumping with synchronized multi-injection.

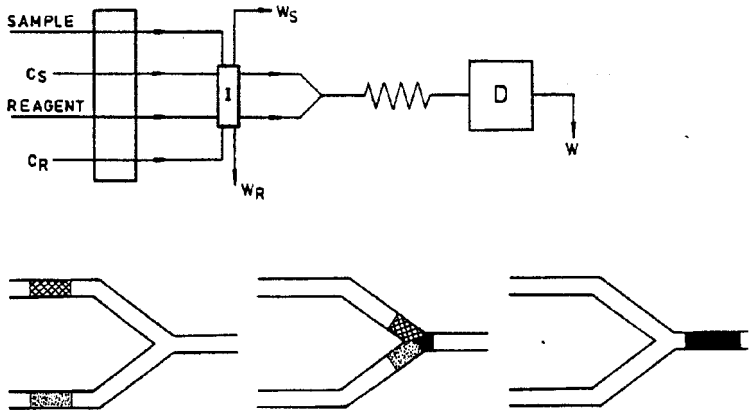

Figure 4.2 Merging zones by synchronized multi-injection. Both loops of the dual injector are filled with sample and reagent, and these are injected simultaneously into separate channels which lead both plugs to a merging point

4.2.6 Zone sampling

A well defined aliquot of a processed sample is introduced into a carrier stream, and after a time span has elapsed (Δt), an aliquot of the sample dispersed zone is withdrawn at a point and

introduced into a second carrier stream. This introduces a second zone which is first processed and measure on a flow detector and then discharged to waste. An example of this mode of FIA is given by the determination of calcium as described by Jacintho *et al.* [18].

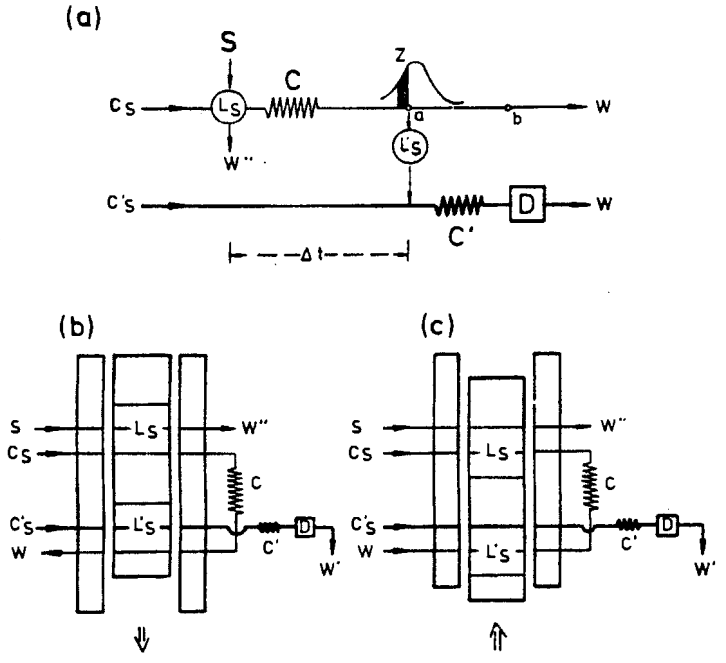

Figure 4.3 Zone sampling or re-injection

4.2.7 Zone trapping

This is a variant of the zone sampling mode where the reacting plug can be separated from the flow for a definite period to allow the reaction to occur until a specific extent is attained without the need for very long reactors.

4.2.8 Reversed FIA

The sequence of events in this mode of FIA are exactly the opposite of the conventional FIA operation. The sample is the one that is continuously circulated through the manifold and the reagent is the one that is periodically injected. The method suffers from two limitations in that it has a low sampling rate and high sample consumption, the sensitivity is however greatly increased.

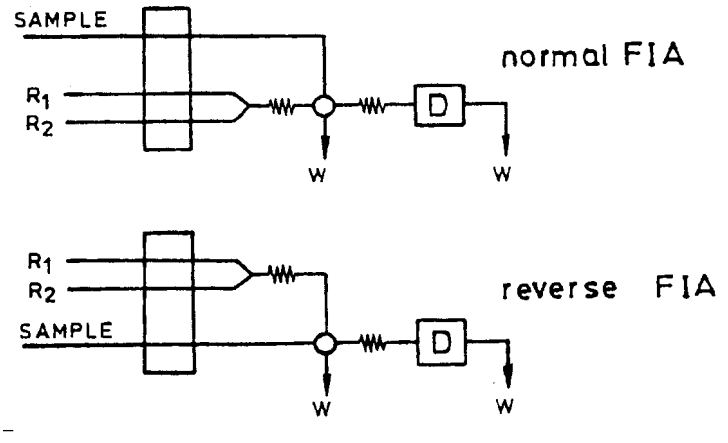

Figure 4.4 Comparison of normal and reversed FIA modes.

4.2.9 Simultaneous multi-determination

The conduits of this type of system are structured in such a way that they can be used to determine a variety of analytes at the same time through a single FIA system. The ideal assembly for multi-determinations should be fairly simple and used reliably, straightforward chemical methods preferably with a single detector and injector. Simultaneous determinations can be classified according to the number and arrangement of the detectors. These detectors can be arranged in series or be parallel to each other. A further distinction is achieved by the number and

mode of injection of the sample, that is whether the injection is sequential or simultaneous. A number of researchers have tried this mode of FIA on a variety of samples.[19-23] A method closely related to multi-determination is speciation which is the determination of individual ionic or molecular forms of a given element in the sample.

4.2.10 Miniature systems

Miniaturisation seems to be the major trend towards which all modern analytical chemistry is directed. This is achieved in one of the two ways, either through capillary FIA or through integrated micro conduits.[24-25] To achieve the reduction of a FIA system the size of some usual elements should be reduced or new micro systems for integration into manifolds should be developed. The capillary system makes use of narrow-bore transport tubes which offers the advantage of a high sampling rate and low reagent consumption. Solid-phase mini reactors, electro-osmotic flow systems, air-pressurized pumps and capillary tubes have made the miniaturisation system possible.[26] Miniature systems use drastically reduced sample volumes and this coupled with their small size facilitates construction of pocket analysers for monitoring of industrial processes or bed side patient monitoring.

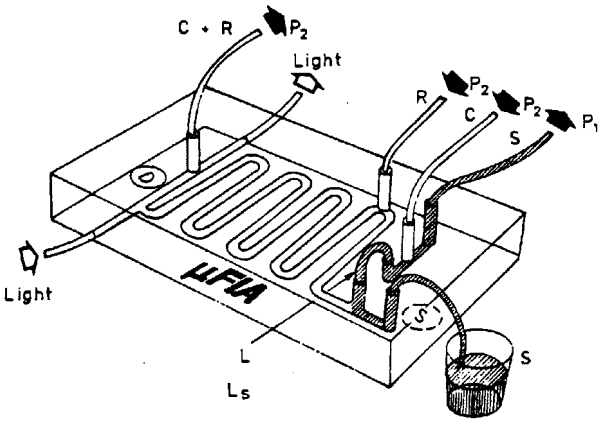

Figure 4.5 Integrated micro conduits for the sample is aspirated through pump P_1 into the sample loop, L_S(20μl), while the solution in the channel operated by pump P_2 are at rest. The stop and go of P_1 and P_2, respectively, sweeps the sample zone towards the straight portion of the conduit, which constitutes the flow cell (D), through which light is transmitted by an optical fibre.

4.2.11 Automated FIA

FIA systems are not strictly automatic, but they can easily be automated. An automatic FIA system is one that has an automatic injection system since all other elements of the FIA system work in a continuous manner. The microprocessor is the " brain" behind an automatic FIA system that can be linked through two ways interfaces: the passive one which is an alternative to signal reception by a pen recorder, it is connected to the detection unit and through it the microprocessor collects the detector output for data handling. It operates by selecting data and relating signal strength as a function of time, run repetitive determination for statistical treatment

from the same sample, and printing of collected and processed data. The active interface is responsible for the microprocessor to fully participate in the automated process. The application of automated systems has been tried in various fields of analysis [27-30].

4.3 FIA gradient techniques

The FIA gradient technique mode was described not very long after the original and conventional method was introduced. There was a just a two year span between the two stated methods. In conventional FIA systems, the trend is to derive analytical information from peak height for quantitative determinations. In gradient techniques, however, this trend is not followed as the information is collected from the rest of the recorded signal and are based on the concentration gradients created at the double interface carrier-sample-carrier. Measurements for this technique are usually made under conditions other than the optimum for achieving maximum sensitivity. The measurements are made on the initial or final portion of the FIA curve. Since measurements are not made from the peak height it follows that dispersion for this mode (D_g) will always be higher than (D_{max}) from the normal FIA. Gradient techniques can be divided into those in which measurements are performed before or after the residence time. In dealing with this mode, timers or microcomputers are required for measurement without significant errors.

4.3.1 Gradient dilution

Diluting the sample is necessary whenever the analyte concentration must be accommodated within the optical range of the instrument's measuring scale. Dilution can also decrease the effect of foreign components. For dilution to be done electronically the measurements must be made at

a delayed time interval (t) which allows dispersion to occur compared with the residence time (T). Since (t) is greater than (T) it follows then that the measurement zone is the portion of the tail of the recorded signal. For the method to be effective there must be a linear relationship between the response and sample concentration, no inhibitory effects, and reagents should be in excess. The method suffers from loss of sensitivity, lower sample throughput, and expensive data collecting equipment.

4.3.2 Electronic calibration.

The main aim of electronic calibration is to relate the signal output to the concentration of a sample. This can only be achieved if there is an established relationship between the peak height, time of measurement and concentration of the sample. Advantages of this method are, fast calibration, high precision, and any deviation that arises due to a chemical reaction can be cancelled out during the sole standard running for calibration. A typical example of this technique has been described by Kuban *et al.* [31].

4.3.3 Stopped flow

This is achieved by intermittent pumping where the pump can be stopped and re-started at exactly controlled times. The development of the reaction can be followed by continuously recording the stopped-flow signal for example in cases where the sample zone is stopped within the flow cell.

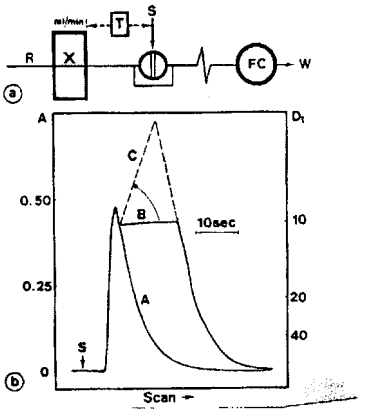

Figure 4.6 (a) Simple stopped flow FIA manifold. When the sample S is injected, the electronic timer T is activated by a microswitch positioned on the injection valve. The time for injection to stopping of the pumping and the length of the stop time can both be preset on the timer. (B) The principle of the stopped flow FIA method as demonstrated by injecting a dyed sample zone into a colourless carrier stream and recording the absorbance by means of a colorimetric flow-through cell. All curves were recorded from the same point (S) by injecting 26 µl of the same dye: (A) continuous pumping; (B) 9 s pumping, 14 s stop, and continuous pumping again; (C) the dashed line indicate the curve which would have been registered if any chemical reaction had taken place within the flow cell during the 14 s stop interval.

The obvious prerequisite for such a reaction rate measurement to be reproducible is that the movement of the carrier stream can be controlled from the operational pumping rate used to complete standstill, and in this manner the same section of the sample zone can always be held reproducible within the flow cell for measurement.

4.3.4 FIA three dimensional scanning

In this mode the detector is dynamic instead of the normal static system. The most important requirement of this system is that the scan should be done very fast. This lead to a series of points instead of a single one. The three dimensional factor comes into focus from time, parameter scanned, and property measured for each series of elements as a function of the instrumental parameter changed. FIA three dimensional scans can be realized in two ways:

(a) with higher scanning speed than flow rate;

(b) if the above is not satisfied, the stopped flow technique is recommended.

The method has the following advantages over the traditional mode, it provides more information, it can be used for 'electronic' calibration with a single standard solution, useful for studying formation of complexes. This mode is not fully explored since its principles are too abstract.

4.3.5 FIA systems with a gradient chamber

The incorporation of mixing chambers between the injector port and detector opens up numerous possibilities for the application of FIA systems. This set-up always lead to a wide concentration gradient in the injected plug. The result is an extremely wide signal so much that a plateau is generally observed. The dispersion for these systems is very high ($D > 15$). The signal is derived from the time increment within which the peak appears instead of the peak height or area as is normally the case in conventional FIA modes. Pardue and Fields [32] came up with a theory to describe the fundamentals of this system. This theoretical model explains the basis and

mathematical expressions from which this mode is derived. The basis for this are:

(i) very fast chemical reactions so that they do not feature at all among the variables in the system;

(ii) sample plug has to reach the chamber without undergoing any sort of dispersion;

(iii) There should be instantaneous mixing within the chamber;

(iv) There must be no dispersion between the chamber and detector.

The model satisfied the rational behind, single channel systems with a chemical reaction and those without a chemical reaction and dual channel systems.

4.3.6 Calibration methods

This is essential for sensors that undergo a change in their sensitivity due to lack of chemical or mechanical robustness or by change of a complex sample matrix. When this occurs the measurement signals will change independently of the real analyte concentration. Calibration can be achieved by addition from one to more internal standards or the addition of background electrolyte. Sometimes the addition of both the internal standard and the background analyte may be necessary. This theoretical mode offers many possibilities of establishing a relationship between the time and the analyte concentration. $\Delta t = t_3 - t_1$ the initial analyte concentration which is the basis for the analyte determination and this is done by making use of the following three plots namely:

(i) direct, $C°_{as} = f(\Delta t_{ep})$

(ii) exponential, $C^{\circ}_{as} = f\left[\exp\left(q/v_g\,(\Delta t_{ep}\right)\right]$

(iii) logarithm, $\ln C^{\circ}_{as} = f(\Delta t_{ep})$

In conclusion this method can be explained from the following points:[33]

(i) plots obtained do not differentiate whether there is a chemical reaction or not;

(ii) if there is no chemical reaction the analyte concentration begin to increase as soon as the analyte enters the chamber. When a reaction occuring there is dead time which correspond to the first stage;

(iii) The maximum concentration in the chamber is higher when there is no chemical reaction.

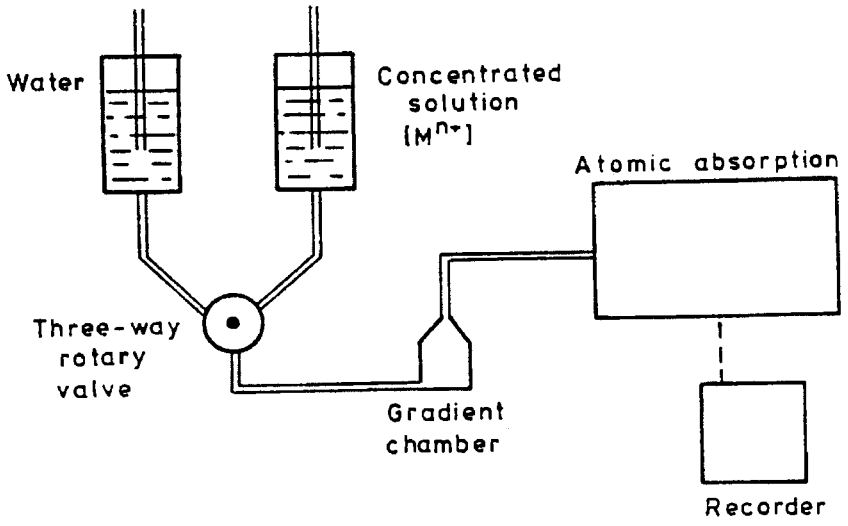

Figure 4.7 Calibration method for detection by atomic absorption spectrometry, with a gradient chamber.

4.3.7 FIA titrations

The classical titration method in analytical chemistry involves the determination of substance A by adding substance B (standardized) until an indicator system shows when both reagents have been added in stoichiometric amounts. The variable -time mode of classical kinetic methods is based on the measurement of a time increment, δt, between two preselected points in a chemical, physical or physicochemical system which has not attained either physical or chemical equilibrium. In this FIA mode the analysis is based on the peak width rather than on the peak height as is normally the case with all other FIA modes. This then dictates a great dispersion of the sample zone. This is because the volumetric methods differ from direct measurements by having, as the base for analytical read-out the consumption of a reagent added into the reaction vessel until the equivalence point is attained. FIA titrations were described by Ruzicka and Hansen [34]. This was

followed by the work of Pungor et al.[35] The method was summarized by Pardue and Fields [36] giving the following intrinsic characteristic:

(i) The reagent and analyte react in a non-stoichiometric proportions;

(ii) The loss of sample or reagent in this dynamic continuous flow is through mass flow rather than through a chemical reaction;

(iii) Similar results are obtained without reagent in the flow system.

The common characteristic of different continuous titrations is that the equivalance point is reached when the product of normality and flow rate for the sample is equal to the product of normality and flow rate for the reagent. The FIA titration has a very simple design, and the titrant is pumped at a constant flow rate through a single line and a gradient is created of the injected sample on its way through the gradient chamber [37]. The reagent and sample are not homogeneously mixed. An example of this mode is described by Gyurcsanyi *et al.* [38].

4.3.8 Scale expansion technique

Stewart and Rosefeld proposed a mode based on exponential dilution [39]. By the use of a gradient chamber of a reasonable size, it was shown that a logarithm plot of the concentration is a function of end-point time instead of FIA plots (concentration *vs.* peak height). This revealed a wider linear analyte concentration range over which the detector response is linear and a higher 'linearity ratio'. This is defined as the quotient between the maximum and the minimum analyte concentration in the linearity range. This proposal suffers from lower sensitivity and lower sampling rate. This type of technique has been evaluated from a single channel up to three

channels. A microprocessor is quite ideal with this type of system as the analytical parameter is the end point time.

4.3.9 Gradient calibration methods

The best explored detector for this technique is atomic absorption spectroscopy. Tyson [40] has tried this technique with success [40]. The mode displays excellent, rapidity, low sample and reagent consumption and elimination of glass ware manipulation. The calibration is made possible by the standard addition method and the determination of the total tolerated interference level. A gradient chamber is used to create exponential gradients. The unknown concentration can then be calculated by a classical mathematical eqaution, from the time needed for a given absorbance value to be attained. The mathematical eqaution is:

$$C = C_0\left[1-\exp\left(-q/v_g t\right)\right]$$

4.3.10 FIA techniques based on a pH gradient

The method is based on the establishment of two non-linear pH gradients corresponding to the two carrier-sample interfaces. When a large sample volume(V_s) is injected at $(pH)_1$ into a carrier at $(pH)_2$ the two gradients are supposed to be of the same magnitude only differing in direction and depending on the degree of mixing between the carrier and the injected plug and consequently on the time span which the latter spend in the reactor. The two gradients described above are dependent on the flow-rate and on the ratio V_s/V_r. This technique was used for simultaneous determination of vanadium(V) and lead [41]. The establishment of a pH gradient has not only

been used for the simultaneous analysis of cation mixtures, but also as a means of overcoming perturbations in certain FIA determinations.

4.4 FIA kinetic modes

Kinetic methods are based on measurements made prior to the attainment of equilibrium. These methods are strictly dependent on time. A kinetic method may be defined as the one from chemical reactions evolve over a measured period while the physical processes are in equilibrium or as one in which the chemical equilibrium has been attained and the physical processes are still evolving. Physical and chemical processes are important in describing a kinetic system. It is generally accepted that kinetic methods are based on chemical reaction rates. Flow injection analysis applies a methodology based on the generation of transient signals resulting from the occurrence of at least two sequential processes(transport & reaction), either of which can be responsible for the kinetic character inherent in the FIA methodology. Sometimes reaction rate measurements are used to describe kinetic methods. They are divided into three categories namely, initial rate, fixed time, and variable time, all these are based on the measurement between the initial slope of the signal time curve, the signal increment between two selected preselected times, and the time increment between two given signals and the concentration. All the methods that fall under this mode have their type of measurement made from the following parameters, slope, peak-to-peak, $\Delta s/\Delta t$, two maximum signals corresponding to two FIA curves recorded at $t_1=T_1$ and $t_2=T_2$.

4.4.1 Stopped flow techniques

These type of systems have been discussed in the elementary FIA mode. The basis of this mode involves the actual stopping of the propulsion system with the result that the sample flow is stopped allowing a reaction to take place with minimal dispersion. There is an increase of the residence time without increasing the reaction coil.

4.4.2 Differential kinetics

These methods are theoretically very viable but they seem to have a subdued impact in practice. The possibility of basing simultaneous determinations on different rates of reaction of the analyte with the same reagent promises a great potential in chemical analysis. This is however not the case practically as it is very difficult to find an ideal situation where two analytes give a sufficient difference under the same conditions. These methods have poor accuracy and reproducibility because of disturbances stemming from the diversity of the samples or from the slightest change in the experimental conditions. The differential kinetics is achieved by using systems with one to more detectors or injections respectively.

4.5 Two phase FIA modes

When the system to be analysed is composed of more than one phase it becomes very difficult to perform a determination because of the inherent interferences associated with the different phases. This problem is solved by introducing a separatory phase into the conduits of the FIA system. The distinction among this mode is based on the nature of the phases involved in the transfer processes.

The incorporation of continuous separation enhances selectivity and opens up a great deal of possibilities for FIA.

4.5.1 Gas-liquid (gas-diffusion)

The main aim with the incorporation of suitable gas-diffusion units into FIA systems is to separate gaseous species from ionic interferents in solution. Various approaches are possible, however, for on-line dialysis. Dialysers are basically incorporated into the conduits of FIA manifolds to achieve one or more of the following objectives. A dialyser as part of the manifold system not only facilitates exact and reproducible means of automated dilution, but the main aim of dialysis so far was the separation of analyte species from underwanted matrix constituents. It is also possible, however, to use dialysis for indirect determinations and for simultaneous determination of components [42].

4.5.1.1 Distillation

These are not normally used in automated analytical methods because they require complicated methods of incorporation. When applied it can be with heating, as in the determination of cyanide using amperometric detection [43] or without heating (isothermal) where a gas is transferred by gas phase diffusion from one liquid to the other at the same temperature this has been used in the determination of nitrogen in plant material [44].

4.5.1.2 Determination in the gas

A variant of distillation involves direct transport of the gas from the donor solution to the detector An example is cited by the determination of bismuth by FIA-AAS with formation of a volatile hydride [45]. In this system the sample is injected into the carrier solution (hydrochloric acid) which merges with a stream of alkaline alkaline sodium borohydride solution , only after suitable dispersion of the sample has been achieved nitrogen stream is then introduced at a second merging point and sweeps the gaseous hydride into the atomic-absorption spectrophotometer. This method compares favourably with other conventional methods.

4.5.1.3 Gas sampling

The best example of this mode is the determination of sulphur dioxide from air as described by Ramasamy and Mottola [46]. This was achieved using two method of sampling one involving intercalating the sample as a gas plug into the reagent stream and the other involves midget impinger.

4.5.2 Gas-Solid

This is not a popular mode of FIA and very little attention has been given to it. A typical principle of this method is the lack of the collecting solution and the determination is based on the photometric measurement. The system operates in the following manner: a reaction between a gas whose concentration is to be determined and a solid takes place resulting in a solid whose chemical nature can be linked to quantitative determination. An example is offered by the following reaction:

$$Br_{2(gas)} + 2NF_{(S)} \quad 2Br{:}NF_{(S)} \quad \text{.....(A)}$$

$$2Br{:}NF_{(S)} + AS(III) \quad 2\,Br_2^- + As\,(V) + 2NF_{(S)} \quad \text{.....(B)}$$

Where NF is α-naphthoflavone[2-phenyl-4H-naphtho(1,2-b)-pyran-4-one] and reactions A and B are gas-solid and solid-solid, respectively. The red-brown species, $Br{:}NF_{(S)}$, generates the transient signal, height of which is directly proportional to the amount of bromine gas present in the injected sample [47].

4.5.3 Solid-Liquid

The earliest example for this type of separation was described by Bergamin *et al.* [48]. This technique is not preferred with FIA because of the loss of sample during the separation step due to the adsorption properties of the ion-exchange resin. The position of the column within the manifold depends on the type of determination that is required. When the merging zone technique is employed with this technique, the sample is continuously flushed across the resin through to waste and the reagent stream flows through a different loop. Then after an electronically set period the loops are switched and the sample carrier stream washes the resin. The confluents from both streams are then merged and the analysis is then carried out. After a series of trials it was determined that the dispersion depends on the length of the resin column rather than on the reaction coil. Anionic interferences may be eliminated by the use of ion exchange, for example, in the determination of calcium by AAS where sulphate and phosphate interfere. The combination of FIA with ion exchange offers the possibility of simultaneous determination of zinc and cadmium [49]. Ion exchange is also used for enhancing sensitivity through preconcentration.

4.5.4 Reducing columns

Reducing columns are rarely used in FIA, but when they are used it can be in one of two ways:

(i) by placing the column before the injection unit , thus reducing a reagent reagent in the carrier stream so that it subsequently act as a reductant on the unknown sample;

(ii) by passing the sample through the column, thereby converting the species of interest into an oxidation state acceptable to the sensor.

The first case is demonstrated by Schothorst *et al.* [50] where they examined the reduction of vanadium(IV) to vanadium(III) by the Jones reductor. To achieve quantitative reactions with these reagents in non-acidic media, complexing agents are added to the solutions to modify the formal potential of the redox pairs involved. This is necessary when chromium has to be reduced from chromium(III) to chromium (VI). The most notable application of the reducing column placed after the injection valve is connected with the determination of nitrate
and nitrite in mixtures [51].

4.5.5 Adsorption

Activated carbon is used as a filter where the presence of organic compounds can interfere with the analysis. This is normally done in photometric determinations with measurement in the ultra violet region. An example of this analysis is described by Slanina *et al* [52] From this work a series of conclusions were achieved at namely:

(i) when there is no water and the sample is directly injected into the perchloric acid is diluted

and for dilute sample solution there may be a negative peak arising from the absorption of light by the perchloric acid;

(ii) The filter used displays memory effect at low concentrations of nitrate small amounts adsorp and the desorption process is greatly reduced;

(iii) higher reproducibility is attained by sample loop and sampling valve rather than by syringe;

(iv) sample consumption is greatly reduced by sampling valve.

4.5.6 Dialysis

Dialysis phenomenon involves the separation of two different phases across a semipermeable membrane. The separation takes place by selective passage of some solutes through the membrane. They separate a given sample from a complex matrix. The principle allows separation of compounds of high molecular weight from those of low molecular weight. Ions or molecular constituents have different mobilities in a liquid phase when transported across a membrane into a second phase. The membrane also acts as a barrier readily permeable to the donor solvent and the smaller analyte solute molecules [53]. The theory of dialysis is based on the following principles :

(i) donor fluid enters the system in laminar flow at uniform concentration;

(ii) the dialysate has a constant bulk concentration at all axial positions in the dialyser;

(iii) the donor fluid is newtonian and steady state exists;

(iv) both fluids are homogenous and have constant physical properties;

(v) mass transport through the membrane is purely diffusive and is Fickian for the donor;

(vi) the membrane permeability and dialysate-side mass transfer coefficients are constant;

(vii) the solute between the fluids and membrane are not the same or equal to unity;

(viii) axial diffusion in the donor fluids is negligible compared to axial convection.

One of the best example of the application of a dialyser inclusion into the conduits of the FIA system is in the determination of chloride from milk samples [54].

4.5.7 Solvent extraction

Liquid-liquid extraction is done to remove interferences and to preconcentrate an analyte. This limits toxic odours from the organic solvents. To automate the extraction process a phase segmentor, extraction coil and phase separator should be built into the conduits of FIA. These systems are classified according to the type of separator employed. There are three types of separators:

(a) The first is based on the density differences between the phases and the other two are based on the different affinities of the phases for the for the tube wall. The first one is made up of a minichamber with an inlet in the horizontal plane for the segmented flow and two outlets;

(b) The second separator is a membrane, based on the nature they prefer one of the phases mostly the organic phase, which can then pass through the membrane and continue on to the detector;

(c) The last one is the T- shaped continuous separator, it operates in the following manner: the segmented flow enters through the central channel, which has a piece of Teflon attached to its wall, the organic phase moces upward by affinity with the Teflon to the upper outlet. The bottom part is a hollow cylinder through which passes the aqueous phase

and the organic phase moves upwards by affinity with the Teflon. Karlberg et al. supplied an example of this type of application [55].

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CHAPTER 5

ON-SITE MONITORING OF CHLORIDE IN MILK WITH A DIALYSER/UV/Vis SPECTROPHOTOMETER/FLOW INJECTION SYSTEM

5.1 Introduction

This chapter describes the determination of chloride in milk with an on-line dialyser using a UV/VIS spectrophotometer as detector.

Quality control of food is of prime importance for human health. Milk is very important for human consumption because of its nutritional value. The ash constituents of milk are partly combined with protein and fat. Most of the phosphorus occurs as calcium phosphate, which is a suspension. Some of the phosphorus is combined with casein to form a phosphoprotein, and a smaller amount is a constituent of certain fat like substances. Ash components of milk include calcium, potassium, magnesium, sodium, phosphorus, chlorine, sulfur, iron, copper, zinc, aluminium, manganese, iodine, and traces of other elements.

Milk has been called the perfect food, probably because it serves as the food for the young of all mammals. Modern research has revealed that although milk is an excellent food from a nutritional standpoint, it has certain limitations if used alone. Its caloric value is significant and it is one of the most valuable sources of proteins.

The concentration of chloride in milk is one of the indicators defining the quality of milk. It has been established that the maximum tolerated level of chloride in milk is 800-1200 mg/ℓ. A high chloride concentration indicates mastitic milk. Mastitis is recognise as one of the major disease problems concerning the dairy industry. It brings down milk yield, shortens the productive life of the cow and causes severe economic losses to the dairy farmer.

Milk containing less than 0.14% chloride is considered normal. Udder infection increases the chloride and decreases lactose in milk, thereby changing the ratio more than the concentration of either of the components individually.

5.2 Properties of chloride

Chloride (Cl) (covalent radii 0.99 x 10^{-10}, atomic mass 35.5) is situated in the periodic table of elements in the 7th main group. It has got the first electronegativity after fluoride. The electronegativity is a result of its electronic configuration:

$$\mathbf{1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^5}$$

It can be easily seen that the chloride atom can easily accept an electron when it became the chloride ion: Cl^-, and also it can easily form a covalent bonds with different elements, as H and O.

The same electronic configuration favours the following oxidation states: Cl^{1-}, Cl^{3-}, Cl^{5-} and Cl^{7-}. The best examples for these oxidation states are the following oxyanions: ClO^- (hypochlorite), ClO_2^- (chlorite), ClO_3^- (chlorate), and ClO_4^- (perchlorate).

Chloride can also occur as a homogeneous molecule in the Cl_2 form (pale green gas). It can also be part of an organic molecule (e.g., chlorobenzen, trichloroethylene, chloromethane, etc.).

The most useful forms of chloride are: HCl, NaCl, HClO, and Cl_2.

5.3 Biochemistry of chloride

Chloride is the anion most commonly combined with sodium in the extracellular fluid and, to some extent, is also found with potassium in the cells. Unlike these bases, chlorine can pass freely between these two fluids through the cell membranes. Usually, movements of chloride between body fluid compartments are compensated by those of sodium. An exception is the movement between plasma and erythrocytes, where chloride rapidly travels in and out of the cells in an exchange of bicarbonate, thereby enhancing the capacity of the red cells to carry CO_2 from the tissues to the lungs and aiding in the maintenance of an acid-base balance.

During digestion, some of the chloride of the blood is used for the formation of hydrochloric acid in the gastric glands; it is secrete into the stomach, where it functions temporarily with the gastric enzymes and is then reabsorbed into the bloodstream along with other nutrients.

Chloride intake and losses from the body may also be a result of an excess of sodium remaining after the loss of gastric contents due to vomiting or suctioning. The loss of chloride from the body fluids is replaced by bicarbonate to maintain electroneutrality, and the resulting alkalosis increases the loss of body potassium. Chloride administration corrects both problems. Excess chloride is readily excreted by the kidney and the skin, mostly as sodium chloride.

The estimated safe and adequate intake of chloride is assumed to be the same as that of sodium and potassium on an equivalent basis (50 - 150 mEq for adults).

Human milk contains more chloride (11 mEq per liter) than sodium (7 mEq per liter) but less than potassium (13 mEq per liter). This results in a sodium plus potassium to chloride ratio of close to 2:1, which is desirable for the regulation of acid-base balance, and is recommended for infant formulas by the nutrition committees all over the world.

5.4 Choice of analytical methods

The most common basic standard manual method used for the determination of chloride is the Volhard titration method.[1] Although this standard method is accurate, it is time consuming and expensive. Ion-selective electrodes have been used for various manual determinations in milk [2-5] including chloride [6-8]. Direct manual measurement of chloride with ion-selective electrodes is not possible [8] and results obtained by De Clercq *et al.* [8] revealed a substantial error. This is caused in part by the interference of casein, although picrate, citrate and lactose interference may also be possibilities. Van Staden [9] eliminated the interferences by developing a rapid and reliable automated procedure for the direct measurement of the chloride content in milk using the

principles of flow injection analysis with a dialyser and a coated tubular inorganic chloride-selective electrode as sensor. The potential of using the dialysis process was also extended in our laboratory [10] to three-component flow injection analysis using two dialysers in series for the simultaneous determination of free calcium, total calcium and total chloride in milk on a single sample injection. The same concept was also applied for the flow injection analysis of high chloride levels in electroplating baths [11]. We also employed electrodes in series for the simultaneous flow injection determination of chloride and pH with ion-selective electrodes [12].

UV/VIS spectrophotometry as detection system in flow injection analysis [13-21] was proposed for the indirect monitoring of chloride which is based on the assay of the red iron(III) thiocyanate complex formed as follows:

$$Hg(SCN)_2 + 2\ Cl^- \rightarrow HgCl_2 + 2\ SCN^-$$

$$SCN^- + Fe^{3+} \rightarrow [Fe(SCN)]^{2+}$$

The incorporation of dilution and separation techniques into flow injection analysis which greatly expanded the scope of application for this method, has been described by several authors [13-21].

5.5 Experimental

5.5.1 Reagents and Solutions

All reagents were prepared from analytical-reagent grade chemicals unless specified otherwise. De-ionised water from a Modulab system (Continental Water System, San Antorio, TX, USA) was used throughout. The water was tested beforehand for traces of chloride. All solutions were degassed before measurements with a vacuum pump system. The main solutions were prepared as follows.

Chromogenic reagent. Dissolve 1.26 g of mercury(II) thiocyanate in 300 mℓ of methanol, add 1 ℓ of distilled water and shake to mix well. Then add 8 mℓ of nitric acid (specific gravity 1.42) and 31 g of $Fe(NO_3)_3$. 9 H_2O. Shake well until dissolved and dilute to 2 ℓ with distilled water. Filter, if necessary. This reagent is stable for several months at room temperature if stored in a dark bottle.

Standard chloride solutions. A stock solution of 10 000 mg/ℓ chloride was prepared by dissolving 16.484 g of dried NaCl and diluting to a total volume of 1 ℓ. Working standard solutions in the range 500-2500 mg/ℓ were prepared by appropriate dilution from the stock solution.

5.6 Apparatus

The following equipment were used: A Cenco peristaltic pump operating at 10 revolutions per minute; a VICI 10-port two-position sampling valve with two identical sampling loops, each

having a volume of 50 µℓ, a Cenco sampler, reaction manifold systems, a laboratory-made dialyser with previously described [17,19], a Pye Unicam SP6-550 UV-visible spectrophotometer equipped with a 10 mm Hellma-type flow-through cell (volume 80 µl). Data acquisition and device control were achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa) and an assembled distribution board (MINTEK, Randburg, South Africa). The *FlowTEK* [22] software package (obtainable from MINTEK) was used throughout the procedure.

5.6.1 Dialyser unit

A slightly modified version of a 12-in Technicon AutoAnalyzer II single membrane dialyser unit was used for the dialysis process. Both the top and bottom parts of the dialyser unit consisted of 160 x 30 x 25 mm modules (laboratory made as modified versions of 12-in Technicon dialysers). The channels on the top and bottom parts of the single dialyser unit consisted of semi-tubular grooves with an i.d. of 0.5 mm (i.e. 0.5 mm wide and 0.5 mm deep) and with a distance (width) of 15 mm between the entrance and exit sides of a channel. These two channels are exact mirrors images of each other to fit precisely. The path lengths of both dialyser units were 300 mm for both the donor and acceptor channels. The dialysis units were equipped with Technicon Pre-mount Dialysis Membranes Type C between the donor and acceptor channels.

5.6.2 Flow system

The sequence of equipment interaction for the determination of chloride in milk is represented in schematically (Figure 5.1).

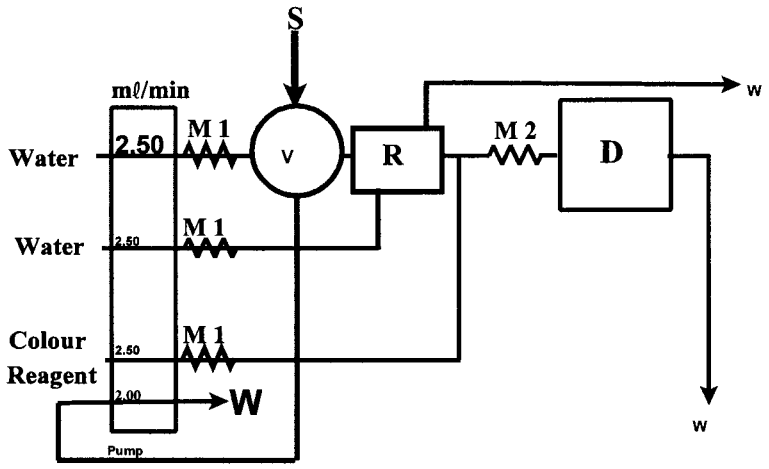

Figure 5.1 Schematic flow diagram for the determination of chloride in milk. S- Sample; M - Mixing coil; D - Detector; W - Waste; V - Valve; M1 = 50 cm; M2 = 150 cm; Tube i.d. = 0.76 mm; R = Dialyser.

The manifold consists of Tygon tubing with an inside diameter of 0.76 mm cut into required lengths and wound around glass tubes with an outside diameter of 15 mm. Two tubes, one leading to the dialyser and the other leading to the valve system, had de-ionised water as carrier propelled at a flow rate 2.5 mℓ/min. The dialyser equipped with a Technicon Pre-mount Dialysis Membrane Type 'C' was connected in series to remove any interfering species. The flow rate for the colour reagent was set at 2.5 mℓ/min. Samples were injected from a 50-$\mu\ell$ sampling loop. The injected samples were mixed with the colour reagent in a 250 cm reaction coil and the resulting product monitored spectrophotometrically at 480 nm.

5.7 Optimization of method.

The follow following parameters were studied in order to find the optimum working conditions for the determination of chloride in milk.

5.7.1 Effect of Flow Rate

A series of preliminary and evaluation experiments were conducted in order to optimize the flow rates of both the donor and acceptor streams as well as the chromogenic reagent stream and also to have the best linear calibration curve. The flow rates were first evaluated in a series of preliminary experiments. In the first of the final evaluation series the flow rates of the carrier reagents (C_A) in the donor and (C_B) in the acceptor channels were maintained at 2.5 mℓ/min and that of the chromogenic colour reagent was varied from 3.9 mℓ/min to 2.5 mℓ/min using the optimum reagent concentrations as established before [14,17]. Linearity was not affected that much, throughout the chosen flow rates. The sensitivity was greatly changed. A flow rate of 2.5 mℓ/min gave the best sensitivity and precision and this was chosen for further evaluation.

The resident time of the solution within the dialyser is dependent on the carrier flow rates of both the donor and recipient streams as discussed before [10, 12, 14-21]. The lower the flow rates the longer the residential period. To vary the residential period within the dialyser the flow rates of both the donor and acceptor streams were evaluated. The flow rate of the carrier reagent (C_A) from the donor stream was first varied from 3.9 mℓ/min to 2.5 mℓ/min at constant flow rates of 2.5 mℓ/min for both the carrier acceptor (C_B) and colour reagent streams. The results obtained are given in Figure 5.2. The data acquired displayed no great change except for the sensitivity with

2.5 mℓ/min giving the best sensitivity. This was followed by varying the flow rate of the carrier reagent from the acceptor stream (C_B) at constant flow rates of 2.5 mℓ/min for both the carrier donor (C_A) and colour reagent streams. It is clear from the results (Figure 5.3) that a flow rate of 2.5 mℓ/min for all three streams gave the best sensitivity and with a precision of better than 1% this was chosen for further work.

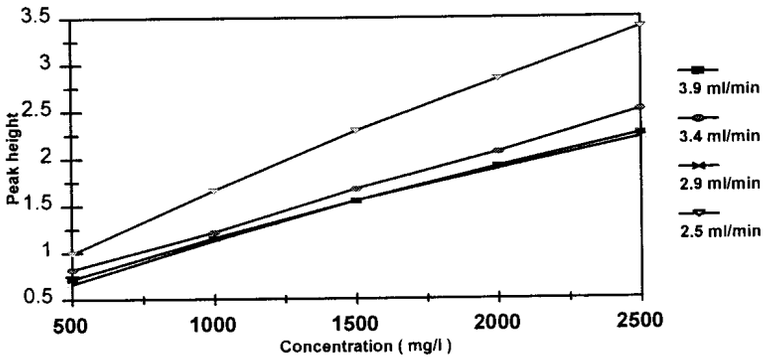

Figure 5.2 Effect of the donor carrier stream flow rate at a constant acceptor and colour reagent stream flow rates of 2.5 mℓ/min.

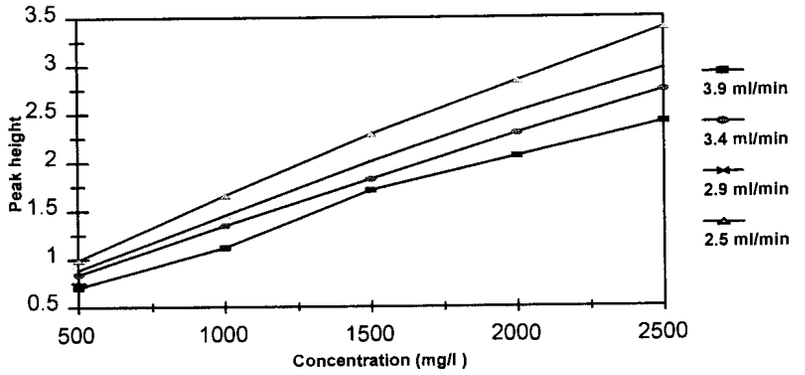

Figure 5.3 Effect of the acceptor carrier stream flow rate at a constant donor and colour reagent stream flow rates of 2.5 mℓ/min.

5.7.2 Effect of Sample Volume

Sample volume influences the linearity of the flow injection system and also the sample throughput. Sample volumes between 50 and 120 $\mu\ell$ were evaluated and the results obtained are outlined in Figure 5.4. A sample volume of 50 $\mu\ell$ was chosen for this determination because it gave the best reproducibility and the best linearity.

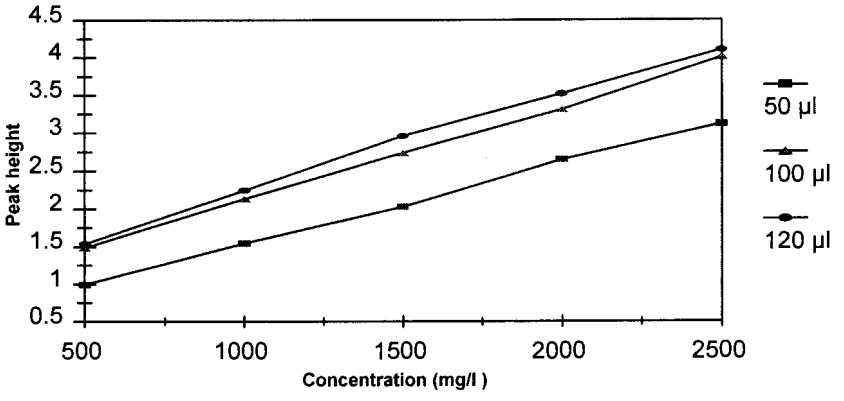

Figure 5.4 Effect of sample volume.

5.7.3 Effect of Manifold Length

The length of the manifold has an influence on the physical and chemical dispersion, the final peak profile and therefore the sensitivity and precision. Various manifold lengths were tested and the results are displayed in Figure 5.5. A manifold length of 250 cm was decided upon since it gave the best results.

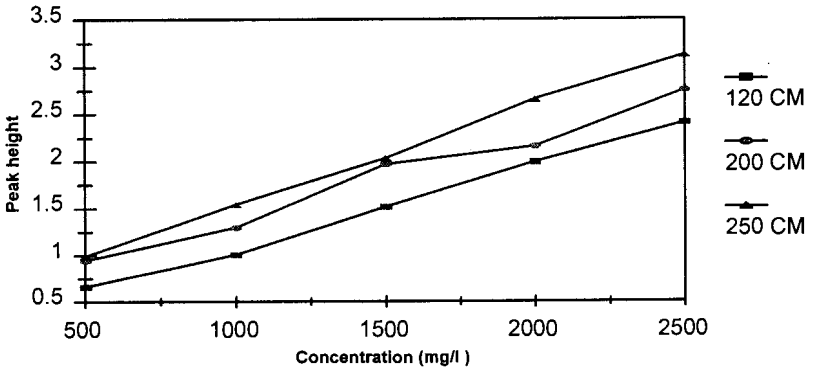

Figure 5.5 Effect of manifold length.

5.8 Method of evaluation

The proposed on-site flow injection analyser was critically evaluated with regard to, linearity, accuracy, precision, sample interaction (carry-over) and sampling rate.

5.8.1 Linearity

The linearity of the proposed FIA system for the determination of chloride was evaluated under optimum conditions. The data collected for the standards yielded a straight line between 500 - 2500 mg/ℓ with the relationship for relative peak height versus chloride ion concentration of:

$$H = 0.00098\ C + 0.3306;\ r = 0.9973$$

where H = relative peak height and C = chloride ion concentration in mg/ℓ. The correlation coefficient (r) indicated that the method was linear for an analyte concentration ranging between 500 - 2500 mg/ℓ.

5.8.2 Accuracy

Real milk samples were analysed with the flow injection system [23]. The accuracy of the proposed FIA analyser was evaluated by comparing the results from real samples of the FIA system with the values obtained by the standard Volhard titration method. The results as shown in Table 5.1 revealed a good correlation between the FIA system and the values obtained by the standard method. From all the milk samples the calculated chloride concentration by both methods was almost the same not differing by more than 2 %, which satisfied the criteria requested for the on-site FIA analyser. The accuracy was also determined by spiking real milk samples with standard chloride solutions. The recovery efficiency of the proposed system varied between 95 and 98% as seen from Table 5.2. The recovery efficiency indicates that only a negligible amount of the analyte was lost when the FIA method was used.

Table 5.1 Performance of the proposed FIA system for the determination of chloride.

Sample name	Manual method in mg/ℓ	Flow injection method in mg/ℓ	% RSD (Manual method)	%RSD (FIA system)
MILK 1	919	915	0.98	0.26
MILK 2	905	903	0.33	0.09
MILK 3	898	897	0.77	0.13
MILK 4	934	933	0.59	0.48

Table 5.2 Recovery efficiency of the proposed FIA system.

Sample name	Spiked milk in mg/ℓ	Expected concentration in mg/ℓ	% Recovery
MILK 5	997	1015	98.22
MILK 6	985	1003	98.15
MILK 7	955	997	95.77
MILK 8	998	1032	96.74

5.8.3 Precision

The precision of the method was determined by performing ten repetitions of each milk sample. The results are listed in Table 5.1. A precision of less than 0.5 % RSD was obtained.

5.8.4 Sample interaction

The interaction (carry-over effect) between consecutive analysis was investigated by analysing a low analyte concentration followed by a high analyte concentration and immediately followed by a low analyte concentration using the equation:

$$\text{Interaction} = \{(A_3 - A_1)/A_2\} \times 100$$

where

A_1 = the true peak height of a sample with a low analyte concentration,

A_2 = the true peak height of a sample containing ten times more analyte and

A_3 = the peak height for an interacted sample containing the same amount of analyte as A_1.

The sample interaction between samples as calculated was less than 1% for the on-site flow injection system for the determination of chloride in milk at a rate of 80 samples per hour which is negligible. Sample interaction is reduced effectively by flushing the manifold with 3 mol/ℓ HNO_3 followed by de-ionised water.

5.9 Conclusions

The proposed flow injection analyser satisfies the requirements needed for the on-site determination of chloride in milk. At a sampling rate of 80 samples per hour the system is suitable to monitor the chloride contents in milk in the range 500 - 2500 mg/ℓ with an RSD better than 0.5%. An outstanding feature of the system is its simplicity and reliability which should be particularly attractive for the dairy industry. Once all the parameters were set the assay was carried out effortlessly and smoothly. The system could be stopped and resumed instantly without any problem.

All the milk samples analysed displayed chloride concentration within the tolerance level. This method worked very well for the determination of chloride in milk, as there is no sample preparation involved. The on-line dialysis saves time and with the high percentage recovery the proposed method is suitable for this type of analysis.

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CHAPTER 6

IMPROVEMENT IN THE FLOW INJECTION SPECTROPHOTOMETRIC DETERMINATION OF ZINC WITH ZINCON THROUGH ELEVATED TEMPERATURES

6.1 Introduction

This chapter describes a proposed flow injection system for the determination of zinc employing the zincon method.

Paracelsius gave to the element with atomic number 30, and with atomic mass 65.38 the name zinc (Zn).

Zinc is a common element, and appears rarely in metallic form, except in the basaltic rocks or banks of the rivers. It is present in small amounts in igneous rocks and sedimentary rocks. Zinc is mostly found in association with some other elements and substances. Zinc ores are widely distributed, and they are common in mines. It is particularly found associated with ores of lead, silver, and copper. Zinc occurs as 0.003% (w/w) in humans.

Zinc was established as an essential element in the 1960's. It occurs abundantly in nature in different forms, is involved in many biochemical processes and contributes significantly to human health. Zinc is essential for the functioning of over 70 enzymes that control protein synthesis and the growth and repair of cells. Zinc forms an integral part in physiological processes such as taste, appetite control and wound healing. It plays a critical role in the environment and in both animals and plants, and is associated with the absorption and activities of vitamins A and B complexes.

Zinc falls under critical nutrients limits which is the minimum amounts necessary to satisfy the body's requirements over a reasonable time period. Without these nutrients the body may develop typical negative responses which may be physical. It is required only in trace quantities in the diet, but is very essential for the normal functioning of many body systems. It is a cofactor in a wide variety of biochemical processes in bacteria, plants, and superior organisms and is, therefore essential for their behaviour. It can be found in the cells in relatively high concentrations. Either an excess or a lower concentration level of zinc can be harmful.

The allowance for zinc is in the order of 10 to 15 mg in food used in the average man's diet. Nutrient availability of zinc is greatly hampered by the diet with a high calorie content, or having a high concentration of fibre. Phytate has a negative influence in the absorption of zinc; as a result only half of the available zinc is absorbed by the body in its presence.

Zinc is used for roofing in galvanized form. It is one of the essential components in galvanic batteries. It is also used in galvanised baths and containers. It is a special ingredient in alloys.

6.2 Properties of zinc

Zinc (Zn) (covalent radii 1.25 x 10^{-10}, atomic mass 65.38) is situated in the periodic table of elements in the 2nd secondary group. It is a gray-white metal, which is fairly malleable and ductile. Zinc melts at 410 °C, and boils at 906 °C. It is a transition element with the following electronic configuration:

$$\mathbf{1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^6\ 4s^2\ 3d^{10}}$$

It can lose two electrons, becoming an cation: Zn^{2+}. Its structure is a hexagonal one.

The pure metal dissolves very slowly in acids and in alkalis. Dissolution occur in dilute H_2SO_4, HCl, and HNO_3.

A typical reaction for zinc is the following:

$$Zn_{(s)} + HCl_{(aq)} \rightarrow ZnCl_{2(aq)} + H_{2(g)}$$

6.3 Biochemistry of zinc

Zinc fulfill a very important role in biochemical processes, e.g., sexual development, physical growth, wound healing and tissue repair, cofactor for enzymes involved with metabolism, metabolism of alcohol, vitamins, test acuity, insulin biotransformation.

Zinc is important as well as the proteins in normal processes of growth and maintenance of body tissue.

For biochemistry of zinc the key is the ligand that bind zinc, e.g., in cow's milk zinc is strongly associated with colloidal calcium phosphate (CCP) in the casein micelles and that CCP removal significantly improves the bioavailability of zinc on cow's skim milk.

The presence of low hair Zn-, high urinary Zn-values and low height for age is demonstrated in children suffering from recurrent upper respiratory tract infection. The interpretation is hazardous: Zn deficiency can be both a cause and an effect of the recurrent infection. Therefore, a controlled metabolic study was done.

Studies in humans have suggested that zinc absorption can be inhibited by milk. Since the zinc content of diets consumed by old woman is reported to be inadequate, increased consumption of food which lower zinc bioavailability could compromise zinc nutritional status.

The absorption of trace zinc from a diet is influenced by low molar mass ligands, e.g., organic acids and peptides promoting or facilitating absorption as well as complexing agents like phytic acid depressing absorption. The absorption of zinc from the cereal based meals varied from 8% (w/w) in an oatmeal meal with phytic acid content of 615 μmol to 27% (w/w) in a meal with a phytic acid content of 100 μmol. From the high protein meals the absorption varied from 19-32 % depending on the zinc and protein content. A close correlation between dialyzable zinc at pH = 8, and the *in vivo* measurement of zinc absorption in humans was observed.

The most reliable sources of zinc is animal food, especially muscle meats, wheat germ, bran.

6.4 Choice of analytical methods

The determination of zinc is of prime importance in an array of fields, such as environmental chemistry, industrial and domestic water control, agriculture, metallurgy, and in the health industry. In all the specified fields the selectivity, sensitivity and rapidity are essential for the determination of zinc. The need for a suitable method for the determination of zinc led to the establishment of three techniques that emerged as standard methods: atomic absorption spectrophotometry [1], EDTA titration [2], and the dithiozone method [3]. The progress made in analytical chemistry particularly the adaptation of flow injection analysis to a broader spectrum of analysis led to the exploitation of a number of methods for the determination of zinc with different workers contributing a series of competing methods [4-9]. A flow injection analysis method using xylenol orange as reagent and thiosulphate as the masking agent for copper and lead has already been described [10]. However nickel still interferes. A severe handicap that greatly hampers the determination of metals is that they interfere so much with each other [11]. As a remedial step towards this persisting problem some researchers exploited the differential de-masking of these metals [12,13], using apo-enzymes [14], metal ion buffers [15], and carbonic anhydrase [16]. One of the most effective and widely used procedures for eliminating interference is by solvent extraction [17]. Interferences can also be eliminated by effectively masking all metal ions followed by selectively only de-masking the analyte zinc, while the other ions remain masked.

For zinc assay, flow injection analysis is proposed in coupling with ion-selective, membrane electrodes [18-20], spectrophotometric methods [21, 22], and fluorimetric methods [23].

6.5 Principle of the reaction between zinc and zincon

The zincon method in flow injection analysis seems to be an excellent example in this regard. It is possible to improve this method by elevating the operational temperature. The rise in temperature increases the kinetic energy and the Brownian motion. The frequency of collision is increased thus enhancing rapid de-masking of the cyanide zinc complex. This is a rate-determining step and its increase consequently speeds up the overall reaction rate leading to a faster analysis process. The analytical reactions involved can be schematically represented as follows:

$$M^{n+} + x\ CN^{-} \rightleftarrows [\ M\ (CN\ )_X\ ]^{(x-n)-} \quad \text{(A)}$$

where M are all the metal ions. Zinc is forming a complex according to the reaction:

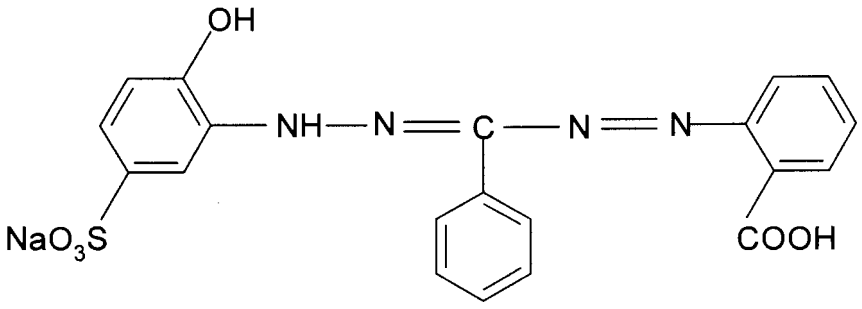
$$Zn^{2+} + 4\ CN^{-} \rightleftarrows Zn[(CN)_4\ ]^{2-} \quad \text{(B)}$$

Only zinc is de-masked by cyclohexanone according to the following reaction.

$$[\ Zn(CN)_4\ ]^{n-} + 4\ C_6H_{10}O + 4\ H_2O \rightleftarrows Zn^{2+} + 4\ C_6H_{10}(OH)CN + 4\ OH^{-} \quad \text{(C)}$$

$$Zn^{2+} + \text{Zincon} \rightleftarrows \text{blue complex} \quad \text{(D)}$$

where zincon has the following structure:



Equation (C) denotes the rate determining step, it is at this point that the Zn^{2+} is released to be available for reaction (D). The raise in temperature favours step (C). Step (D) is also heat dependent allowing the development of the blue indicator colour. The intensity of this colour is directly proportional to the zinc concentration.

6.6 Experimental

6.6.1 Reagents and solutions.

All reagents were prepared from analytical-reagent grade chemicals unless specified otherwise. De-ionised water from a Modulab system (Continental Water system, San Antonio, TX, USA) was used throughout. The water was tested beforehand for traces of chloride. All solutions were degassed before measurements with a vacuum pump system The main solutions were prepared as follows:

Borate buffer solution. (0.5 mol/ℓ, pH = 9.0) A stock 0.5 mol/ℓ borate buffer solution was prepared by dissolving 30.9 g of boric acid and 8.4 g of sodium hydroxide in 900 mℓ of water and adjusting the pH with 5 mol/ℓ NaOH or HCl respectively. The solution was then diluted to 1000

mℓ with water. A 0.05 mol/ℓ borate buffer working solution was prepared from the stock borate buffer by appropriate dilution and pH adjustment as required.

Zincon stock solution. (0.1 % m/v) 0.2 g of zincon (2-carboxy-2′- hydroxy-5′ sulphoformazylbenzene) was dissolved in 200 mℓ of water. This solution is stable for about 7 days.

Composite reagent A (0.01 mol/ℓ cyanide - 0.1mol/ℓ ascorbate buffer, pH 9.0). 0.65 g of KCN and 17.6 g of ascorbic acid were dissolved in 1 litre of borate buffer (0.05 mol/ℓ) solution and the pH adjusted accordingly to 9.0.

Composite reagent B (10 % v/v cyclohexanone - 0.016 % m/v zincon buffer, pH 9.0. 50 mℓ of pure cyclohexanone and 80 mℓ of zincon stock solution were mixed and diluted to 500 mℓ with borate buffer (0.05 mol/ℓ).

Zinc stock solution. (1000 mg/ℓ) 4.397 g of $ZnSO_4$ .7 H_2O were quantitatively dissolved and diluted to 1000 mℓ of de-ionised water to give a 1000 mg/ℓ stock zinc solution. Standard working zinc solutions (1 - 10 mg/ℓ) in dilute 0.05 mol/ℓ borate buffer were freshly prepared as required.

6.6.2 Apparatus

Composite reagents (A & B) were propelled into the flow system at different flow rates of 1.6 mℓ/min and 3.9 mℓ/min respectively using a Cenco peristaltic pump operating at 10 revolutions

per minute (Figure 6.1).

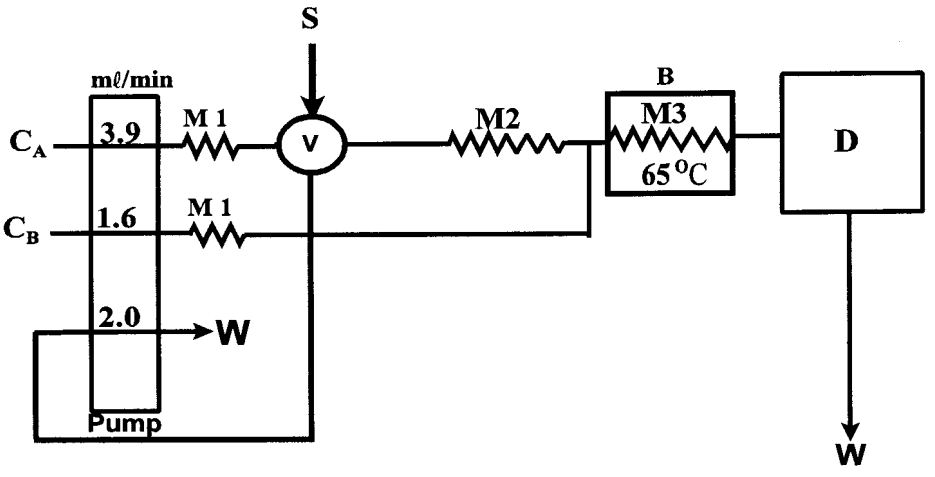


Figure 6.1 Schematic flow diagram for the determination of zinc. S - Sample; M1 - coil (50 cm); M2 - coil (150 cm); M3 - reaction coil (250 cm); D - Detector; W - waste; V - Valve; B - Water bath; i.d. tube = 1.14 mm; C_A - Composite reagent A; C_B - Composite reagent B.

A VICI 10-port two-position sampling valve with two identical sampling loops, each having a volume of 50 $\mu\ell$, was used to inject samples into the carrier stream. The manifold consisted of Tygon tubing with an i.d of 1.14 mm. Intermediate coils of the same diameter were incorporated between the valve system and the pump to reduce pulsations. A Unicam 8652 UV-VIS spectrophotometer equipped with a 10 mm Hellma flow-through cell (volume 80 $\mu\ell$) was used to monitor the coloured product at 620 nm. Data acquisition and device control were achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa) and an assembled distribution board (MINTEK, Randburg, South Africa). The FlowTEK [24,25] software package

was used throughout the procedure.

6.6.3 Sample preparation

A. Liquids

Liquid samples containing solid particles, were filtered through a 0.45 pore size filter paper, pre-cleaned with 1% v/v HNO_3, followed by de-ionised water. The pH of the filtrate was then adjusted to pH 9.0 with 0.5 mol/ℓ of borate buffer.

B. Alloys

0.5 g of the solid sample was dissolved in 10 mℓ of concentrated HNO_3 and the solution was then slowly evaporated with constant stirring to half of its original volume over a water bath. The resultant solution was then filtered through a No.42 Whatman filter paper pre-cleaned as previously described. The filter paper was washed with 1% v/v HNO_3. The filtrate was the diluted to 100 mℓ with water and treated as described in A above.

6.7 Results and Discussion.

The reaction is kinetically dependent, and increasing the temperature pushes the equilibrium towards the products thus speeding up the reaction rate. The reaction is not changed, but only stimulated to occur faster and eliminating excess reagents that would normally be used. The

residence period of the reagents within the manifold is greatly reduced without compromising the performance of the method. The reduction of the residence time reduces interferences associated with the instability of the cyanide complexes in the room temperature analysis. This proposed method would fit well in analytical problems that require short periods of analysis. The novel idea of improving a well-established method like the zincon method by only temperature manipulation opens up a whole new spectrum of analysis with only minor additions to equipments. The fact that the reagents and equipments are all retained save for the addition of the thermostated waterbath introduces improvement in performance to be effected quickly with minimal financial costs.

6.7.1 Method of optimization

Key parameters that influence performance of the proposed method were studied in order to establish the optimum working configurations.

6.7.1.1 Influence of temperature

Temperature is a major parameter in the reaction. It is evident that not only did the response increase when the temperature was increased, but also that the system became more precise as seen from Table 6.1. A temperature of 65 °C gave the best results and this temperature was used throughout.

6.7.1.2 Effect of chromogenic flow rate (C_B).

Figure 6.2 clearly demonstrates the influence of varying the colour reagent stream (C_B) flow rate.

It was established that the decrease in flow rate is inversely proportional to the analytical signal. A lower flow rate yielded a higher sensitivity and a very low % RSD of 0.58%. This behaviour can better be explained in that the slow flow rate supplies a low volume which is however enough for the reaction to occur. The steady state thus established allows enough time for a reaction on which reasonable assumptions can be made. The formation of the blue colored complex favors sufficient residence time. If the flow rate is high this flushes the system prior to a sufficient colour development. The slow stream increases the residence period. A flow rate of 1.6 mℓ/min gave the best response and precision and was chosen for the proposed system.

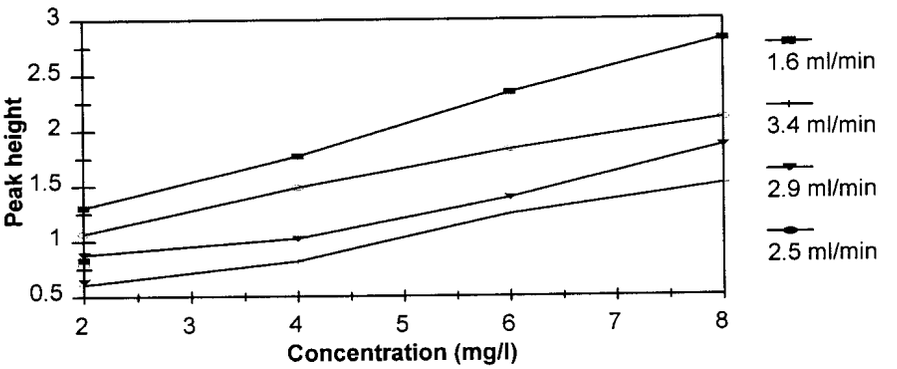


Figure 6.2 Effect of the colour reagent flow rate (C_B).

Table 6.1. Influence of temperature on response and precision

Temperature	Statistics	Peak height in mV				
		2 mg/ℓ	4 mg/ℓ	6 mg/ℓ	8 mg/ℓ	10 mg/ℓ
25 ^{0} C	$\bar{x}$	0.83	1.31	1.8	2.3	2.79
	% RSD	0.4	0.28	0.19	0.2	0.66
30 ^{0} C	$\bar{x}$	0.85	1.6	2.15	2.62	3.2
	% RSD	0.52	0.43	0.04	0.29	0.16
35 ^{0} C	$\bar{x}$	0.91	1.86	2.7	3.61	4.5
	% RSD	0.38	1.18	0.15	0.16	0.08
40 ^{0} C	$\bar{x}$	0.96	1.95	2.9	3.8	4.61
	% RSD	0.3	0.1	0.18	0.19	0.09
45 ^{0} C	$\bar{x}$	1.16	2.14	3.1	3.98	4.81
	% RSD	0.08	0.15	0.37	0.43	0.13
50 ^{0} C	$\bar{x}$	1.17	2.22	3.11	4	4.9
	% RSD	0.12	0.59	0.54	0.33	0.09
55 ^{0} C	$\bar{x}$	1.23	2.34	2.23	4.21	5.2
	% RSD	0.16	0.15	0.31	0.16	0.18
60 ^{0} C	$\bar{x}$	1.24	2.38	3.34	4.3	5.3
	% RSD	0.72	0.37	0.97	0.08	0.18
65 ^{0} C	$\bar{x}$	1.24	2.38	3.27	4.3	5.3
	% RSD	0.01	0.01	0.07	0.04	0.01
70 ^{0} C	$\bar{x}$	1.24	2.38	3.33	4.3	5.3
	% RSD	0.3	0.3	0.68	0.08	0.15

6.7.1.3 Effect of buffer reagent flow rate (C_A).

The pattern of higher flow rate, low sensitivity was reversed with the manipulation of this parameter (Figure 6.3).

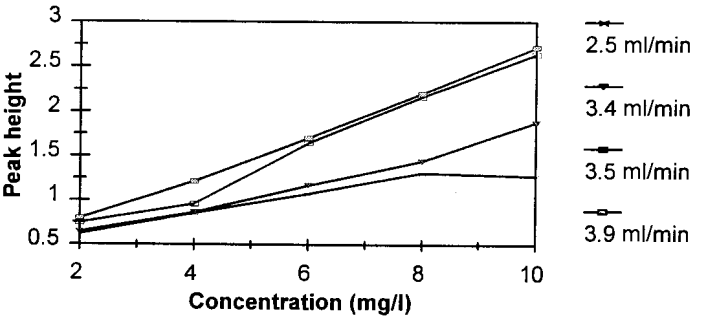


Figure 6.3 Effect of the carrier stream flow rate (C_A).

A new phenomenon emerged from the study of buffer flow rate variation. An increase in the flow rate resulted in a higher sensitivity. This can be explained with one of the following reasonable conclusions. With faster flow rates the dispersion decreases, resulting in sharper peaks and thus higher response. Higher flow rate also feeds the system with a larger volume of the buffer allowing effective buffering, or this introduces enough cyanide for masking. Effective buffering at pH 9 limits interferences that would inevitably reduce the sensitivity. A flow rate of 3.9 mℓ/min gave the best response, linearity and precision and this was used for further work.

6.7.1.4 Effect of reaction coil length.

The method is based on the masking of the different metal ions with cyanide, effective de-

masking of only zinc by cyclohexanone and the final complexation of zinc with zincon. Reaction coil length therefore had an important influence on the response and precision and particular attention was given in the optimisation of this parameter in the system design. Various reaction coil lengths with an i.d. of 1.14 mm were evaluated (Figure 6.4). A 250-cm reaction coil length was chosen since it displayed the best sensitivity and lowest % RSD among all the various line lengths.

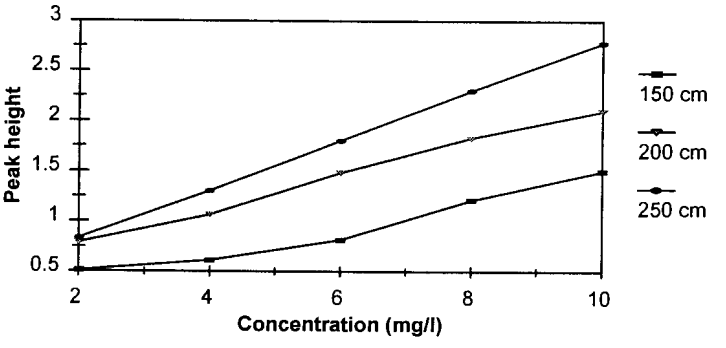


Figure 6.4 Effect of reaction coil length.

6.7.2 Evaluation of the method.

The proposed temperature enhanced method was critically evaluated with regards to linearity, accuracy, precision, sample interaction, interferences and sampling rate.

6.7.2.1 Linearity.

The linearity of the proposed FIA system for the determination of zinc was evaluated under optimum conditions. The data collected evaluated yield a straight line between 2 - 10 mg/ℓ with

the relationship for relative peak height versus zinc ion concentration of:

$$H = 0.2454C + 0.33412; r = 0.9976$$

where H = relative peak height and C = zinc ion concentration in mg/ℓ. The correlation coefficient (r) indicates that the method is linear for an analyte concentration ranging between 2 - 10 mg/ℓ.

6.7.2.2 Accuracy

Real samples from soft drinks, beer energy drinks, fertilizers and steel wool were analysed with the proposed flow injection system. The accuracy of the proposed FIA Analyser was evaluated by comparing the results from real samples of the FIA system with those obtained with a standard ICP procedure. The results as shown in Table 6.2 revealed a good correlation.

6.7.2.3 Precision

The precision of the method was determined by performing ten repetitions of the each sample at 25 °C and 65 °C . The results listed in Table 6.2 show that the proposed system is more precise at 65 °C.

TABLE 6.2 Comparison of the results of the proposed FIA system at 25 °C and 65 °C with a standard ICP procedure.

Samples	25 °C		65 °C		ICP
	% RSD	$[Zn^{2+}]$ as mg/ℓ	% RSD	$[Zn^{2+}]$ as mg/ℓ	$[Zn^{2+}]$ as mg/ℓ
Sample 1	0.41	0.256	0.32	0.262	0.26
Sample 2	0.4	0.286	0.28	0.291	0.291
Sample 3	0.52	0.314	0.4	0.321	0.32
Sample 4	0.39	0.866	0.31	0.914	0.911
Sample 5	0.48	12.36	0.3	12.85	12.81

6.7.2.4 Sample interaction

The interaction (carry-over effect) between consecutive analysis was investigated by analysing a low analyte concentration followed by a high analyte concentration and immediately followed by a low analyte concentration using the equation:

$$\text{Interaction} = \{(A_3 - A_1)/A_2\} \times 100$$

where A_1 = the true peak height of a sample with a low analyte concentration,

A_2 = the true peak height of a sample containing ten times more analyte and

A_3 = the peak height for an interacted sample containing the same amount of analyte as A_1.

The sample interaction between samples as calculated was less than 1% for the proposed flow injection system for the determination of zinc at a rate of 120 samples per hour which is negligible.

6.7.2.5 Interferences.

Tables 6.3 and 6.4 display results of different metal ions whose presence may affect the determination of zinc. It is clear from the Tables that the influence of all tested metal ions is tolerable with the highest % error for Fe^{2+} at 3.2 with the proposed system at 65 °C (Table 6.4) giving the best results.

Table 6.3 Influence of interferences at 25 °C.

Metal ion	Concentration of interferent as mg/ℓ	Zinc found as mg/ℓ	% Error
Fe^{3+}	50	51.8	3.8
Cu^{2+}	50	50.4	0.8
Ni^{2+}	50	52.3	4.6
Pb^{2+}	50	51.2	2.4
Co^{2+}	50	51.2	2.4

Table 6.4 Influence of interferences on the proposed system at 65 °C.

Metal ion	**Concentration of interferent as mg/ℓ**	**Zinc found as mg/ℓ**	**% Error**
Fe^{3+}	50	51.6	3.2
Cu^{2+}	50	50.1	0.2
Ni^{2+}	50	51.3	2.6
Pb^{2+}	50	50.9	1.8
Co^{2+}	50	50.6	1.2

6.8 Conclusion

After the method was evaluated and comprehensively tested. it became evident that this proposed method performed to the required standard. The introduction of the water bath allows the shortening of the manifold as compared to the room temperature one. Temperature manipulation fine-tuned the normal Zincon method into an excellent tool for zinc analysis. The only set back with this method is that it still uses cyanide which is not environmentally friendly. This is however lessened since only minute volumes are used. This proposed method is rapid, sensitive, precise, and accurate with a high sample out-put.

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CHAPTER 7

FLOW INJECTION SPECTROPHOTOMETRIC ASSAY OF α-AMYLASE ACTIVITY

7.1 Introduction

Every enzyme catalyses a reaction which is both substrate-specific and product-specific; because of this, enzymes are extremely valuable as analytical reagents. In particular, they can be used for the estimation of specific substances, possibly present at very low concentrations, in the presence of other, chemically similar, substances. Ordinary chemical reagents might not be able to distinguish between several of the components of a sample, and costly separation procedures might be required before a satisfactory analysis can be carried out; even then, several different products might be formed because of side-reactions, thus reducing the sensitivity of the method. In contrast, enzyme-based methods of analysis are both selective and sensitive, which means that little or no sample preparation is required in many cases.

Another characteristic of enzyme-catalysed reactions which makes them suitable for analytical applications is that they proceed under relatively mild conditions (e.g., at certain pH and at around

room temperature): hence they are usually simple to set up, and can be used for the analysis of substances which would be unstable under more extreme conditions.

On the other hand, enzymes themselves are quite unstable. For this reason, identical enzyme preparations may be found to have different activities, as may the same preparation on different occasions, particularly if correct storage and handling procedures are not strictly adhered to. However, such variations in activity should not affect the results obtained by enzyme-based methods of analysis, provided the activity present in each case is a reasonable approximation to that specified, and provided appropriate calibration experiments are always carried out.

Another disadvantage in the use of enzymes is that they are often expensive and sometimes difficult to obtain. Hence it is important that they are not used in a wasteful fashion. One way of minimizing wastage is by the use of immobilized enzymes, which are now becoming increasingly available: these can be recovered intact at the end of a procedure and used again. Enzymes in this form are sometimes more stable than in free solution.

Enzyme-based analytical procedures may be designed to determine the concentrations of substrates, coenzymes, activators and inhibitors.

Enzymes have been used in certain industrial processes for centuries, their precise role, or even their identity, was not known over most of this period: they were often utilized as components of intact cells (e.g., yeasts in the baking and brewing industries) or as extracts containing a mixture of many enzymes (e.g., malt, containing amylase and proteolytic enzymes, also used in baking and brewing). Despite the importance of enzymes from animal and plant sources, their

large-scale extraction poses technical, economical and possibly ethical problems.

α-Amylase is widely distributed in nature, i.e., in mammals, fungi, bacteria, most of the plant kingdom, etc. [1]. Its activity is very important in both clinical and food chemistry. In clinical chemistry the activity of α-amylase indicates the presence of parotitis, pancreatitis, and reveals occlusion of the pancreas. For food industry it is important for the production of saccharin and glucose, being extensively used in the sweet factory. The production of ethanol relies heavily on the usage of the hydrolysis product from α-amylase activity. The brewing and confectionery industries both depend on the activity of α-amylase. Therefore the determination of the activity of α-amylase is of prime importance.

7.2 Biochemistry of α-amylase

In baking bread, the preliminary process involves the mixing of wheat flour (mainly starch and protein) with yeast and water. Starch consists of D-glucose units linked by α-1,4 glycosidic bonds, with α-1,6 bonds at branching points; the enzymes α-amylase and β-amylase present in flour cleave some of the α-1,4 bonds, the eventual products being glucose, maltose and some oligosaccharides which cannot be broken down further because of the presence of α-1,6 bonds. Glucose and maltose can then be metabolized by the yeast, and carbon dioxide is formed which distends the protein framework of the dough, ready for baking. However, wheat flour often has a low α-amylase content, so it may be supplemented with malt flour or, a more recent development, by fungal α-amylase (from *Aspergillus oryzae*). Wheat α-amylase is very heat stable, so it continues to act for a time during the baking process; this may lead to too much starch breakdown taking place, and cause the bread to be somewhat soggy. For this reason, wheat α-

amylase is sometimes inactivated by a brief treatment with superheated steam prior to supplementation of the flour with the more heat-labile fungal α-amylase.

In the industrial production of glucose from starch, the latter is first solubilized and partly degraded by bacterial α-amylase and then treated with fungal amyloglucosidase. This can cleave both types of glycosidic bond found in starch, so gives a good yield of glucose.

7.3 Choice of analytical methods

Due to its high impact in diagnosis and in the quality of food the methods proposed for the assay of α-amylase activity must be characterised by objectivity and high selectivity. The objectivity can be obtained by using flow analysis techniques. An overview concerning the utilisation of FIA in the determination of enzyme activity is proposed by Luque de Castro and Fernadez-Romero [2]. Generally two techniques are recommended to be used in FIA for the assay of the enzyme activity:

(i) the immobilization of the enzyme on a support;

(ii) RFIA with the injection of the enzymatic solution into the system or by using the merging zones approach.

Usually the immobilization on a support has as a result a decrease in the activity of the enzyme. It follows that the data obtained will not correspond to the real values. The second technique is more appropriate for this kind of analysis. Several techniques are proposed for the assay of α-amylase [3]. Among these are an FIA system employing an enzymatic reaction proposed by Emneus *et al.* [4-6]. Different types of inorganic supports were tested for the immobilization of

α-amylase [4].

The utilisation of sensors [5] in FIA systems improved the quality of analytical information [7,8]. A spectrophotometric technique based on the degradation of starch by α-amylase and measurement of the residual starch iodine-complex is also used in a FIA system [9]. Fourier-transform-infrared spectroscopy is also recommended as a detection method of a FIA system for the assay of the activity of α-amylase [10]. Min *et al.* [11] proposed a SIA technique for the assay of α-amylase activity. The detection is based on the monitoring of the de-colouration of the starch-iodine complex.

7.4 Principle of α-amylase assay

The α-amylase assay is based on the assay of maltose obtained in the hydrolysis of starch at 37^{0}C [12]. Practically, the method relies on the reaction of 3.5-dinitrosalicylic acid with maltose at high temperatures (96 °C). Maltose is the major component of the amylase hydrolysis reaction. It occurs as 70 % of the product and can thus safely be used as the indicator for the diastasis activity. The colour that results from the reaction between the maltose and the acid develops fast enough to be effectively used in flow injection analysis.

7.5 Experimental

7.5.1 Reagents and solutions

All reagents used were prepared from analytical grade chemicals. De-ionised water was used

throughout. The water was tested beforehand for any traces of other ions that may exist after treatment. All solutions were degassed before any measurements with a vacuum pump system. The main solutions were prepared as follows:

Chromogenic reagent. 1 g of 3,5 dinitrosalysilic acid (Fluka. Bucsh SG, Switzerland) was dissolved in 20 ml of 2 mol/ℓ NaOH solution, followed by the addition of 30 g of sodium-potassium tartrate and diluted to 100 mℓ with de-ionised water. The solution was protected from CO_2 by covering with a tight fitting cap.

Phosphate Buffer. 9.470 g of Na_2HPO_4 (Merck, Germany) and 9.208 g of NaH_2PO_4 (Merck, Germany) were each dissolved in sufficient de-ionised water before they were individually diluted to 1 litre. To prepare 500 mℓ phosphate buffer (pH 6.89), 200 mℓ of Na_2HPO_4 were mixed with 300 mℓ of NaH_2PO_4 and 2.3 g of dried NaCl. This was followed by the adjustment of the pH to 6.89 with either 2 mol/ℓ HCl or 2 mol/ℓ NaOH as required.

Starch. The starch solution was prepared by dissolving 0.270 g of dry starch (Merck) in 200 mℓ of 6.89 phosphate buffer and gently boiling for a minute. The solution was slowly cooled to room temperature. Phosphate buffer was used for the dilution of the solution to 400 mℓ.

α-Amylase working standard solution. An α-amylase (from Bacillus licheniformis, 2.0 U/mg) (Fluka, EC 3.2.1.1, Buchs, Switzerland) solution was prepared by addition of the appropriate mass of the pure α-amylase to a specific volume of de-ionised water to prepare a standard stock solution (0.05 FAU). The other working α-amylase solutions (0.01-0.04 FAU) were obtained by serial dilutions from the standard stock solution.

7.5.2 Apparatus

The following equipment were used: A Cenco peristaltic pump operating at 10 revolutions per minute; a VICI 10-port two-position sampling valve with two identical sampling loops, each having a volume of 50 mℓ; reaction manifold system (Figure 7.1); three thermostat water baths set at 37 ^{0}C, 100 ^{0}C and 25 ^{0}C; a Pye Unicam SP6-550 UV-visible spectrophotometer equipped with a 10 mm Hellma-type flow-through cell (volume 80 μℓ); PC30-B interface board (Eagle Electrics, Cape Town, South Africa) for data acquisition and device control; a distribution board (MINTEK, Randburg, South Africa).

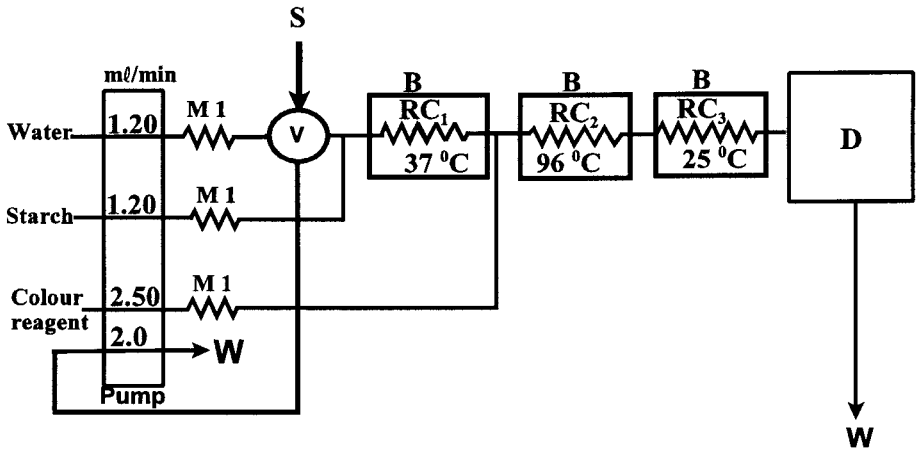

Figure 7.1 Schematic flow diagram for the determination of α-amylase activity. S - Sample; M1 - coil (50 cm); D - Detector; W - Waste; V - Valve; B - Water bath; Tube i.d. = 1.14 mm; RC_1 - reaction coil (100 cm); RC_2 - reaction coil (100 cm); RC_3 - reaction coil (60 cm).

The *FlowTEK* [13, 14] software package was incorporated into the system.

7.5.2.1 Flow System

The sequence of equipment interaction for the determination of α-amylase activity is represented in a detailed schematic outlay in Figure 7.1. The manifold consists of Tygon tubing with an inside diameter of 1.14 mm cut into appropriate lengths and wound around glass rods with an outside diameter of 15 mm. The manifold consisted of three tubes one leading to the valve system and propelling de-ionised water at 1.2 mℓ/min. The next one propelled starch solution in phosphate buffer at 1.2 mℓ/min that mixed with the sample before leading into a 37 ^{0}C water bath. The last tube propelled the colour reagent at 2.5 mℓ/min to mix with the hydrolysis product before leading into a 96 °C water bath. (Figure 7.1)

The main reaction (catalysed by α-amylase) is taking place in the first bath at 37 ^{0}C. The reaction between maltose and 3,5 dinitrosalysilic acid occurred at the high temperature of 96 °C. The final product was cooled to 25^{0}C in a water bath before monitored spectrophotometrically at 540 nm.

7.5.3 Sample preparation

Seeds were germinated for three days, then harvested and dried in an oven. They were then ground and 2 % peptone solution was added, followed by stirring for 2 hours, centrifugated and filtered using Whatman filter paper. The supernatant was collected and analysed for α-amylase activity. For powdered samples the sample preparation method was followed after the addition of 2% peptone solution and carried on from this step as described above for seed treatment.

7.6 Optimization of the method

In order to obtain a reliable assay of α-amylase activity, the following parameters were optimised:

7.6.1 Effect of flow rates

A series of preliminary and evaluation trials were conducted in order to optimise the flow rates of the colour, carrier and starch solutions. The experiments proved that the method performs better when the flow rates of the carrier and starch were similar and that the total of both these flow rates was almost equal to that of the colour reagent. This leads to either varying the starch-carrier flow rate and keeping the flow rate for colour constant or vice versa.

7.6.2 Effect of starch flow rate when a constant colour reagent flow rate (2.5 mℓ/min) is used

Figure 7.2 displays the effect of the flow rate of starch solution. It is evident from Figure 7.2 that a flow rate of 1.2 mℓ/min gives a maximum sensitivity and a straight calibration curve. As stated earlier the system operated better when the total starch-carrier flow rate almost equal the colour flow rate. Therefore a total of 2.4 mℓ/min feeds the system with a steady volume of the reagents which is stoichiometrically sufficient for amylase hydrolysis and colour development.

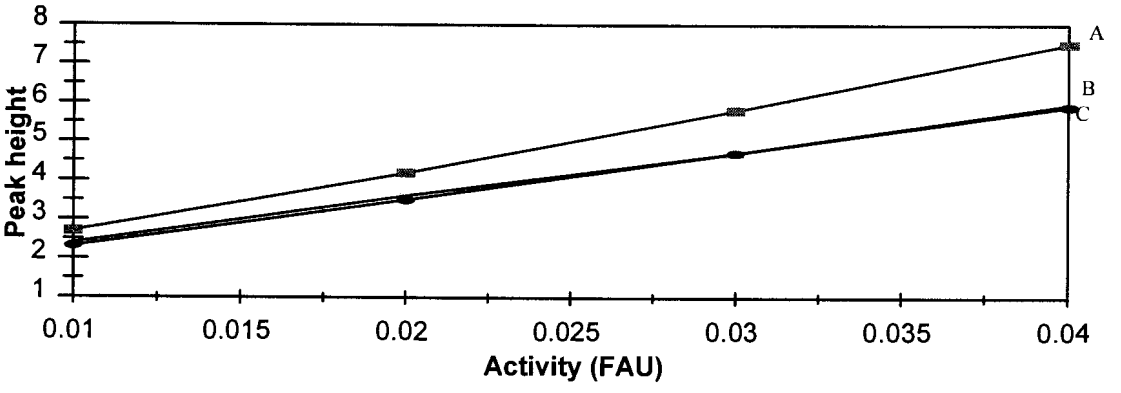

Figure 7.2 Effect of carrier-starch flow rate at constant colour reagent flow rate (2.5mℓ/min). A - 1.2 mℓ/min; B - 1.0 mℓ/min; C - 1.6 mℓ/min.

7.6.3 Effect of colour reagent flow rate when a constant starch flow rate (1.2 mℓ/min) is used

A flow rate of 2.5 mℓ/min for the colour reagent yielded the best sensitivity and precision with a

straight calibration curve (Figure 7.3). With higher flow rates the residence time of reagents in the reaction coil (RC_2) decreases to such an extend that the 3.5-dinitrosalicylic acid has not sufficiently bound to the maltose for full colour development and the response dropped as seen from the Figure 7.3. The sensitivity and also precision decreased for lower flow rates due to insufficient reagents to react with the colour reagent as only minimal quantities will be propelled.

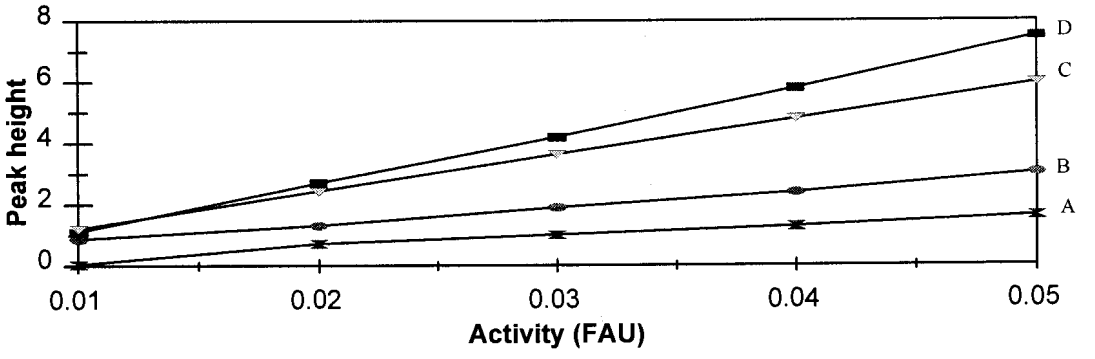

Figure 7.3 Effect of colour reagent flow rate at constant carrier-starch flow rate (1.2mℓ/min). A - 1.0 mℓ/min; B- 2.0 mℓ/min; C - 2.5 mℓ/min; D - 2.9 mℓ/min.

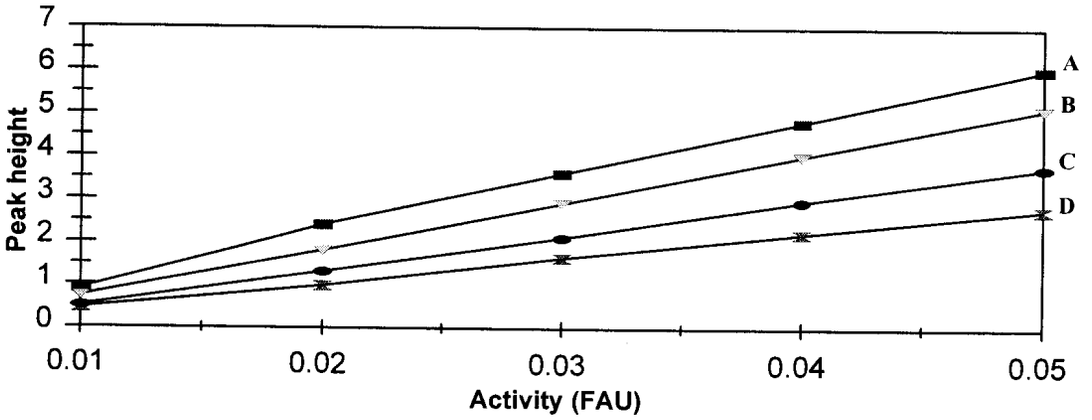

Figure 7.4 Effect of reaction coil length. A - 100 cm (hollow support); B - 100 cm (solid support); C - 150 cm; D - 200 cm.

7.6.4 Influence of the different reaction coil lengths

There are basically three different operating conditions at different temperatures involved in the flow injection spectrophotometric assay of α-amylase activity. As each temperature has a different function to fulfill, care should be taken to use the optimum working temperature, to make sure that this temperature should be reached as fast as possible and that the optimum product is formed. The main reaction, that is catalysed by α-amylase, is the hydrolysis of the 1, 4 glycosidic bond from starch. The optimum operational temperature for the α-amylase activity is body temperature of 37°C . The residence time as a function of reaction coil 1 (RC_1) was evaluated and it was found that a reaction coil of 100 cm gave the best sensitivity and precision. The second reaction between maltose and 3.5-dinitrosalycilic acid is temperature dependent and is much more pronounced at 96 °C. The following parameters play a very important role in this

part of the reaction and was evaluated; the reaction should reach 96 °C as fast as possible, residence time and dispersion that is dependent on coil length. The reaction coils were wound around solid rods and also around hollow tubes with outside diameters of 15 mm. Figure 7.4 displays this trend of results, however a hollow support for the reaction coil seemed to yield the best results as it exposes the reaction coil to a larger volume of hot water at 96 °C than does a solid cylindrical support. A reaction coil (RC_2) of 100 cm on a hollow support gave the best response and precision and was used for further work. The formation and accumulation of air bubbles in the flow cell is one of the main problems for routine use of the proposed flow injection system. At the temperature of 96 °C the system is more prone for this. It was therefore necessary to cool down the manifold to 25 °C before detection. It was found that a 60 cm cooling coil was sufficient enough for this purpose.

7.6.5 Effect of NaOH addition into the colour reagent solution.

Through preliminary experiments it was discovered that the addition of 2 mol/ℓ NaOH as percent fraction of the total volume of colour reagent solution had a marked influence on the sensitivity of the reaction between the maltose and the 3.5-dinitrosalycilic acid. Various volume fractions of the 2 mol/ℓ NaOH as fraction of the total volume of the colour reagent solution were evaluated and the results are given in Figure 7.5. The best results were obtained with a 0.33 % (v/v) NaOH and this was used for further work.

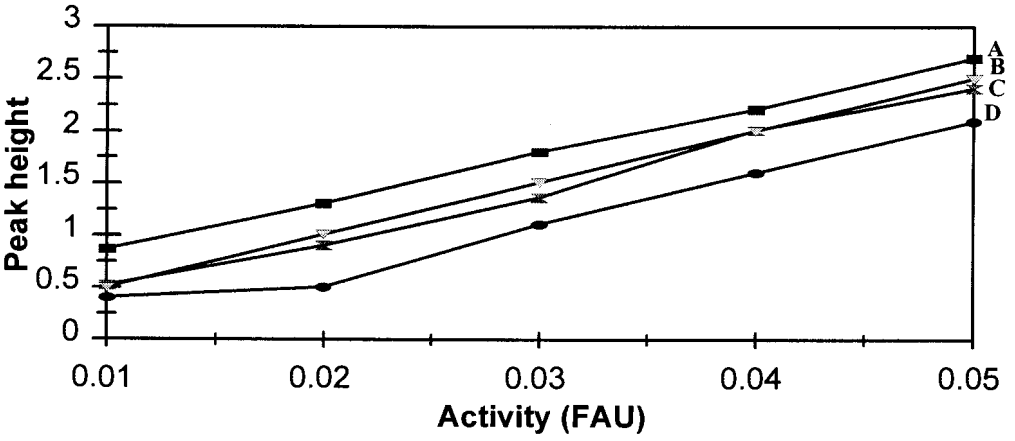

Figure 7.5 Effect of 2 mol/ℓ NaOH solution. A - 0.33 %; B - 0.50 %; C - 0.67 %; D - 0.00 %.

7.6.6 Effect of starch concentration

The concentration of starch has a significant bearing on the sensitivity of this method. It also gives the working range for this analysis since it is the substrate for this enzymatic reaction. Enzymatic studies proved that the substrate should always be in excess compared with the enzyme concentration. It was determined that 0.1 % (w/w) and 1 % (w/w) gave results that went beyond the chosen sensitivity range for some chosen amylase concentration. A 0.01 % (w/w) of starch was chosen after it showed consistent results for all the standards. (Table 7.1)

7.6.7 Effect of colour reagent concentration.

Maltose, the major component of the α-amylase hydrolysis reaction, and 3,5 dinitrosalycilic acid reacts at high temperatures to form a brick red coloured complex that is monitored spectrophotometrically at 540 nm. Therefore the influence of the concentration of a 3,5 dinitrosalysilic acid solution in 2 mol/ℓ NaOH were evaluated and the results are given in Table 7.2. Although the best response was obtained with a 1.5 % (w/w) 3,5 dinitrosalysilic acid (Table 7.2), the best linearity and precision was obtained with a 2 % (w/w) concentration and this was used for the proposed system.

Table 7.1 Effect of starch concentration (%, w/w) on the peak height for different α-amylase activity (FAU)

Starch concentration % (w/w)	α-amylase activity (FAU)				
	0.01	0.02	0.03	0.04	0.05
0.01	Peak Height				
	1.0986	2.711	4.2034	5.8031	7.4086
0.1	2.8423	4.6048	6.4026	8.2049	-
0.15	2.9611	4.8779	6.5098	8.2997	-
1	3.0259	5.8837	8.0089	-	-

7.6.8 Influence of temperature of reaction coil (RC_2)

Temperature is a major role player in the reaction between maltose and 3,5 dinitrosalycilic acid.

It is evident that the response increase dramatically when the temperature is raised to 96 °C (Table 7.3) and this temperature was used throughout.

Table 7.2 Effect of colour reagent concentration on the peak height for different α-amylase activity (FAU)

Colour reagent concentration % (w/w)	α-amylase activity (FAU)				
	0.01	0.02	0.03	0.04	0.05
0.1	Peak height				
	0.3811	0.7178	0.8149	0.9211	1.2863
0.5	0.4834	0.8876	1.0117	1.5235	1.8336
1	1.0986	2.711	4.2034	5.8031	7.4086
1.5	0.6336	1.0897	1.6975	2.0701	2.7878
2	0.5344	0.9117	1.3098	1.7845	2.0109

Table 7.3 Effect of temperature of the colour development water bath for different activities of α-amylase

Temperature ^{0}C	α-amylase activity (FAU)				
	0.01	0.02	0.03	0.04	0.05
60	Peak height				
	0.3676	0.3985	0.4309	0.4511	0.4568
80	0.4774	0.5118	0.6101	0.6101	0.6448
96	1.0986	2.711	4.2034	5.8031	7.4086

7.7 Evaluation of the method

The sensitivity and performance of the method were evaluated under optimised conditions with regards to detection limit, linearity, accuracy, precision, sample interaction, and sample rate.

7.7.1 Detection limit.

It is very important that any new method should be evaluated for its detection limit to be sure which concentrations of the analyte can be determined with confidence and that the results obtained can be trusted. The detection limit for the method was calculated using the following formula:

$$DL = \frac{[3S_b + I_b] - k}{m}$$

where: DL is the limit of detection;

S_b is the standard deviation of the background signal;

I_b is the relative peak height of the background signal;

k is the intercept of the calibration graph and

m is the slope of the calibration graph.

The detection limit was found to be 0.0048 FAU

7.7.2 Linearity

The linearity of the method was evaluated under optimum conditions for the system. The data

collected for the standards yielded a straight line between 0.01 and 0.05 FAU. The regression coefficient for this system is:

$$H = -0.47 + 157.12a; r = 0.9998$$

where H is relative peak height and a is the enzymatic activity (FAU). The correlation coefficient (r) indicated that the method is linear for the chosen working standards.

7.7.3 Precision

The precision for this method was tested by performing ten repetitions of the analysis on each of the different samples. In all the analysis carried out the % RSD was found to be less than 2.7%. (Table7. 4).

Table 7.4 Comparison between the results obtained for the α-amylase activity (FAU) with the proposed FIA method and with two standard methods [12]

Number of sample	Proposed FIA method	FIA iodine method	Falling no. Method
Sample 1	0.034	0.035	0.032
	% RSD = 0.17	% RSD = 0.64	% RSD = 1.49
Sample 2	0.006	0.006	0.006
	% RSD = 0.26	% RSD = 0.75	% RSD = 2.64
Sample 3	0.042	0.044	0.042
	% RSD = 0.42	% RSD = 0.96	% RSD = 2.47

7.7.4 Accuracy

The results from analysis by this method were tested for accuracy by comparing them with those from well established and standard methods, which are the iodine and the falling number methods. It is evident from Table 7.4 that these results did not differ much from those obtained with the other methods and that the results compared excellently.

7.7.5 Sample interaction

The influence of sample interaction seemed to be insignificant as there was no observable change or shift from the baseline with each successive analysis. This is due to the fact that at some stage the method employs very high temperature which aids in the cleaning of the system. However it was necessary to rinse the system with copious amounts of the buffer after the analysis was completed and just before another batch could be determined.

7.7.6 Sampling rate.

The proposed method is faster than the other FIA methods presented in the literature [7,8]. The sampling rate was found to be 60 samples per hour.

7.8 Conclusion

The proposed flow injection analysis method satisfies all the requirements for an analytical method of analysis. It is reliable with precision and accuracy clearly demonstrated. When compared to

other known and tried methods it stands out as a simple and fast one. The enzyme is not immobilized on a support, therefore the enzymatic activity determined is accurate. The precision obtained is better than the one given by the other FIA systems [7,8].

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CHAPTER 8

DETERMINATION OF PARACETAMOL USING A FLOW INJECTION ANALYSIS / SPECTROPHOTOMETRIC SYSTEM

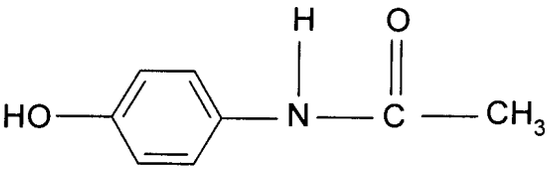
8.1 Introduction

Pharmaceutical analysis encompasses a wide variety of substances that are used as raw materials for the manufacturing of commercially available preparations. Legislation is becoming increasingly strictly regarding purity and contents. Pharmaceutical preparations are not only analysed for the content of a given active principle for excipient in samples from a manufactured batch, but also for consistency within the batch concerned. A large number of routine analysis are thus involved with an increasing demand of automatic methods for efficient quality control in the pharmaceutical industry.

Automatic equipment has also been incorporated into pharmaceutical laboratories in response to the large amounts of data demanded by governmental agencies on newly developed pharmaceuticals. These relate to bioavailability, transport across biological membranes, receptor accessibility, metabolism, and excretion - data which are usually available to control

manufacturing as well [1].

Paracetamol (N-acetyl-p-aminophenol)



is used as an alternative to aspirin because of its analgesic and antipyretic activity. It has been formulated in many commercial tablets as a sole active ingredient or with other active drugs (e.g., caffeine, codeine phosphate, folcodine, phenylepherine). Overdoses of paracetamol causes hepatic necrosis, probably owing to its metabolite, N-acetyl-p-benzoquinone [2].

Diagnosis must be quick and rapid methods for the determination of paracetamol are therefore needed.

The metabolism of paracetamol has been studied extensively using NMR spectroscopy and it is known that the two major metabolites are glucuronic acid and sulfate conjugates at the phenolic hydroxyl group [3].

8.2 Choice of analytical methods

Due to its high impact on the quality of the body, the assay of paracetamol must be characterised by objectivity and high selectivity. The objectivity can be obtained by using flow analysis techniques.

Spectrophotometric methods are recommended for the assay of paracetamol. 2-Iodylbenzoat [4], 8-hydroxyquinoline [5], and on the utilization of the oxidative minicolumn before detection [6,7]. are recommended to be used in the spectrophotometric method. A fluorimetric method [2] is also recommended for the assay of paracetamol.

FTIR in coupling with FIA systems for the assay of paracetamol gave very good qualitative results, and satisfactory quantitative results [8,9].

A voltammetric flow-through cell has also been designed to be used in the assay of paracetamol [10].

8.3 Principle for the assay of paracetamol

The principle selected for the FIA/spectrophotometric assay of paracetamol is based on the reactions proposed by Martínez Calatyud *et al.* [11].

The reactions involved, are as follows: paracetamol is oxidised with hexacyanoferrate (III). The reaction product is is reacting with phenol, to form a blue product.(Figure 8.1)

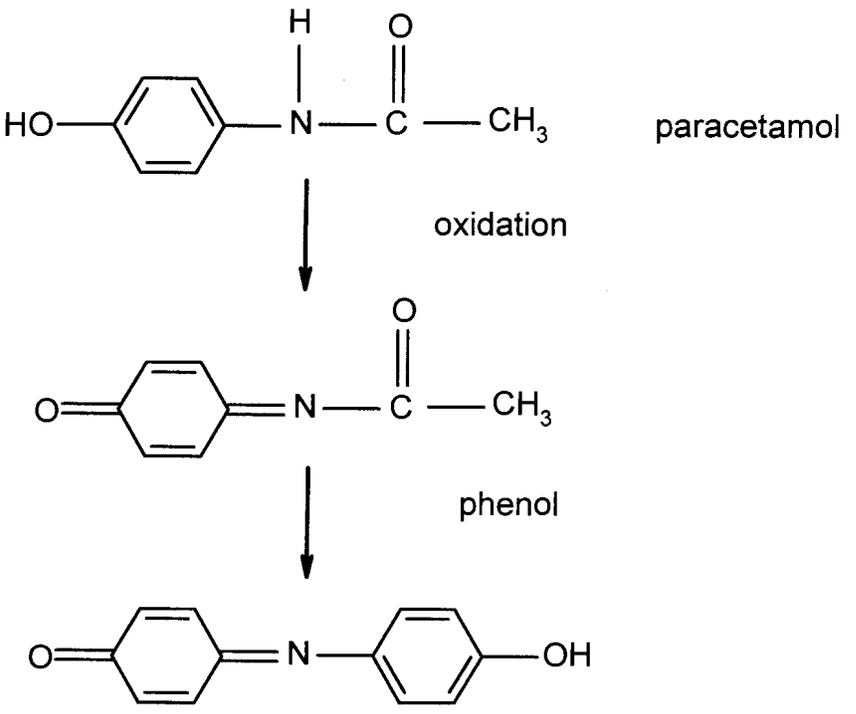


Figure 8.1 N-[p-hydroxyphenyl]-p-benzoquinoneimine (blue colour)

Colour development for the assay of paracetamol

8.4 Experimental

8.4.1 Reagents and solutions

All reagents used were prepared from analytical grade chemicals. De-ionised water was used throughout. The water was tested beforehand for any traces of other ions that may exist after treatment.

All glassware were soaked overnight in 25% (v/v) nitric acid, and thoroughly washed with ultra

pure water from a Modulab system (San Antonio, Texas, USA).

The main solutions were prepared as follows:

Phenol solution. 7.5 g of phenol (Merck, Germany) were dissolved in 250 mℓ of deionised water, followed by dilution to 500 mℓ with de-ionized water.

Potassium Hexacyanoferrate (III). 1 g of potassium hexacyanoferrate (III) (Sigma, Aldrich) were dissolved in 250 mℓ de-ionised water. The solution was diluted to a final volume of 500 mℓ with de-ionized water.

Paracetamol. 0.05 g paracetamol (Merck, Germany) were dissolved in 250 mℓ de-ionized water, the equivalent volume of ammonia solution added to obtain a concentration of the solution in ammonia of 0.4 mol/ℓ. The solutions necessary for calibration were obtained by serial dilutions from the stock solution.

8.4.2 Apparatus

The following equipment was used: A Cenco peristaltic pump operating at 10 revolutions per minute; a VICI 10-port two-position sampling valve with two identical sampling loops, each having a volume of 100 μℓ; reaction manifold system (Figure 8.2); two thermostat water baths set at 80^{0}C and 20^{0}C; a Pye Unicam SP6-550 UV-visible spectrophotometer equipped with a 10 mm Hellma-type flow-through cell (volume 80 μℓ); PC30-B interface board (Eagle Electrics, Cape Town, South Africa) for data acquisition and device control; a distribution board (MINTEK,

Randburg, South Africa).

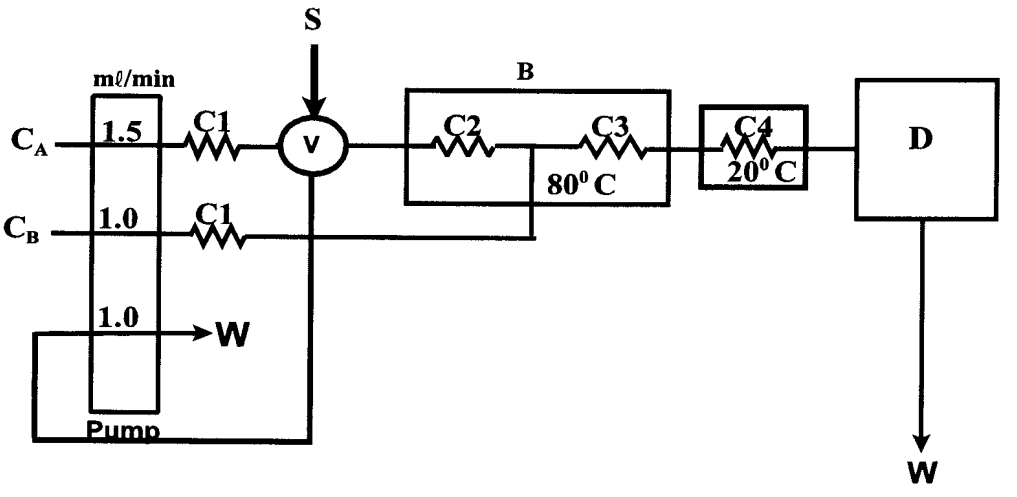


Figure 8.2 Schematic flow diagram for the determination of paracetamol. S - Sample; C1 - coil (50 cm); C2 - reaction coil (140 cm); C3 - reaction coil (400 cm); C4 - cooling coil (100cm); D - Detector; W - Waste; V - Valve; B - Water bath; Tube i.d. = 0.76 mm; C_A - Hexacyanoferrate (III); C_B - Phenol.

The *FlowTEK* [12, 13] software package was incorporated into the system.

8.4.2.1 Flow System

The flow injection system used, is schematically represented in Figure 8.2. The manifold consists of Tygon tubing with an inside diameter of 0.76 mm cut into appropriate lengths and wound around glass rods with an outside diameter of 10 mm. The manifold consists of two tubes into which 100 µℓ of sample is injected, one leading to the valve system, propelling hexacyanoferrate (III) solution at 1.5 mℓ/min in C_2 at 80 °C. The next tube propelled phenol solution at 1.0 mℓ/min.

(Figure 8.2)

The oxidation and colour development reactions are occuring in the first bath at 80^{0}C. The final product was cooled to 20^{0}C in a water bath before monitored spectrophotometrically at 630 nm.

8.4.3 Sample preparation

Ten tablets were weighted and grounded with a pestle and mortar. The powder tablet was quantitatively transferred into a beaker with 50 mℓ de-ionised water, and throughly stirred. The solution was filtered through a No. 4 Whatman filter paper. The solid particles on the filter paper were washed three times with 20 mℓ de-ionised water, and the filtrate was diluted to a final volume of 500 mℓ with de-ionized water into which was added the equivalent amount of NH_3 to obtain a final solution of 0.4 mol/ℓ in ammonia.

8.5 Optimization of the method

In order to obtain a reliable assay of paracetamol, the following parameters were optimised:

8.5.1 Effect of flow rates

A series of preliminary and evaluation trials were conducted in order to optimise the flow rates of both phenol and hexacyanoferrate (III) solutions. It was found that a flow rate of 1.5 mℓ/min for the hexacyanoferrate (III) solutions gave the best sensitivity and RSD (0.76 %) values.(Figure

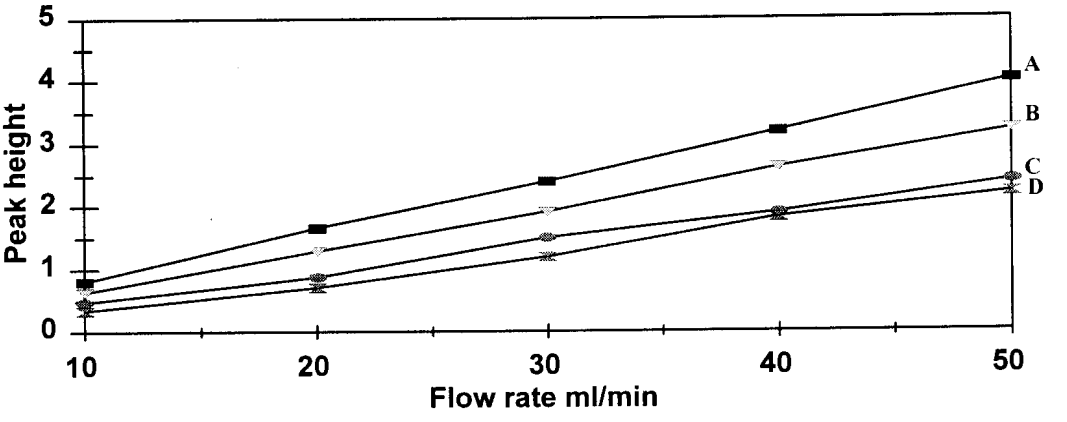


Figure 8.3 Effect of hexacyanoferrate (III) solution flow rate, at constant phenol flow rate (1.0 mℓ/min). A - 1.5 mℓ/min; B - 2.0 mℓ/min; C - 2.5 mℓ/min; D - 3.0 mℓ/min.

8.3) For phenol stream, the best results in terms of sensitivity and RSD were obtained for a flow rate of 1.0 mℓ/min. (Figure 8.4) These flow rates were chosen for further work.

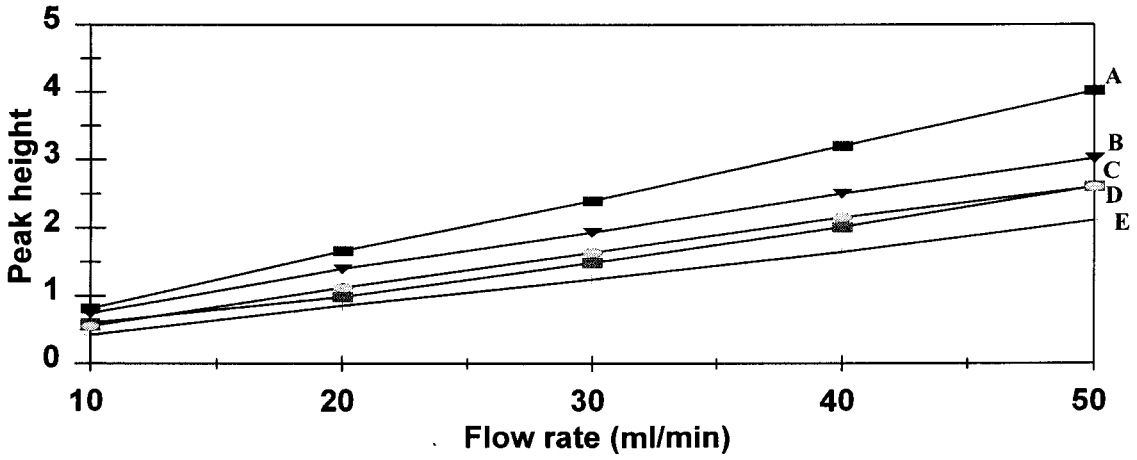


Figure 8.4 Effect of phenol solution flow rate, at constant hexacyanoferrate (III) solution flow rate (1.5 mℓ/min). A - 1.0 mℓ/min; B - 0.5 mℓ/min; C - 1.5 mℓ/min; D - 2.0 mℓ/min; E - 2.5 mℓ/min.

8.5.2 Influence of the different reaction coil lengths

There are basically two different operating conditions at different temperatures involved in the flow injection spectrophotometric assay of paracetamol. As each temperature has a different function to fulfill, care should be taken to use the optimum working temperature, to make sure that this temperature should be reached as fast as possible and that the optimum product is formed. Reaction coil 1 (RC_1) is the one where the oxidation process is taking place. The residence time as a function of reaction coil 1 (RC_1) was evaluated and it was found that a reaction coil of 120 cm gave the best sensitivity and precision. (Figure 8.5) The second reaction

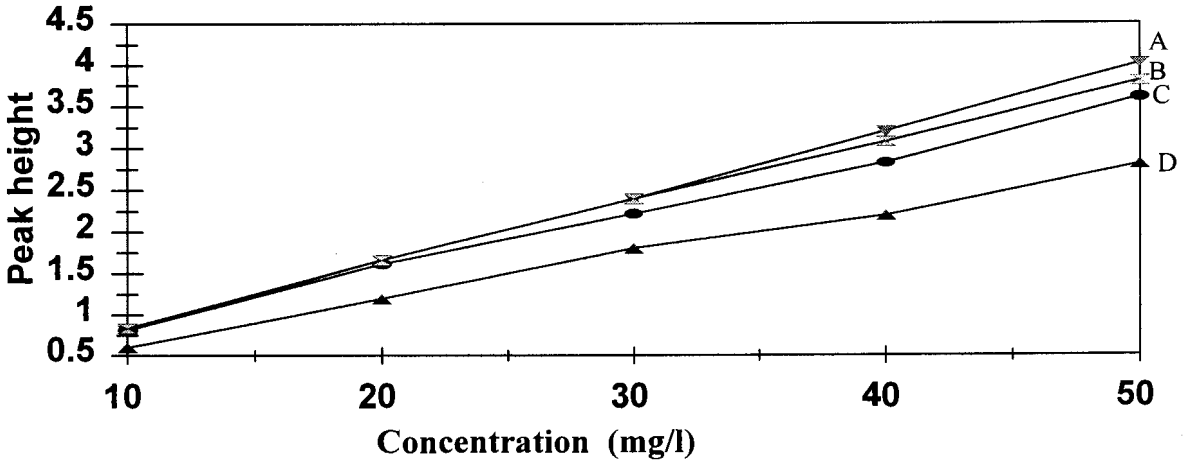


Figure 8.5 Effect of reaction coil length 1. A - 160 cm; B - 140 cm; C - 120 cm; D - 100 cm.

coil where the colour is developed is also temperature dependent and is much more pronounced at 80°C. The following parameters play a very important role in this part of the reaction and was evaluated; the reaction should reach 80°C as fast as possible, with residence time and dispersion that is dependent on coil length. A reaction coil (RC_2) of 450 cm gave the best response and precision and was used for further work. (Figure 8.6) The formation and accumulation of air bubbles in the flow cell is one of the main problems for routine use of the proposed flow injection system. At the temperature of 80°C the system is more prone for this. It was therefore necessary to cool down the manifold to 20°C before detection. It was found that a 100 cm cooling coil was sufficient enough for this purpose. (Table 8.1)

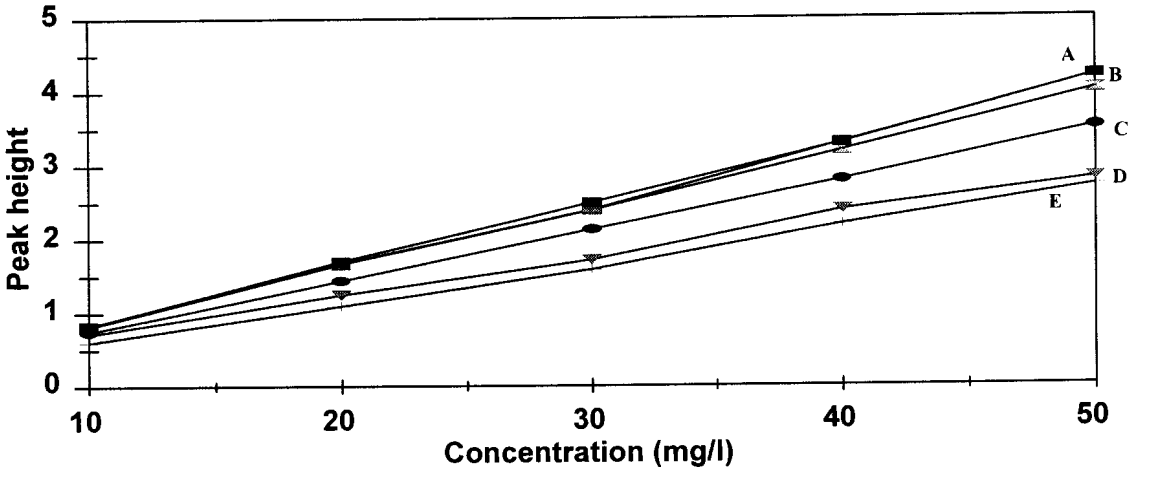


Figure 8.6 Effect of reaction coil length 2. A - 500 cm; B - 450 cm; C - 400 cm; D - 350 cm; D - 300 cm.

Table 8.1 Effect of cooling reaction coil 3 length (cm)

Coil length (cm)	Statistics	Peak height in mV				
		10 mg/ℓ	20 mg/ℓ	30 mg/ℓ	40 mg/ℓ	50 mg/ℓ
50	$\bar{x}$	0.8122	1.6035	2.2020	3.0204	4.0148
	% RSD	1.33	1.85	1.98	2.63	2.37
100	$\bar{x}$	0.8147	1.6632	2.3967	3.2009	4.0144
	% RSD	0.75	1.03	0.86	1.10	0.66
150	$\bar{x}$	0.8014	1.6075	2.2024	3.0985	3.6935
	% RSD	0.86	1.31	0.94	0.72	1.01
200	$\bar{x}$	0.7735	1.5406	2.1448	2.8011	3.8945
	% RSD	0.63	1.08	0.96	1.01	1.35

8.5.3 Effect of ammonia concentration at constant hexacyanoferrate (III) (2 g/ℓ) concentration.

It was discovered through preliminary experiments that the addition of 0.4 mol/ℓ ammonia as percent fraction of the total volume of sample solution had a marked influence on the sensitivity of the reaction between paracetamol and hexacyanoferrate (III). Various concentrations of ammonia as fraction of the total volume of the sample solution were evaluated and the results are given in Figure 8.7. The best results were obtained with a concentration of 0.4 mol/ℓ ammonia of the sample solution.

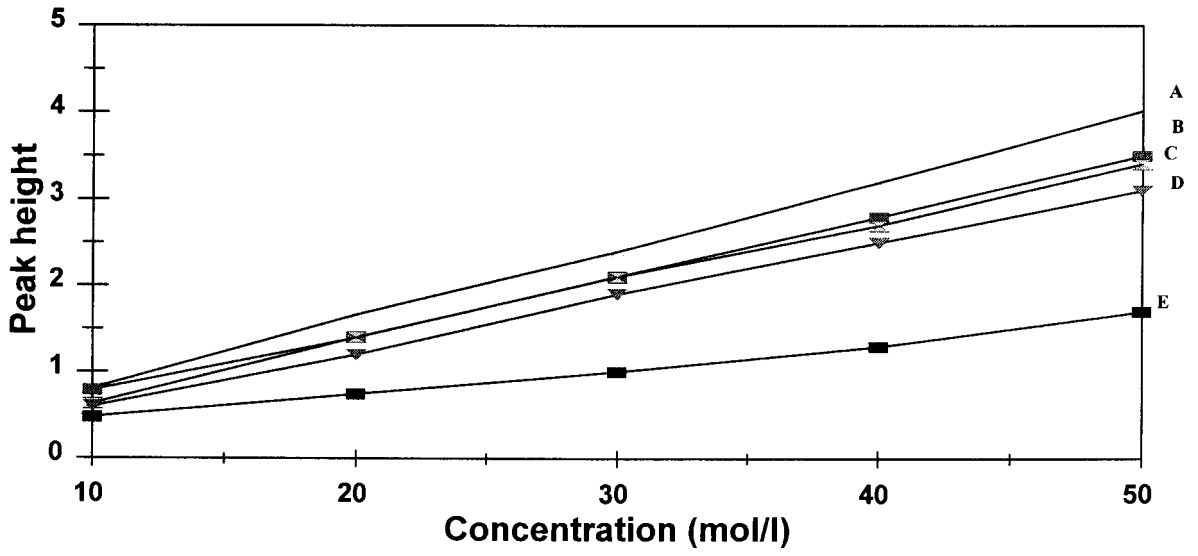


Figure 8.7 Effect of ammonia solution concentration. A - 0.4 mol/ℓ; B - 0.6 mol/ℓ; C - 0.8 mol/ℓ; D - 1 mol/ℓ; E - 0.2 mol/ℓ.

8.5.4 Effect of hexacyanoferrate (III) concentration at a constant ammonia concentration (0.4 mol/ℓ)

The concentration of hexacyanoferrate (III) has a significant bearing on the sensitivity of this method. It was determined that 2 mg/mℓ gave the best results for the sensitivity and RSD (0.76%). (Figure 8.8)

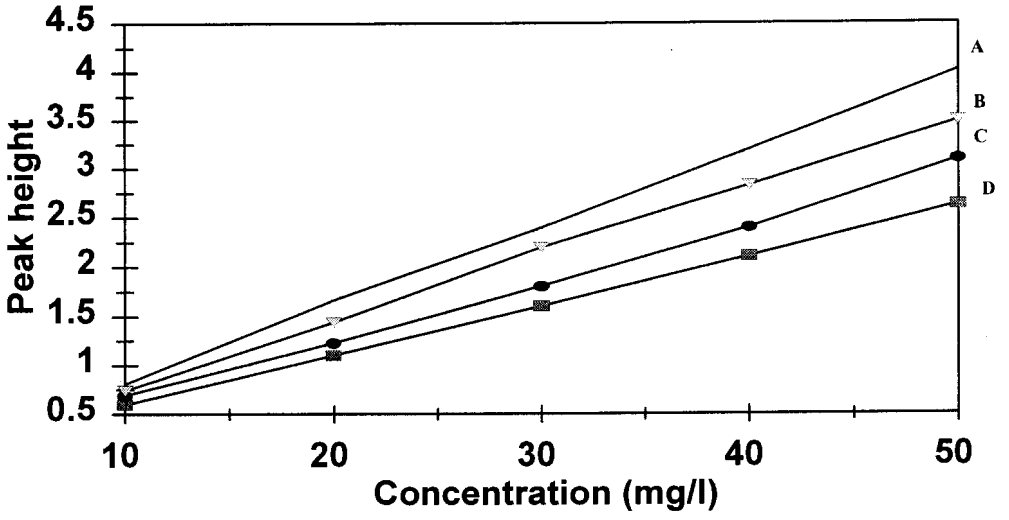


Figure 8. 8 Effect of hexacyanoferrate (III) concentration. A - 2 mg/ℓ; B - 4 mg/ℓ; C - 3 mg/ℓ; D - 1 mg/ℓ

8.5.5 Effect of phenol concentration at constant hexacyanoferrate concentration (2 mg/ℓ).

Different concentrations of phenol ranging from 7.5 to 30 mg/mℓ, were evaluated, through a series of experiments. It was found that the concentration of 15 mg/mℓ gave the best results with the higher sensitivity and lower RSD (0.68%). (Figure 8.9)

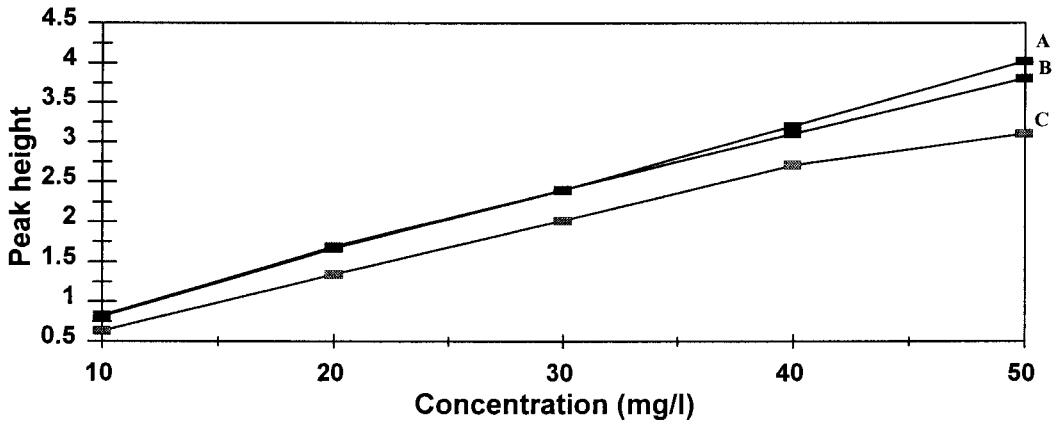


Figure 8. 9 Effect of phenol concentration. A - 15 mg/ℓ; B - 30 mg/ℓ; C - 7.5 mg/ℓ.

8.5.6 Influence of temperature of reaction coils 1 and 2 (RC_1, RC_2)

Temperature is a major role player in the oxidation of paracetamol as well as in the development of the colour reaction. It is evident that the response increase dramatically when the temperature is raised to 80°C (Figure 8.10) and this temperature was used throughout.

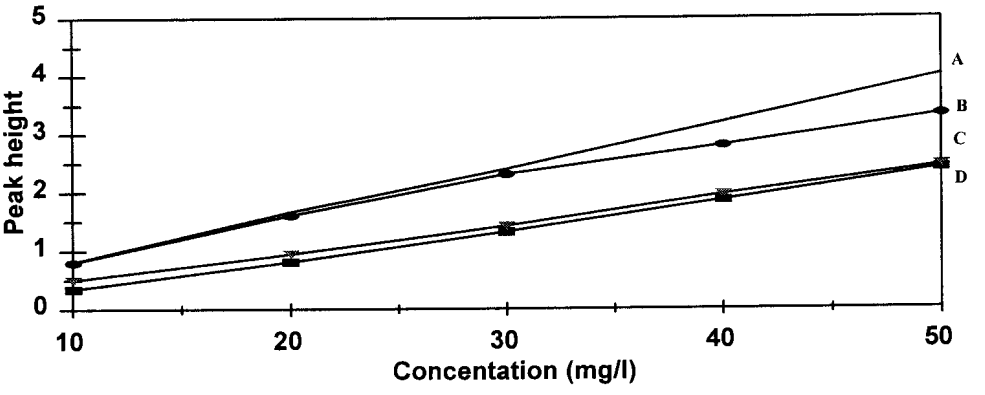


Figure 8.10 Effect of temperature of reactions coil 1 and 2. A - 80°C; B - 95°C; C - 60°C; D - 40°C.

8.5.7 Effect of sample volume

Table 8.2 shown that a sample volume of 100 μℓ is giving the best results in terms of sensitivity and RSD (%).

Table 8.2 Effect of sample volume

Sample volume (mℓ)	Statistics	Peak height in mV				
		10 mg/ℓ	20 mg/ℓ	30 mg/ℓ	40 mg/ℓ	50 mg/ℓ
50	$\bar{x}$	0.4295	0.8101	1.2401	1.6385	2.0145
	% RSD	0.78	1.43	1.62	1.02	1.35
100	$\bar{x}$	0.8147	1.6632	2.3967	3.2009	4.0144
	% RSD	0.75	1.03	0.86	1.10	0.66
150	$\bar{x}$	0.8098	1.6859	2.5065	3.3010	4.2610
	% RSD	1.20	0.85	1.02	0.98	1.21
200	$\bar{x}$	0.8135	1.6975	2.5698	3.3964	4.3111
	% RSD	1.32	1.52	1.44	1.09	0.96

$\bar{x}$ is an average of ten repetitive measurements

8.6 Evaluation of the method

The sensitivity and performance of the method were evaluated under optimised conditions with regards to detection limit, linearity, accuracy, precision, sample interaction, and sampling rate.

8.6.1 Detection limit.

It is very important that any new method should be evaluated for its detection limit to be sure which concentrations of the analyte can be determined with confidence and that the results obtained can be trusted. The detection limit for the method was calculated using the following formula:

$$DL = \frac{[3S_b + I_b] - k}{m}$$

where: DL is the limit of detection;

S_b is the standard deviation of the background signal;

I_b is the relative peak height of the background signal;

k is the intercept of the calibration graph and

m is the slope of the calibration graph.

The detection limit was found to be 0.25 mg/ℓ.

8.6.2 Linearity

The linearity of the method was evaluated under optimum conditions for the system. The data collected for the standards yielded a straight line between 0.01 and 0.05 FAU. The regression coefficient for this system is:

$$H = 0.04 + 0.08\ C;\ r = 0.9996$$

where H is relative peak height and <C> = mg/ℓ is the concentration of paracetamol. The correlation coefficient (r) indicated that the method is linear for the chosen working standards.

8.6.3 Precision

The precision for this method was tested by performing ten repetitions of the analysis on each of the different samples. In all the analysis carried out the % RSD was found to be less than 1.0%. This value is in agreement with that requested by the US Pharmacopoeia XXII [14] (Table 8.3).

Table. 8.3 Comparison of the results obtained by using the proposed FIA/spectrophotometry and standard method (500 mg paracetamol/tablet)

Formulation	Statistics	Recovery, mg paracetamol	
		FIA	Standard method
Panado (500 mg paracetamol per tablet)	$\bar{x}_1$	485	477
	% RSD	0.63	2.44
Syndol (450 mg paracetamol per tablet)	$\bar{x}_2$	438	435
	% RSD	0.77	2.35
Grandpa (500 mg paracetamol per tablet)	$\bar{x}_3$	489	470
	% RSD	0.85	1.87

8.6.4 Accuracy

The results obtained for the uniformity content test of three kinds of formulations of paracetamol (panado, syndol, grandpa) using the proposed FIA/spectrophotometric system were compared with those obtained from well established and standard methods. It is evident from Table 8.3 that these results did not differ much from those obtained with the other methods and that the results compared favourably with the content in paracetamol of the tablets.

8.6.5 Sample interaction

The influence of sample interaction seemed to be insignificant as there was no significant change or shift from the baseline with each successive analysis. This is due to the fact that at some stage the method employs very high temperature which aids in the cleaning of the system. However it was necessary to rinse the system with copious amounts of the buffer after the analysis was completed and just before another batch could be determined.

8.6.6 Sampling rate.

The proposed method is faster than the other FIA methods presented in the literature [4-7]. The sampling rate was found to be 60 samples per hour.

8.7 Conclusion

The proposed flow injection analysis method proved to be reliable for the uniformity content test of paracetamol formulations (panado, syndrol, grandpa). The accuracy and precision obtained are in concordance with the requests of US Pharmacopoeia.[14]

Furthermore, the method is rapid, and can be used for on-line monitoring of paracetamol - raw material during the technological process.

8.8 References

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CHAPTER 9

CONCLUSIONS

With the achievement and improvement of flow injection analysis as a general analytical laboratory technique, on-site monitoring and process analysis becomes a reality. It introduces a great improvement in the objectivity of analysis, accuracy of analytical information and significantly enhances the rate of the analytical process. This technique owes its wide application and popularity to its simplicity and the fact that it is very convenient to be used. Quality control of food and pharmaceutical products is of prime importance for human health. Reliable, rapid and accurate analytical methods are therefore necessary.

The future of FIA is excellent as sampling handling system. In fact in at-line monitoring there is only a limited number of other techniques that can compete. Although the on-line monitoring field should also not be so difficult to penetrate, the prerequisites needed to be implemented as real process in the actual situation is very tough as the task to be fulfilled is very demanding under certain circumstances and at the present moment FIA struggle to succeed. It is very difficult to see it employed as in-line process analysers in the near future. It is therefore not a surprise that the number of genuine on-line and even in-line applications of FIA remains small in spite of the

attractive features associated with them.

The understanding of the concept of dispersion in FIA made it possible to find the optimum conditions for the analysis of chloride, zinc, α-amylase, and paracetamol in foodstuff and pharmaceutical products, respectively.

The limit of detections obtained for chloride, zinc, α-amylase, and paracetamol assay are very low; the proposed FIA/Spectrophotometric systems are very sensitive, achieving high accuracies and precision for low concentration levels.

The proposed FIA systems are very simple and rapid. They can be successfully used for on-line monitoring of chloride in milk, zinc in foodstuffs, and paracetamol - raw material in technological processes. For α-amylase activity, a special sampling procedure must be followed for solid food samples.

The main advantages over other techniques in the literature are: simplicity in operation (see Addendum A), rapidity, and high reliability (high accuracy, high precision).

APPENDIX

APPENDIX A

Publications and Presentations

Publications

1. *On-site monitoring of chloride in milk with a dialyser/UV/Vis spectrophotometer/flow injection system*

   J F van Staden and **L V Mulaudzi**

   S.Afr.J.Chem., **52** (1999) 145.

2. *Improvement in the flow injection spectrophotometric determination of zinc with zincon through elevated temperatures*

   J F van Staden and **L V Mulaudzi**

   S.Afr.J.Chem., In Press.

3. *Flow injection spectrophotometric assay of α-amylase activity*

   J F van Staden and **L V Mulaudzi**

   Anal.Chim.Acta, Submitted.

Presentations

1. *Determination of α-amylase activity by flow injection analysis*

   LV Mulaudzi

   Young Chemists Symposium: The Gauteng South Section of the South African Chemical Institute. Johannesburg. South Africa. 9 June 1999.

2. *Flow injection spectrophotometric assay of α-amylase*

   L V Mulaudzi and J F van Staden

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

ADDENDUM

Method Construction

The need for rapid, accurate and precise results from analysis led to the introduction of automation in analytical laboratories [1]. Computers are an integral part of automation, hence their world wide application in automated systems. Microprocessors are the controlling key feature for device control and data acquisition. The popularity and wide application of Flow Injection Analysis and Sequential Injection Analysis is largely due to their automation. FIA and SIA depend on precisely scheduled operations which take place in a definite and well preprogrammed interconnected sequence. The devices are controlled in a well timed sequence that is in series. All the experimental work chapters 5-8 in this project were done using FIA. FlowTek is a software program that was developed by Marshal [2] specifically for the control of FIA and SIA. The work described in this dissertation was partially dependent on automation and thus on the use of personal computer. The technical aspects and operational guidelines for the FlowTek program may be obtained from the reference manuals supplied by Mintek [3,4]. The analytical cycle (experiment) used in the determination of the various analytes as described in this dissertation consisted of the following operations: The sample was first loaded into the sample loop of the injection valve, then injected into the carrier stream and then propelled towards the detector after passing through the reaction coil in which the main reactions occur. Each run was

separated from consecutive runs by 30 seconds to allow the rinsing of the system by the carrier reagent. The only device that was operated by the FlowTek program for this project was the injection valve for the load and inject manipulation. The peristaltic pump operated continuously at constant speed and only in the forward direction and it was therefore not necessary to couple it to the computer. Each run was specifically controlled by the computer. To illustrate the FlowTek program in operation, chapter 5 was chosen to serve as an example.

A 1 Setup of the FlowTek program for device control and data collection

A 1.1 Detector

For all the analysis work carried out in this project, the UNICAM 5625 UV/Vis Spectrophotometer was used. When the computer is switched **on** the main menu appears on the screen. Dataverwerk is chosen. This leads to a submenu and FowTek is then chosen. The FlowTek page then appears and upon pressing Return the FowTek main menu appears. **S** from **Setup** is chosen. This gives a submenu with different options. **D** for **Detector** is keyed in. The Questions in a flow chart fashion Chart fashion should be answered as follows:

Enter number of detectors:	**1**
Enter signal transformation for detector:	**NON**
Enter analog input point for detector:	**1 (This is the position at which the detector is connected to the distribution box)**

The baseline was set by adjusting the relevant screw within the PC-30 distribution board.

A 1.2 Injection valve

When the setup menu is Quit, the main menu reappears and on the main menu, the Method Submenu is chosen. This submenu has a series of options.

Number of devices: **1**

Type of device: **IV (injection valve)**

Digital output for device 1: **1 (position on the interface board)**

Then the Numlock is activated and Insert option or Delete option were used as follows:

Insert

Number of device: **1**

Type of event

Inject: **I**

Load: **L**

After you have keyed in the option you want, you go to the file submenu, and the sequence of instructions are as follows: Save (S), Retrieve (R), Delete (D), and then you will chose the correct option. For S, the name of the method should be keyed in.

Exit the menu until to the very first main menu and choose the Once (O) option to initiate a single run.

References

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