CHAPTER 1
INTRODUCTION

1.1 Introduction of Sequential Injection Analysis, SIA [1],[2]

Sequential injection analysis, SIA as a new technique for automatic sample analysis was reported by Ruzicka and Marshall in 1990, based on the same principles as flow injection analysis (FIA). The new technique offered advantages over the original in that hydrodynamic variables could be accommodated simply and easily with instrumentation and it should have been a readily accepted choice by researchers, industrial analysists and in-line quality control engineers.

Normally FIA uses a multichannel pump and unidirectional forward flow, in contrast SIA uses a single-channel pump to move the fluid zone in forward and reverse steps through a system consisting of a holding coil, a multiposition valve and a detector (Figure 1.1). The multiposition valve acts as a central distributor through which required volumes of liquid segments are sequenced by aspiration into the holding coil (HC) and then flushed by a flow reversal into the detector. Either a reaction-rate measurement may be made by stopped flow or a transient peak can be recorded during continuous flow. The sampling cycle time in the SIA mode is equal to the occupancy time that the injected zone spends travelling from the injector throughout the system and detector. Because only one pump is used to move the composite zone through the system, the sampling frequency of SIA is always lower than the multichannel pump FIA method. The SI system uses a smaller number of moving parts than a comparable FIA system and uses at least an order of magnitude less of reagents in the order of microlitres but it requires more complex software. Both FI and SI systems are based on identical underlying principles: sample injection, controlled dispersion and reproducible timing. Thus the design of the sequential systems must follows the established rules. The injected sample zones, the dispersion coefficients of both sample and reagent(s) have to be carefully considered when planing an assay. This allows the proportion of reagent to be selected by choosing a delay time t_d that produces optimum conditions for reaction rate measurement (Figure

1.2) when performs during the stopped flow. When the sample zone moves through the detector without stopping, a peak similar to that obtained with first generation FI is observed (heavily shaded area in Figure 1.2). Note, however, that such a peak is narrower than the sum of the two injected zones, as the sample and reagent zones can never overlap completely.

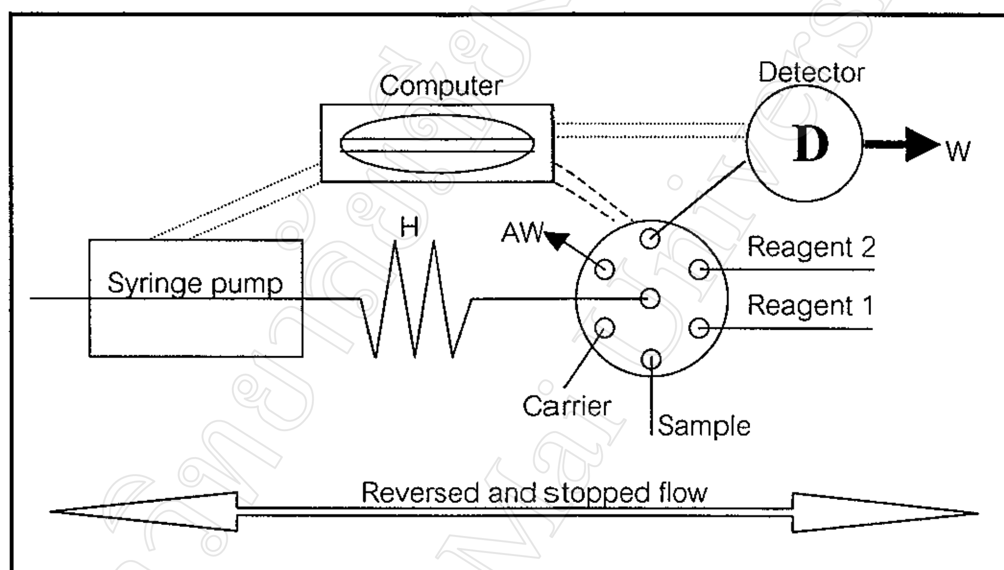

Figure 1.1 Typical sequential injection system utilizing programmed forward, reverses and stopped flow. HC, Holding coil: AW, auxiliary waste: and W, waste [2]

The most pertinent characterization of SI is its versatility and computer compatibility. Once designed, in contrast to the FI system, changes in the essential parameters such as flow rates, sample and reagent volumes, reaction times and reactant ratios do not require physical reconfiguration. Both can be adjusted on the computer keyboard. This allows the future facility of simple optimization, interactivity and automation, This flexibility means that SI may well become the preferred tool for exploratory computer optimized research into reagent based chemistries.

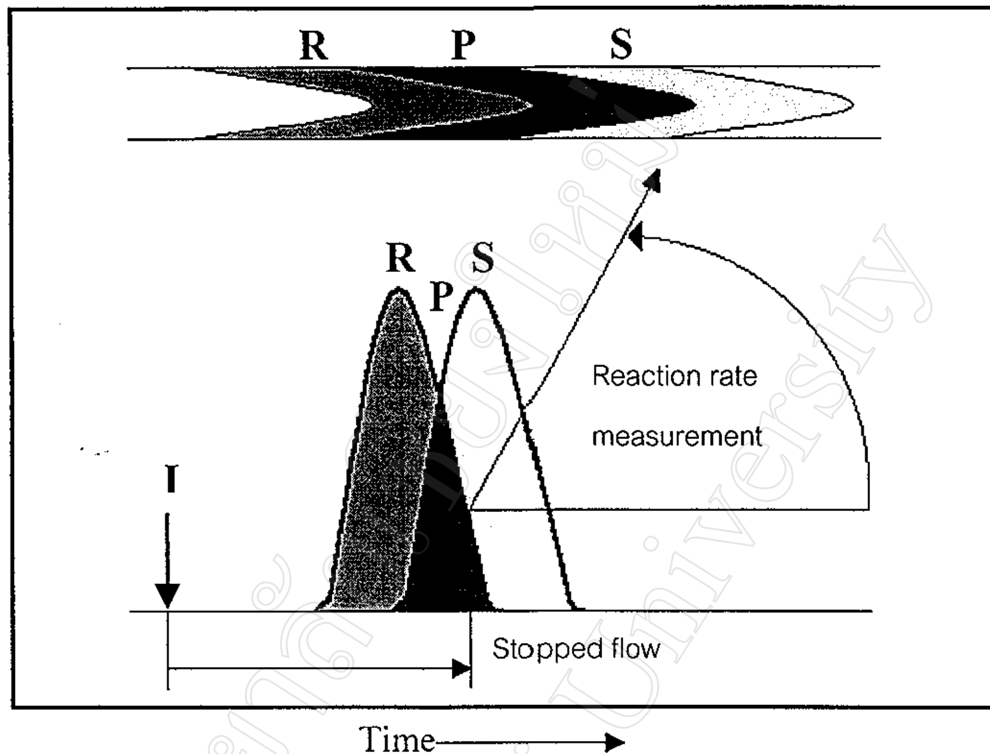

Figure 1.2 Sequenced zones of reagent (R) and sample (S) intersperse during flow reversal while the detectable product (P) is formed. On arrival at the detector a suitable section of the product zone is captured within the observation field of the detector by selecting a stop delay time t_d for reaction-rate measurement [2]

1.2 System configuration [3]

A sequential injection system has been designed and is shown in Figure 1.3. Assuming the system has been washed, each measurement starts by aspirating a carrier fluid, followed by sample and reagent aspiration. The pump is then refilled with carrier fluid and in force pump mode pushes. The carrier fluid into the system pushes the sample reagent of the mixture into the mixing coil where further dispersion takes place and then the mixture is pumped through the detector to waste. The arrows adjacent to the pump show the direction in aspiration and force pump mode. The design promotes well-defined zones which travel from the holding reservoir (H) through the valve and mixing or reaction coil and then via the flow-through detector

(D) to waste. This is the termination of the measurement cycle. The system is now full of fresh carrier fluid ready for the next measurement cycle.

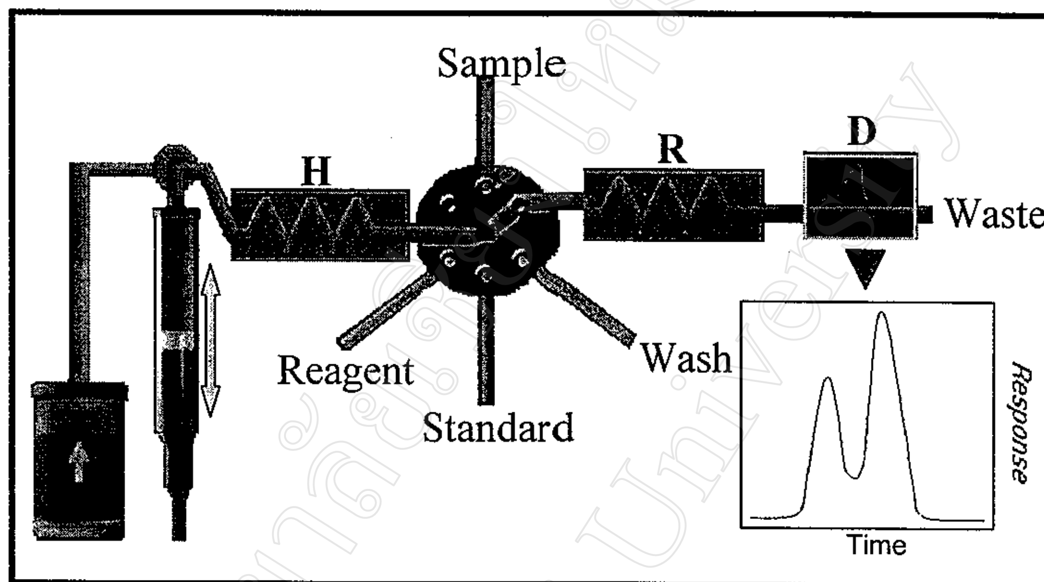

Figure 1.3 Principle of sequential injection technique showing manifold and readout. The plunger of the syringe pump moves in discrete steps to aspirate carrier fluid, sample solution and reagent solution into a channel, which consists of hold-up (H), reaction (R), detection (D) and sections. The selector valve provides sequential aspiration of the solutions into the channel through preprogrammed steps controlled by the plunger movement. The record shows a typical doublet peak, the trough of that is located at the time when the flow is about to leave the detector.

1.3 Application of SIA system [4]

SI is far simpler than conventional FI as it uses only a single double direction force pump, single valve and single channel. The flow path does not need to be reconfigured to accommodate changes in aspiration volume, reagent or sample volumes. Changes of the pump stroke volumes, flow-rate and stopped-flow period can readily alter these parameters when controlled by computer. Additional reagents, reactors and detectors could be clustered around the selector valve, the apparent limit

being only the number of available ports. Multi-reagent chemistries and multi-detector assays can in principle be carried out in a single system.

An example of a flow arrangement which consists of multiple detection and two different reagents assays is shown in Figure 1.4 (A). In fact, the majority of solution-handling operations which have been automated by FIA can in principle be accommodated by SI. Dilution of concentrated analytes, so useful in process control applications, can be performed via a special selection in the system shown in Figure 1.4 (B). The same flow arrangement could possibly be used as a means of automated calibration using a single-standard solution. If an injection valve is clustered with the sequential valve (Figure 1.4(C)) and a column is placed in the loop conduit, preconcentration and matrix removal for atomic absorption spectrometry (AAS), inductively coupled plasma (ICPs) or mass spectrometry (MS) can be performed [4].

The afore mentioned arrangement could be useful for interfacing SI systems with a detector whenever the main stream carrying the analyte is incompatible with the detector performance (water interference in Fourier transform measurements, flow-rates incompatibility for FI-MS or FI cytometry. Interaction of the reactor with the detector, using the well-recognized optosensing scheme is well suited for the SI configuration (Figure 1.4 (D)). Using a large reservoir of acceptor reagent and a reactor/detector with a very small internal volume, the amount of reagent spent and its contribution to the baseline increase will be negligible when the lower piston during its forward and reverse movement replenishes the acceptor within the detection chamber. Stopped-flow operation and the absence of product dilution will compensate for the short optical path through which the reflected light is travelling.

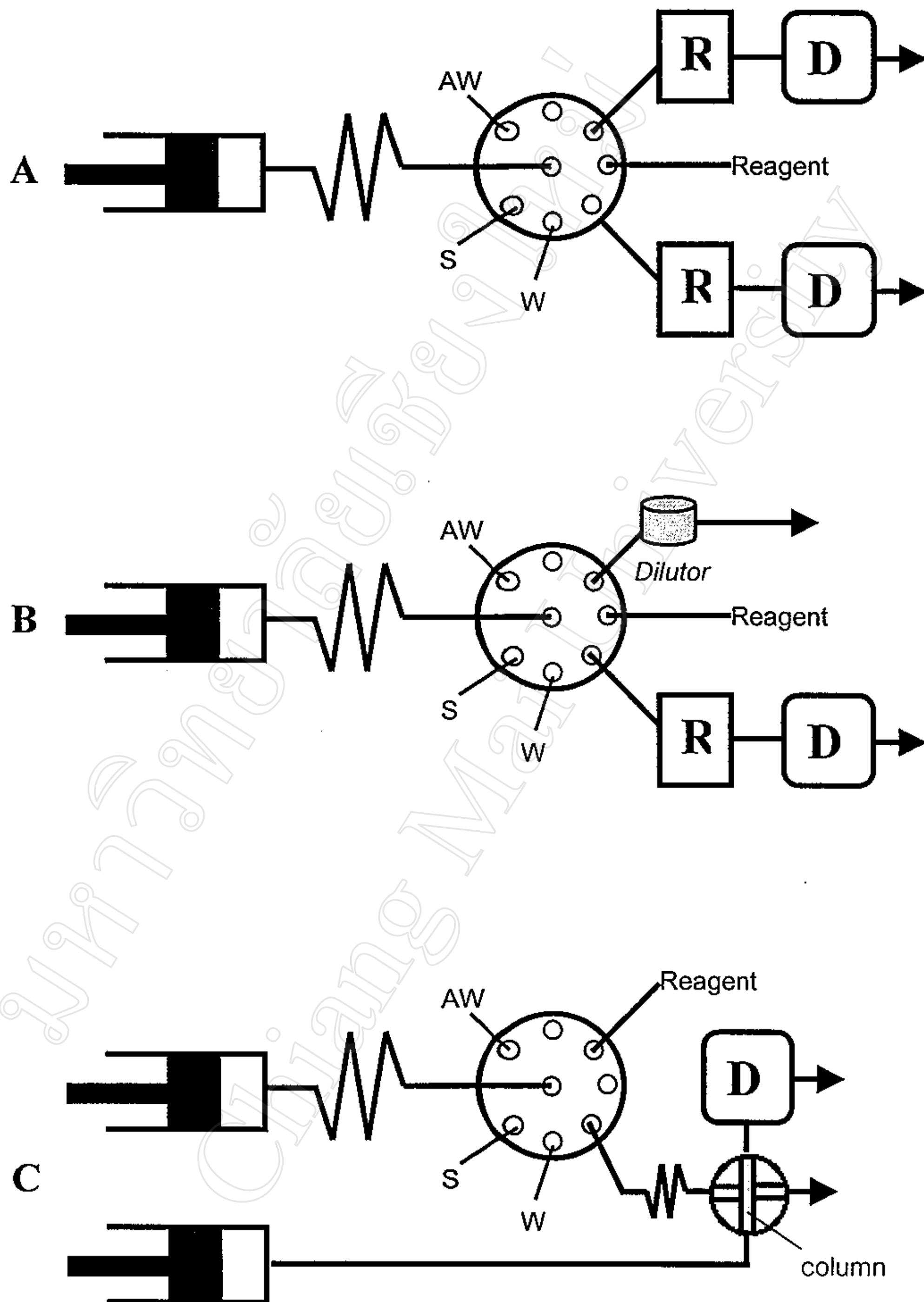

Figure 1.4 Sequential injection manifolds designed to accommodate (A) two different assays, (B) sample and standard dilution, (C) analyte preconcentration and matrix removal and (D) gas diffusion and optosensing [4].

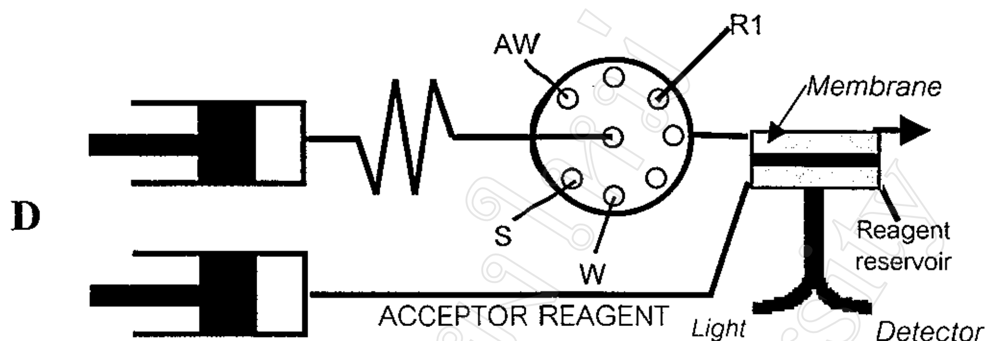

Figure 1.4 (Continued) Sequential injection manifolds designed to accommodate (A) two different assays, (B) sample and standard dilution, (C) analyte preconcentration and matrix removal and (D) gas diffusion and optosensing [4].

1.4 Sequential injection analyzer [3]

The concept of sequential injection analysis (SIA) is based on the mixing of the sample with a reagent in order to produce a measurable reaction. This necessitates solution-handling operations such as sample and reagent aspiration, sample and reagent mixing and detector flushing and reconditioning. These operations, in addition to sequential addition of other reagents and recalibration are currently effected in automated analyzers and can be performed by a sequential injection analyzer such as that shown schematically in Figure 1.3. This apparatus operates by executing a number of preprogrammed plunger movements (see Figure 1.5) synchronized with the positions and movement of the multi-port selection valve.

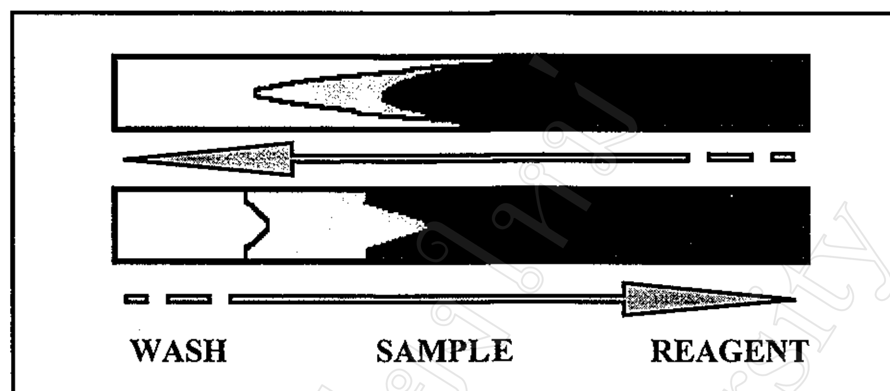

Figure 1.5 Concentration gradients, formed from sequentially injected zones of wash, sample and reagent in the flow channel at forward and reversed flow [3].

Reproducible reactions require homogeneous mixing of reactants for the chemical reaction to occur. The classical batch method is to prepare a homogeneous mixture and then either measure the reaction rate or wait until equilibrium has been reached. The flow injection method exploits the production of reproducible concentration gradients and enables the transient measurement of the resulting products. It is important to understand that either batch or flow injection is adaptable to sequential injection, the homogeneous mixing of reactants is achievable by replacing the reactor coil (R) with a mixing chamber and by allowing the chemical reaction to reach equilibrium. This approach is however more complex and time consuming than the flow injection mode.

Effective mixing is achieved by the use of a suitable number of steps (reversals), n, of length *l*. In the system under investigation, this is fundamentally a relationship between the pump stroke length, number of flow reversals and sample and reagent zone volumes. Increasing the number of repeated strokes will result in increased zone penetration and more throughly mixing. The pump is then charged with carrier fluid and propels the reactant mixture through the detector, allowing a complete wash of the sampling conduit, reactor coil and holding conduit. A system can be designed to allow flexible programming of a number of variations of pumps and valve actions and to ensure there is no sample carryover.

The effect on mixing by changing the sample and reagent volumes will be first investigated. Once suitable volumes are established, the reproducibility of the

dispersion and residence times in the analyzer is determined. To establish these parameters the mutual zone penetration of sequentially injected zones should be first investigated by using a non-reactive dye, bromothymol blue, in a series of flow-reversal and stopped-flow trials.

Reproducibility of zone dispersion is the prime factor in FIA and is equally important in SIA. It can be investigated by sequentially injecting two adjacent zones together into a tubular conduit and subjecting them to a unidirectional flow. As the central streamline in such a conduit travels at twice the mean flow velocity due to the boundary layer the zones will penetrate each other due to turbulance, forming characteristic concentration gradients (Figure 1.6). These gradients will contain a section of pure component A (sample), pure component B (reagent) and their mixtures (A+B) in a continuous range of ratios. As $1/D_A+1/D_B=1$ where D_A and D_B being dispersion coefficient of A and B respectively and therefore $C_A^0=C_B^0(D_A-1)$. the proportion of sample and reagent in any element of fluid can be calculated for any delay time, and an estimate can be made of the delay time that will provides sufficient reagent excess to allow complete conversion of the analyte to the desired product, Thus, whereas an adequate excess of reagent exists for a delay times greater than t_{A+B}, even if the originally injected concentrations of reagent and sample were equal ($C_A^0=C_B^0$), at t_3 and prior to it, the original reagent concentration will have to be increased until ultimately at time t_2, an excess of reagent cannot be maintained, owing to insufficient penetration of the reagent zone. Indeed, the leading edges of the sample zone will always remain reagents free and the tailing edge of the reagent zone will remain sample free. An increasing number of flow reversals of sufficient amplitude, l, will eventually lead to compensation of the initial peak skewness and, more important, thorough mixing of sample and reagent at their adjacent interfaces. This is shown in a series of injections (Figure 1.7), where sample zone alone and reagent zone alone were injected to one, two and four flow reversals. Note that the concentration gradient obtained in this way is similar to that obtained with unidirectional flow in a flow-injection manifold (Figure 1.6) except that the flow reversal, which occurs after peak maximum has been registered (i.e. part t_3 in Figure 1.6), results in a doublet peak. This flow reversal facilitates the flushing of the reaction products and reactants from the system and subsequent washing of the

various conduits in preparation for the next measurement cycle. Although an increasing zone penetration is observed with increasing numbers of flow reversals, it follows from these injection profiles that a single flow reversal (which is necessary to flush the system) produces a section of mutually penetrated zones, where the reaction product will be monitored with sufficient reproducibility. It also follows that it is futile to increase the sample volume with a view to increasing the peak, height, beyond the minimum required to ensure that the portions into which the reagent and sample zones overlap is adequate. In SI, on the other hand, as shown for pH measurement, the injected sample volume can be increased at will as the injected zone contacts and penetrates the reactive material held in the sensor cavity. This is the principle advantage of heterogeneous versus homogeneous reagent addition.

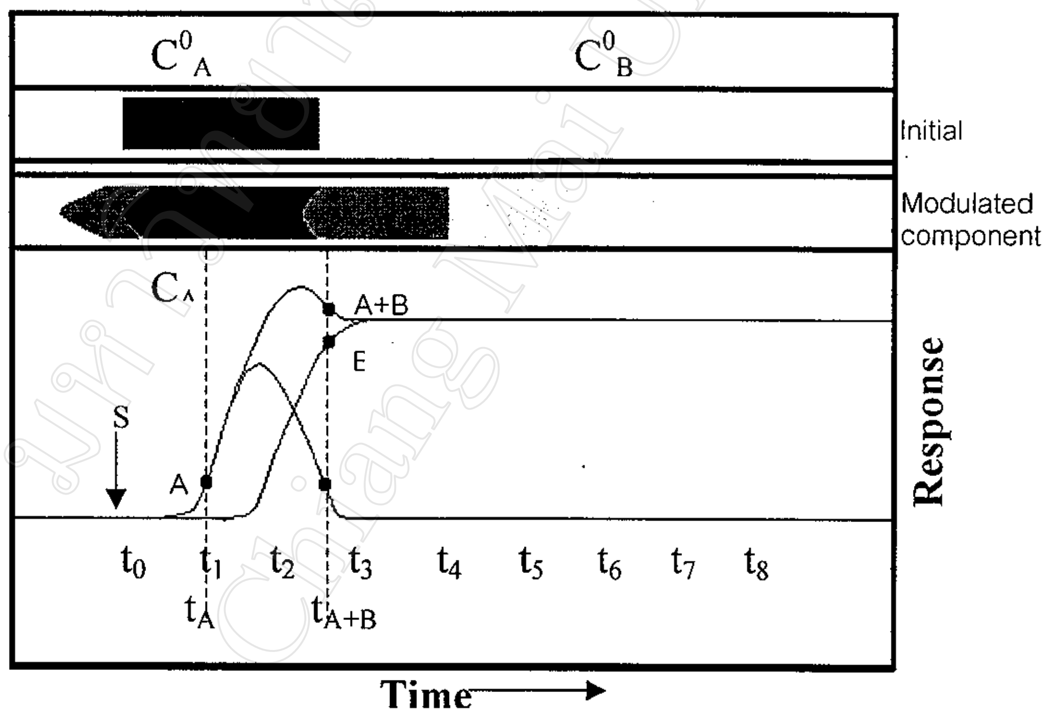

Figure 1.6 Sequential zone injection and their mutual penetration, as it take place during their forward flow in the channel (top). Individual (A, B) peaks of sample and reagent and their composite profile (A+B) show (below) the degree of mutual penetration and a region where A will react with B. Note that reversing of the flow past t_3 will yield typical double peaks obtained in this work [3].

As the beneficial effect of an increasing number of step reversals is well documented by theory and experiment, the reproducibility of reversals was investigated. This is justifiable because any higher number of reversals with result in a larger degree of zone penetration and better mutual sample reagent mixing.

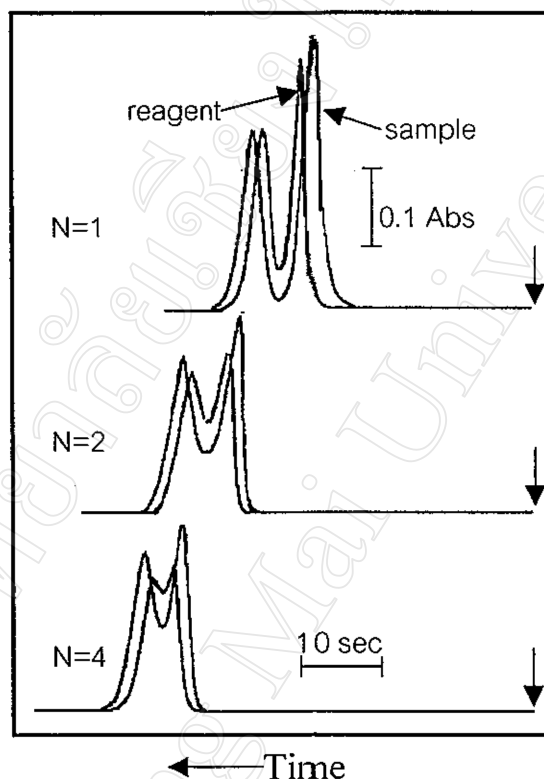

Figure 1.7 Influence of repeated reversal (N) on mutual penetration of sample and reagent zone, obtained by injecting separately a non-reactive dye as a sample zone and then a reagent zone, and by performing multiple flow reversals in the auxiliary reactor. Since the dye zones entered the detector only during the final reversal period, only a single double peak is observed in all experiments. Note that whereas the residence time increases with the number of reversals, the peak height decreases and the peaks become progressively more symmetrical, yet the leading (sample) edge remains reagent free [3].

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1.5 Ascorbic acid [5]

Vitamin C is perhaps the best known vitamin. Advertisers have had a heyday with it, and almost any synthetic drink touts that it contains this vitamin. Vitamin C is also L-ascorbic acid. Vitamin C has a very simple structure.

This chemical is widely distributed in the plant and animal world. Only guinea pigs, some fish, fruit bats, and primates cannot synthesize this vitamin. These organisms lack the enzyme L-gulono-g -lactone oxidase. Ascorbic acid is a relatively strong reducing agent. Thus, with this ability, it functions in the body as an electron carrier. Loss of a hydrogen atom yields the L-ascorbate free radical, whose extra electron is stabilized through the oxygens and the conjugated bonds. Various plant and animal enzymes can reduce this free radical back to L-ascorbic acid. In addition, L-ascorbic acid can be reversibly oxidized to dehydro-L-ascorbic acid, forming a useful redox system.

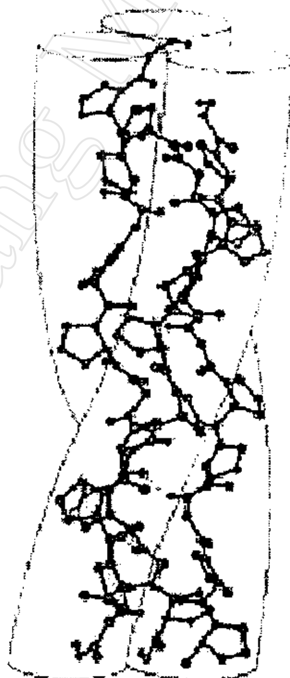

Figure 1.8 A collagen triple helix [5].

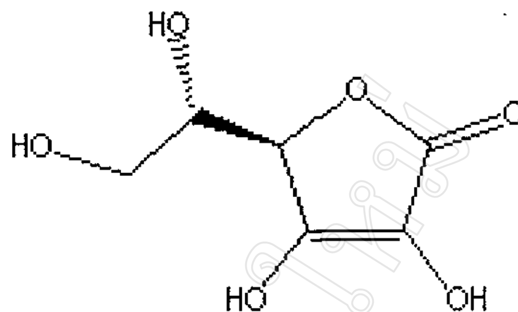

Figure 1.9 The structure of Vitamin C [5].

Ascorbic Acid can be oxidized in the electron transport chain to provide potential energy for ATP production, but is also used for the hydroxylation of proline residues. This role is incredibly important to animals dependant on collagen as a connective and structural protein. This protein binds cells to one another. The potentially fatal condition, scurvy, is a result of not enough vitamin C hydroxylating proline. Units of collagen fibers have the quaternary structure of three polypeptide strands wrapping around one another to form a triple helix held together with hydrogen bonds.

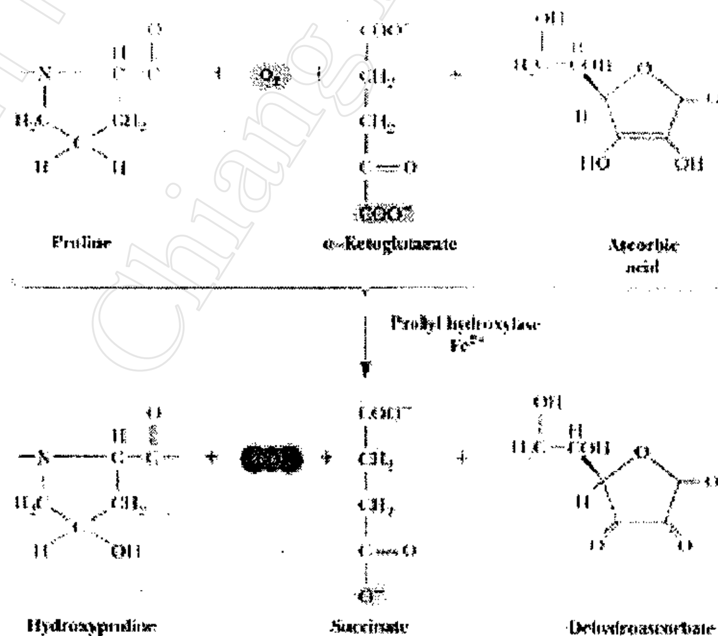

Figure 1.10 Formation of ascorbic acid and its derivative [5].

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The polypeptide strands have a sequence of gly-x-y, where x is often pro, and y is often hydroxyproline. Interchain bonding occurs between the N–H of glycine to the C=O of the x residue. The chain is further strengthened with the help of hydrogen bonds involving hydroxyproline, the hydroxylated form of proline. Since this hydroxylation reaction is dependant on vitamin C, which keeps the enzyme prolyl hydroxylase active, a lack of this chemical will cause the chain to lose strength, and begin to degrade. Vitamin C also plays a role in the hydroxylation of lysine in collagen, which is also important to maintaining This causes a weakening of the collagen fibers in the body, which causes the symptoms of scurvy, which include bleeding gums, rotting teeth, slow healing, sores and blotches on the skin. Searches for a cure for this common disease led in part to the discovery of vitamins.

1.5.1 The methods of ascorbic acid analysis [6]

Owing to the wide use of ascorbic acid, many analytical techniques have been proposed for the determination in different matrices and at different concentrations [7-16]. Conventional methods can handle only a limited number of samples owing to the requirement for time consuming procedures involving extraction, reaction and analysis. Such is the case of the AOAC standard method and the method described in the British Pharmacopoeia monograph. Only a few chromatographic methods enable simultaneous assay of several water-soluble vitamins in complex matrices, so separate assays are needed. The problems with these methods are related to sample preparation and to the sensitivity of the detection method that is used. There is a need for fast, selective and automated methods for ascorbic acid determination, particularly for routine analysis. Flow injection analysis was used widely as a tool to solve analytical problem, and is was presented as an interesting alternative for use in ascorbic acid determinations when only one analyte is determined in a large number of samples. Therefore, in many instances chromatographic methods can be replaced by fast flow injection procedures. Furthermore, the advantages provided by the FI methodologies include: high sample throughput, low sample and reagents consumption, high

reproducibility, simple automated operation, low contamination risks, possible enhancement in selectivity by applying kinetic discrimination, and very limited laboratory bench space and utensils required. Due to these motives, many researchers have focused their efforts in developed inexpensive, rapid and automated analytical methods of ascorbic acid determinations involving FI procedures.

1. 5. 2 Spectrophotometric methods

The direct UV spectrophotometry is also used, since ascorbic acid absorbs in the UV region presenting a maximum absorbance at 243 nm in strongly acid media and at 265 nm in neutral media. As many compounds presented in samples also absorb in UV region and matrix effects need to be eliminated, several strategies have been proposed. Making a second measurement generally solves this effect. Furthermore, after ascorbic acid has been decomposed, by means of sodium hydroxide, a column packed with modified silica that retains interference by ionic pair formation between amine groups and interfering compounds is used. An anion exchanger, placed in an adequate quartz flow cell, that retains the ascorbate anion in a flow-through solid phase technique, where the carrier itself acted also the eluting agent, is an other alternative to eliminate interference.

Visible spectrophotometry is more frequently used. These methods involve redox reactions with ascorbic acid in which a colored compound is formed or decomposed in a redox reaction. The methods using chloramine T in the presence of starch-KI solution, the titration with 2,6-dichlorophenolindophenol, the formation of Fe(II)-phenanthroline, Fe(II)-ferrocine or Cu(I)-bathocuproine complexes previous metal ions reduction by ascorbic acid have been reported. Measurement of decrease in colour intensity was also employed by reduction of cerium(IV), reduction of triiodide, reduction of Co(III)-EDTA complex or photochemical reduction of methylene blue. Solid phase reactors using copper(II) phosphate and $Fe(OH)_3$ immobilized in a polyester resin have been employed to provide an

alternative to obtain faster and more efficient sample conversion than is possible with conventional procedures, where the reagents are introduced into the flow system as a solution.

There have been many methods proposed for the determination of ascorbic acid in vitamin C tablets [17-19] and many flow injection procedures have been proposed. One method investigated is based on the reduction properties of ascorbic acid [19]. Using the fact that the colour of potassium permanganate is decreased by the redox reaction between ascorbic acid and potassium permanganate. The change of colour intensity can be monitored by using spectrophotometry. The procedure is simple, versatile, economic and rapid.

1.6 Acetic acid in Vinegars [20]

The quality of vinegar is usually to a standard from each brewery, and variations from the standard are slight. Where possible, however, vinegar should be purchase on sample. It should be clear, with a sparkle when poured, have a deep golden brown colour and a sharp but round flavour. There should be no appreciable bitterness, but the so-call malt flavour must be definite and pleasant. The acidity will vary from 4 to 6 percent acetic acid, 20 grain 5 percent avid and 24 grain 6 percent acetic acid. Specific gravities are between 1.017 to 1.022.

Cider vinegar and other fruit vinegars are subject to the same faults as malt vinegar. They are used when the strong malt flavour is not required and they enter occasionally into specific recipes. They should be clear and bright, almost or quite colourless, of pleasant sharp flavour with an acidity around 4 percent.

Spirit vinegar, or alcohol vinegar is not "natural" in the sense that it is not produced from grain or fruits. It is in fact obtained from molasses by fermentation with yeasts and subsequent acetification with vinegar bacteria. It is flavorless except, of course, for the very pronounced acidity which is between 10 and 13 per cent and is water white and bright. It suffers from none of the defects of malt or fruit vinegars, and will store satisfactorily almost indefinitely.

Distilled vinegar, as its name implies, is malt vinegar from which the colour and soluble solids have been removed by distillation. The product retains much of the distinctive flavour of malt vinegar and is used in products where colour is an important factor.

Edible acetic acid is the product of chemical processes and has no relation to brewed products. Its acidity is 80 per cent and when diluted it has a raw flavour.

1.6.1 Analysis of vinegars [21]

An attempt to determine organic acids in wine vinegar has been carried out by reverse-phase high-performance liquid chromatography (RP-HPLC), using two C18 columns (arranged in tandem), UV detection at 210 nm and sample filtration through Sep-Pack C18. However, the complete separation of the organic acid was not achieved with this procedure. Ion-exclusion chromatography has also been used in order to determine organic acids. It is a valuable technique for separating neutral and weekly acidic of basic substances from ionic compounds based on an Ion-exclusion mechanism rather than Ion-exchange. Thus Ion-exclusion chromatography has been used to determine organic acids in vinegars. Ashoor et.al. used an Aminex HPX8 column with a diluted sulphuric aqueous solution as eluent and UV detection at 210 nm in order to perform a rapid monitoring of acetic acid in various foods, among them vinegars. Non-volatile organic acids in various types of vinegars have been determined using a Dionex (HPICE/AS 1) column, volatile acids being previously separated and determined in the distillate. The sample is subject to a pretreatment consisting in decoloration with active carbon and subsequently filtering.

The simply and cost effective analysis, for acetic acid content in vinegar was proposed by K. Grudpan and et.al. [22], using the flow injection method. This was based on neutralization and was monitored by either spectrophotometry or conductometry.

1.7 Aims of the research

The aims of this research work are summarized as follows:

1. To develop sequential injection spectrophotometric procedure for the determination of ascorbic acid in vitamin C tablet samples.
2. To develop sequential injection spectrophotometric procedure for the determination of acetic acid in vinegar samples.

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