Flow Injection Analysis - A Superior Alternative to Air-Segmented Continuous Flow Analysis

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Flow analysis means the measurement of analyte concentration in a flowing stream. Continuous flow procedures were adopted as a result of increasing work load in clinical chemistry, environmental studies and quality control. Such procedures differ considerably from batch analysis and offer clear advantages of being rapid, reliable, reproducible and economic. Steady state and segmented continous flow analysis (CFA) have been the primary techniques used commercially. In steady state methods reagent and analyte are mixed in a merging stream until a maximum signal plateau is obtained. Although greater sensitivity is possible, this method was soon neglected because of carry over of material from one sample to the other. To eliminate this difficulty Skeggs in 1957 [1] introduced a novel manner of sample-reagent mixing and transport to the detector. The samples from an autosampler are aspirated by a peristaltic pump. The moving stream is segmented by introduction of air. The reagent stream which is introduced after segmentation join the sample segments at strategic points. By passage through mixing coils the sample and reagent solutions are homogeneously mixed. Near the detector the stream is deaerated. As shown in Fig.I the output from the detector is in the form of peaks which show a plateau at the top indicating the complete mixing of sample and reagent and thus the steady state signal. Technicon Corporation has marketed Auto-Analysers based on this principle, which became the most successful tool for automation of wet chemical assays and had resulted in the widespread use of continuous flow systems.

The Skeggs idea of air segmentation was widely accepted and almost unchallenged for about 20 years and nearly every one had assumed that air-segmentation and complete mixing of sample and reagent were essential for carrying out continuous flow analysis. It was not until the middle of the 1970s when two independent groups of research workers, Ruzicka and Hansen from the Technical University of Denmark [2] and Stewart and co-workers in the United States [3] demonstrated some unnecessary aspects of segmented continuous flow analysis. They demonstrated that unsegmented continous flow with discrete sample injection has clear advantages of high sampling rate, low reagent consumption, negligible carry over and also simplicity and versatility. The new technique was named "Flow Injection Analysis (FIA)" by Ruzicka and Hansen. Already several reviews have been published on this new analytical technique (4-12), and the inventors of the technique have written a book [13] on FIA.

The aim in writing this article is mainly to introduce this technique

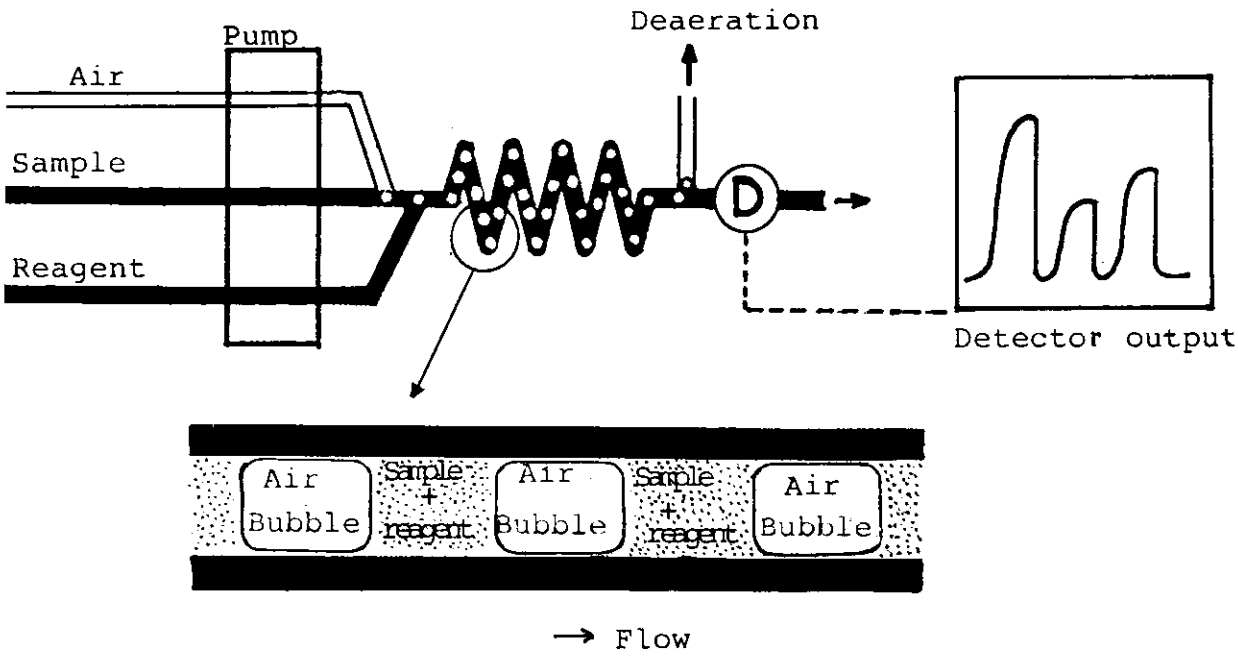

Fig.1: A simple manifold illustrating the principle of air-segmented continuous flow analysis (Auto Analyzer).

and stimulate its interest in teaching, research and industry in this country.

The FIA Principle:

FIA is simply illustrated in Fig.2. It is based on the introduction of an exact volume of sample into an unsegmented carrier/reagent stream. The injected sample forms a well-defined zone (Fig.3), which is pushed towards the detector equipped with a flow-through cell. On its way to the detector the sample mixes with reagent to obtain a chemical reaction between sample and reagent. The reaction product is measured while it passes through the detector cell. As shown in Fig.2, the analytical readout is in the form of a transient peak, the height (H) of which is related to the concentration of the analyte. The reaction product passes the detector long before steady state conditions are established. That is the main difference between FIA and other continous flow systems, in that measurements are not necessarily made at equilibrium, thus less time is needed for analysis resulting in a high sample throughput.

Why and how flow injection analysis works can be easily understood by means of the three fundamentals on which FIA is based: (1) precise sample injection, (2) reproducible timing and (3) controlled dispersion.

Sample Injection:

The purpose of sample injection is to introduce a sample zone into the continuously moving unsegmented stream in such a way that the movement of the stream is not disturbed. This process should be very precise so that each time the volume and length of the injected zone can be reproduced from one injection to the other. The precise sample

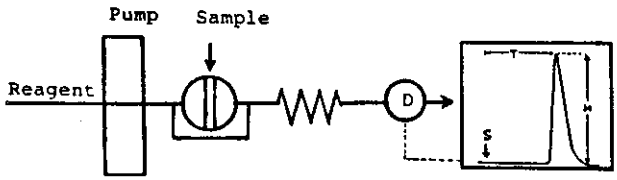

Fig.2: A simple single stream FIAmanifold. "D" denotes the flow-through detector.

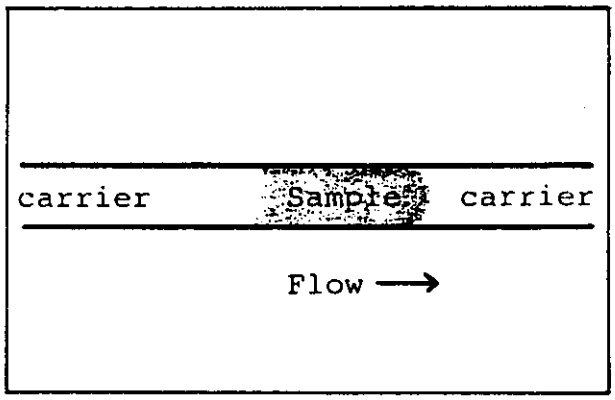

Fig.3: Schematic representation of the flow pattern in an FIA system, just after sample injection.

injection in FIA differenciates it from air-segmented continuous flow analysis and allows the concept of the steady state signal to be abandoned. At the same time it economises on sample consumption, which is very important when dealing with biological samples.

Reproducible Timing:

In figure 2, the 'T' represents the residence time, which is the time taken from injection to the appearance of the peak maximum. As the analytical readout is obtained from peak height (H), reproducible residence time of the injected sample in the system is essential. In FIA such a condition is readily achieved, as the flowing stream is nonsegmented, hence totally non-compressible. But the pump should provide a constant flow rate to ensure precise sample residence time in the mainfold. Any variation in the flow rate will affect the residence time of the sample in the system, and this gives imprecise peak heights. Typical residence times are 10-30s. In some cases this can be increased to give the required sensitivity.

The shorter residence time suggests that most analytical methods used in practice are based on fast reactions, otherwise this would have been a limitation of the flow injection system as $\simeq$20% of the chemical reaction may not have occured in the chosen residence time. The second reason may be the lesser dilution of the sample zone under controlled dispersion as compared to manual methods where the sample is much diluted prior to measurement. Therefore the precise combination of the dispersion and the residence time is essential to exactly suit a particular chemical reaction.

Controlled Dispersion:

Controlled dispersion is the most important aspect of FIA. The success of an FIA system lies in the exact manipulation of this dispersion so that it suits the requirements of that particular system. Dispersion or dilution of the sample occurs as the sample zone travels along the narrow-bore tube (usually less than 1.0 mm i.d) towards the detector. The understanding of dispersion processes in FIA is mainly based on the work of Taylor [14]. A liquid when travels along a tube generally moves under conditions of laminar flow thus adopting a bullet-shaped form (Fig.4a). But if this is the situation then by the time the sample reached the detector it would have completely

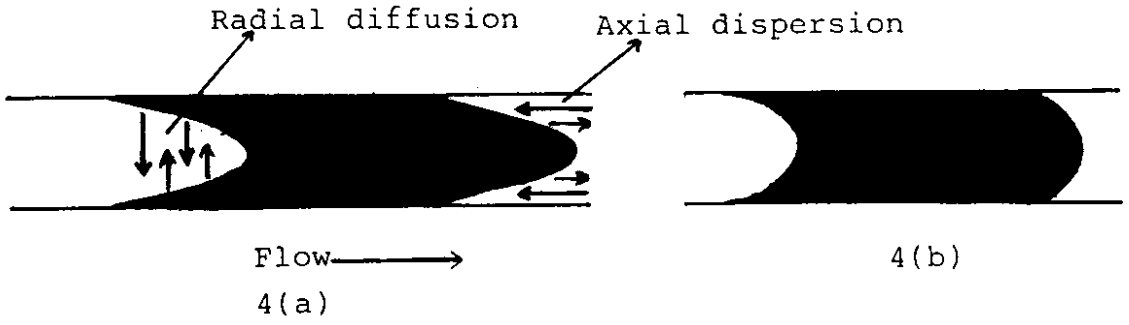

Fig.4: Velocity profile of a sample slug in a flowing stream (a) Laminar flow (b) Modification caused by radial and axial dispersion.

dispersed in the reagent, thus making the dispersion uncontrollable. But actually this is not the case. The bullet-shaped tip of the sample zone is greatly modified by radial diffusion, in which the tip of the zone diffuses outwards into the slower moving layer of reagent stream and thus preventing the slug from bearing a sharp tip. At the same time the very concave tail of the slug diffuses inwards into the fast moving layers of the reagent stream and is thus accelerated resulting in a modified shape of the slug (Fig.4b). This process is quite rapid owing to the small diammeter of the tube used. Apart from radial dispersion a second type of dispersion also occurs called axial or longitudinal dispersion. This type of linear dispersion can readily be observed by rapidly scanning an FIA peak. A peak theoretically reaching the baseline in 10 sec reaches the baseline in ca.20s due to some longitudinal dispersion. Therefore practically longitudinal dispersion of the sample slug should be kept to a minimum for sharp signals and high sampling throughput. The best way to achieve this is to use coiled rather than straight tubes [15]. Dispersion of the sample can be controlled by varying the various parameters of the flow injection system.

The best way to change the dispersion is by changing the sample volume. Small samples disperse more than large samples. Fig.5 shows the increase in peak height (hence a decrease in dispersion) with increasing sample volume, ultimately reaching the steady state as a result of an infinitely large sample volume. Of course this condition has to be avoided, because it is a waste of sample and reagent solutions and also of time to try to reach the steady state response.

In the design of an efficient FIA system it is desirable to use the shortest possible manifold (the tubing connecting the injection port with the detector) so as to minimize the dispersion, which normally increases with an increase in tube length. The diameter of the manifold tube also contributes to the overall dispersion in the system. Tubes with smaller diameters give very small dispersion, which is why Tijssen [15] suggests the use of coiled capillary tubing to give a high sample througput. Manifold tubing with 0.5 mm i.d. is standard in FIA; 1.0 mm i.d. is normally the upper limit. With a large diameter it becomes difficult to maintain the integrity of the sample slug.

The residence time of the sample zone in the system is very important.

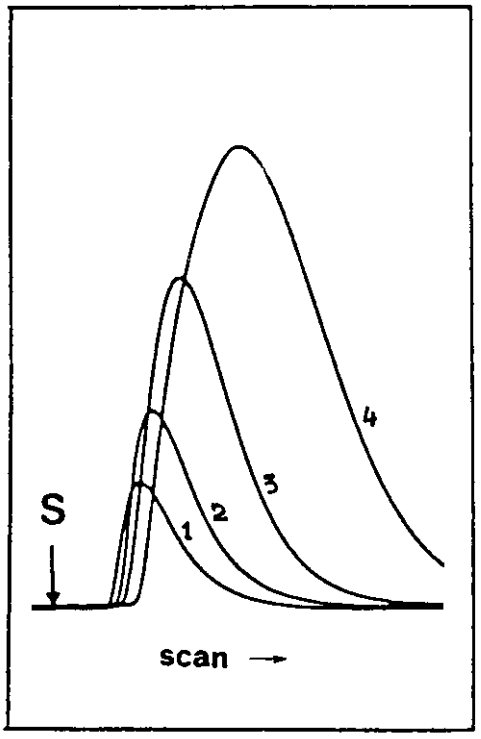

Fig.5:Effect of sample volume, on peak height. "S" denotes the point of sample injection. Sample size increases in the order 1 2 3 4.

To increase the residence time, it would be appropriate to decrease the flow rate rather than increase the length of the reaction coil in the manifold, as the latter leads to an increase in dispersion while smaller flow rates give less dispersion. This provides the basis for stopped-flow systems in which case the flow-rate is zero, therefore dispersion is negligible.

The other major components which contribute to the overall dispersion in the system are the use of columns in the manifold and the dead volume of the flow-through cell. A flow-through cell with least dead volume should be used.

In practice, the dispersion of the sample in the system can easily be determined. Fig.6 shows the practical

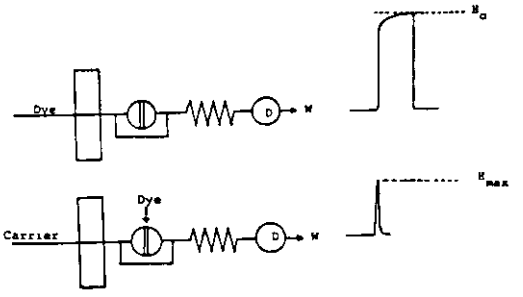

Fig.6: FIA manifold with corresponding output curves from a spectrophoto- metric detector for the determination of dispersion.

way to do it. As the peak height gives the analytical readout in FIA, Ruzicka and Hansen [16] have defined the dispersion coefficient 'D' as the ratio of the concentrations of the sample before and after the dispersive process, i.e. the ratio of the steady state signal (H_o) to the analytical peak height (Hmax.). i.e. $D=H_o/H_{max}$

Depending on the purpose of the flow system, different degrees of dispersion may be desirbale. These are:

Limited dispersion.	D= 1-3
Medium dispersion.	D= 3-10
Large dispersion.	D 10

This division has been done for convenience and the flow injection systems are designed accordingly to accomodate the various analytical procedures.

If the purpose of the flow system is only to transport a sample to the detector without any reaction, then the dispersion should generally be limited in order to avoid unnecessary dilution, as with electrochemical and atomic absorption detection. In Fig.7 is shown a simple FIA system, using limited dispersion for rapid H_2O_2

measurement, using an electrochemical flow-through cell as a detector [17]. Over 300 samples h^{-1} were analysed.

The dispersion should be medium if a chemical reaction has to take place, in order to obtain a measurable product from the species to be determined. Such systems are the most interesting from an analytical viewpoint and cover a majority of procedures involving the mixing of sample with reagents to produce a detectable species based on colorimetry, fluorimetry, chemiluminescence etc. Such types of procedures need the exact manipulation of dispersion by controlling all the parameters described above.

On three occasions large dispersion becomes desirable. When a reaction is slow, a certain residence time is required to allow the reaction to proceed until a signal of sufficient magnitude is obtained; when the sample is too concentrated and need on-line dilution, or the sample is complex such as blood and need the separation of low molecular weight analytes from larger molecular weight components such as proteins by using on-line dialyzers. The system should be designed to have a large dispersion if a concentration gradient extending over a well-defined period of time is desired.

BASIC COMPONENTS OF AN FIA SYSTEM:

An FIA system can easily be assembled from the components already present in an analytical laboratory. The basic requirements are a pump, an injection valve, flexible plastic tubes to connect the injection valve to the detector equipped with a flow through small volume cell and a chart recorder. A brief introduction to these various components is given below.

Peristaltic pumps which can easily accomodate 2-3 channels (streams) are most commonly used to propel the carrier stream at a constant steady flow rate. There are flexible pump tubes of various diameters to give the required flow rate, similar to those used in air-segmented system. Typical flow rates in FIA range from 0.5-3ml/min. Although liquid chromatography pumps can be used, a separate pump is needed for each channel.

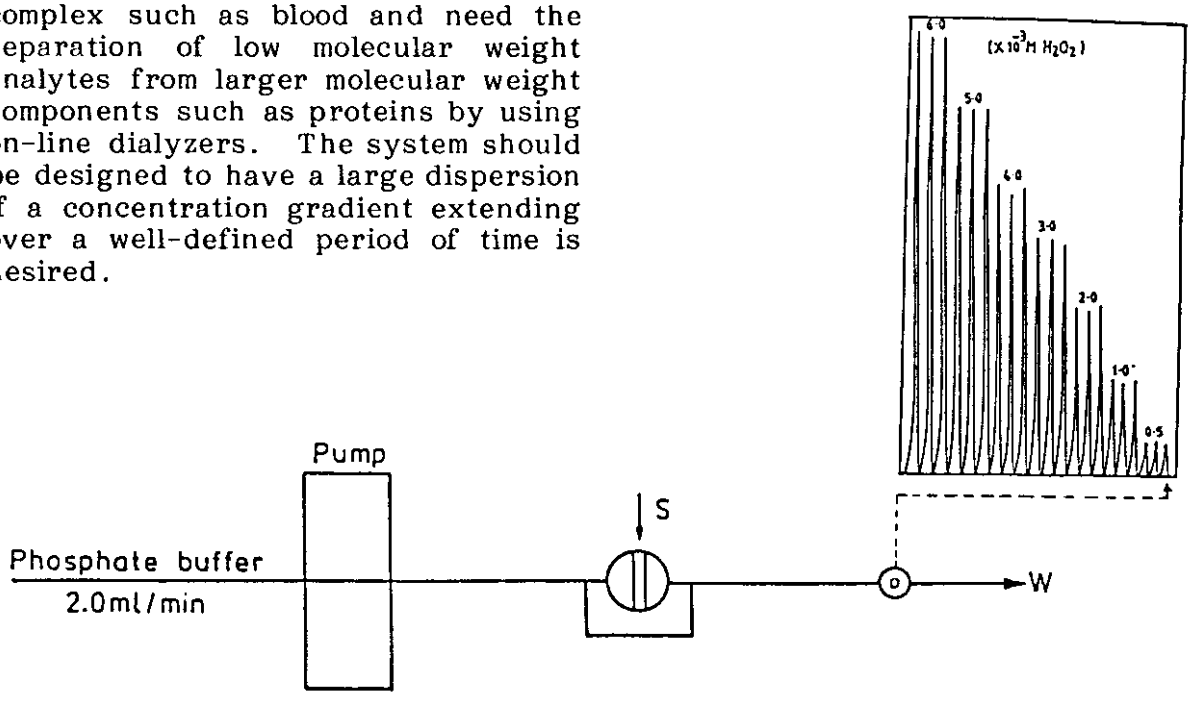

Fig.7: FIA manifold for hydrogen peroxide determination, and typical FIA peaks (detector output signals).

The injection valve which provides a means of injecting an accurately known volume of the sample in a very reproducible manner, into the carrier stream after the pump (to avoid an uncontrolled dispersion of sample) has made the simple FIA system more complicated, and is one of the main obstacles to the commercialization of FIA. Sample injection in FIA has been carried out in a variety of ways. The earliest and the least elegant type involved simply injection from a syringe through the wall of the reaction tube or through a simple injection block [18,19]. But being a tedious and less reproducible method, it is no longer used. By far the commonest means are rotary valves [20,21] with a volumetric bore of constant volume, or the Rheodyne by-pass valves of the type used in HPLC [22]. The former is now available commercially for flow injection application [23]. Ruzicka and Hansen have proposed an alternative method of sample introduction, which they have named "hydrodynamic injection" [24]. Riley et al. [25] have adopted a different approach which they call flow injection analysis without injection.

The manifold consists of flexible polyethylene tubing with uniform inner diameter. Reaction coils are made by winding the appropriate lengths of tubing around small methacrylic cylinders. These and other mainfold components are glued to small Lego blocks which in turn are then attached to a Lego board.

Almost any flow-through detector for wet chemical reactions can be used in FIA. The flexibility of FIA with regard to the detection system is one of the main reasons for the broad applicability of FIA. A wide range of detection methods has been used with FIA, among which are UV/visible spectrophotometry (most widely used), fluorimetry [26], chemiluminescence [27,28], atomic absorption [29], ICP [30], MECA [31], electroanalytical detectors [32,33] diode array detectors [34], turbidimetry/nephlometry [35] and refractometry [36].

The output from the detector can be fed to a chart recorder in order manually to evaluate the FIA results by measuring the peak heights, but nowadays the use of microprocessors for result evaluation in such systems is becoming more common.

Additional application modes developed for Basic FIA:

Apart from numerous types of analyses carried out with simple FIA, a number of modes have been investigated to explore the versatility of the technique. These are briefly discussed here:

Stopped Flow:

In this mode the pump is stopped and the residence time increased without a significant change in dispersion. There are basically two ideas behind stopped flow. One is to increase the residence time of the sample and hence give a longer time for the reaction to produce the detectable species upon which the analysis is based, but without greatly increasing the dispersion. This greatly increases the sensitivity and is applied to slow reactions. The other is when kinetic studies are required. The sample plug is stopped in the flow-cell of the detector and the rate of the reaction is measured as the rate of product formation or reactant consumption.

The increase in peak height during the stop interval is used analytically (Fig.8). This offers the

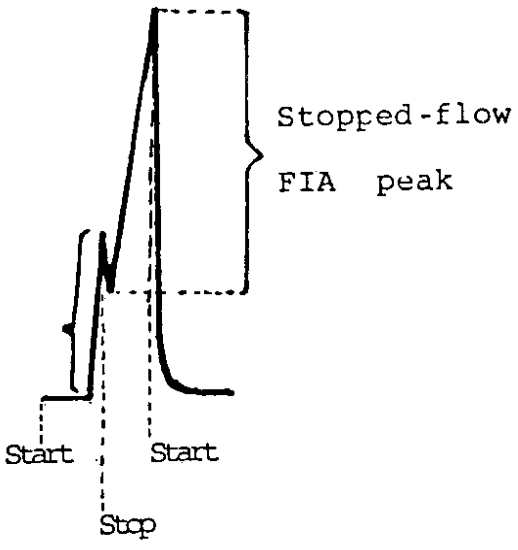

Fig.8: A stopped-flow FIA curve illustrating the principle of stopped-flow FIA technique.

advantage that the background problem is readily circumvented, which is a serious problem while dealing with samples of blood and food [47,37,38].

Merging Zones:

One of the major drawbacks of all continuous flow systems is the high reagent consumption, where a reagent continuously flows through the system even when no sample is present in the system. This becomes an appreciable factor when expensive reagents like enzymes are involved. To avoid this uneconomic use of reagents, Bergamin et al. [39,40] introduced the idea of merging zones in FIA. In this technique two carrier streams are pumped. Sample and reagent are injected simultaneously into their respective streams, which join downstream at a Y-Junction (Fig.9). The combined zone passes through the rest of the manifold to the detector cell. Another advantage of merging zones as illustrated by Ranger [41] is the elimination of high blank values and negative peaks while analyzing dilute samples. By use of the merging zone procedure the reagent immediately reaches the centre of the sample zone and thus results in an increase in sample throughput and high sensitivity [42].

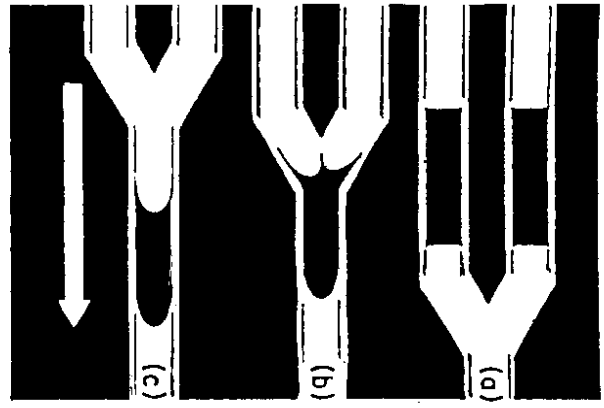

Fig.9: Schematic representation of merging zones in FIA.

A combination of stopped-flow with merging zone has resulted in an economical means of analysis [43], especially for enzymatic reactions where reaction rate is measured after stopping the merged zone in the detector cell [44-45]. Masoom et al. [46] reported a microprocessor-controlled FIA system, based on this concept for the assay of enzymes.

Yet another approach of conserving reagents, developed recently for FIA is that of "intermittent pumping", first established by Ruzicka and Hansen [44]. This is based on the use of two independently operating pumps programmed so that one pump operates first until the peak maximum has been reached, when the second pump is activated to wash the system, while the first pump is stoped. The advantage of this method is that sample throughput can be increased when a wash cycle is performed by a separate

pump of somewhat higher pumping rate. Thus expensive reagent can be saved by using one pump for reagent addition only when required. The applications and advantages of the approach have been summarized elsewhere [47,48].

FIA Titrations:

Titration of an analyte by the classical method is a tedious process. Titration by FIA has simplified the operation and improved it in terms of precision and sensitivity [49].

In conventional FIA titrations the sample is injected into a carrier stream and fed to a mixing chamber to effect a large dispersion. A well-formed concentration gradient is thus produced. Downstream the sample gradient is mixed with a continuously flowing stream of titrant of fixed concentration. The proportions of sample and titrant will vary across the entire sample gradient. A specific proportion value on the rise part of the sample gradient is also obtained on the fall part. The distance in time between these two points depends on the total concentration of titrand in the sample, which is reflected in peak width. The larger the concentration, the larger will be the peak width and hence quantitation of analyte is based on measuring peak width (i.e. time) rather than peak height (Fig.10).

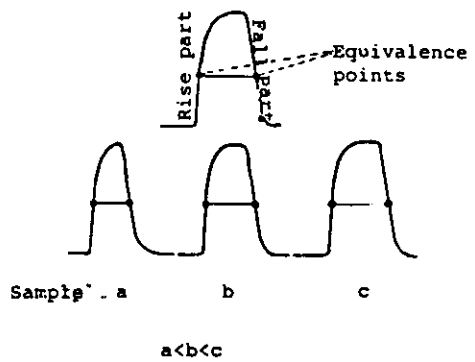

Fig.10: FIA titration curves.

Gradient Techniques in FIA:

When a sample is injected into the carrier stream, it disperses as it travels through the system, resulting in the formation of a concentration gradient, with a concentration between zero and maximum. These concentrations can be characterized by different residence times. Thus any level can be selected and exploited for analytical purposes. This concept has led to designs involving gradient dilutions and calibration [50], stopped flow reaction rate procedures [51], gradient scanning [52] and pH gradients [53].

On-Line Liquid-Liquid Extraction:

It was a tremendous effort originally by Karlberg et al. [54] to adapt on-line liquid-liquid extraction to FIA. The extraction apparatus consisted of a phase combination unit, the extraction coil and then the phase separation unit. Such an extraction aparatus promotes contact and an efficient separation of two immiscible liquids. A typical application has been the determination of thiamine (vitamin B_1) [55]. Samples are injected into an aqueous stream of buffered potassium hexacyanoferrate-III. The stream is combined with chloroform to give alternating aqueous and organic segments as it flows through the extraction coil. The phases are separated, and a portion of the organic phase carried through the fluorimetric detector. A sampling frequency of 70 h^{-1} was achieved. An improvement in the phase separation was achieved by applying a porous PTFE membrane, which is permeable to the organic phase but impermeable to the aqueous phase [56].

On-line dialyzers have been used with FIA. These are the important parts of clinical Auto-Analyzers. They

are used to separate low molecular weight analytes from larger molecular weight components such as proteins in samples [57]. The active part of a dialyzer is a semipermeable membrane which separates the sample injection line physically from the detector line in the FIA system. The analyte dialyzes through the membrane, whereas larger molecules are excluded. FIA systems with on-line dialyzers have been applied to the determination of chloride and phosphate [58], and glucose in serum [59]. Urinary sulphate has been turbidimetrically determined after its dialysis into a stream of barium chloride [60].

A gas diffusion unit has also been incorporated into the FIA manifold. In this case the gas generated from the sample in one stream passes through a membrane into a collector stream in which its concentration is measured. Baadenhuijsen et al. [61] used a simple manifold for the determination of carbon dioxide in plasma. The sample is injected into a carrier stream of sulphuric acid and the carbon dioxide released diffuses through the membrane into a buffered cresol red indicator stream which is detected colorimetrically, with a sample throughout of 90 h^{-1}.

Incorporation of columns in FIA Manifolds:

Small columns filled with particles of a reactive material have been used successfully in FIA. These reactor columns include ion-exchange resins [62,63,84], immobilized enzymes [64-65], and reducing materials [66-67,85]. Solutions carrying the analyte can be made to pass through and react with the material in the reactor. In the case of immobilized enzyme reactors in the flow system, the enzyme is retained for use over and over again, while the product is carried to the detector. The advantage of these reactors is that almost any detector system can be used which can record the concentration of substrate, product or coenzyme.

Recent Advances in FIA:

Today luminenscence itself is a very fast evolving field. Coupling of luminescence with FIA has resulted in a very rapid and sensitive system which has enabled in some cases picomolar concentrations of some analyte to be detected. The very common chemiluminescent reaction between luminol and hydrogen peroxide when catalysed by copper (II) was first adapted to FIA by Rule and Seitz [68] for the determination of hydrogen peroxide. The light generated by the reaction was read using a photomultiplier at a rate of 360 samples per hour. Faizullah et al. [69] constructed a light-tight detector as a part of the FIA-chemiluminescent system and used it for trace metal analysis and for hydrazine determination. More recently Worsfold and Nabi [70] used a similar type of system for the bioluminescent determination of ATP (adenosine triphosphate). Abbott and Townshend used an FIA-chemiluminescent system for the determination of >1f mol of morphine [83].

Ion-selective field effect transistors (ISFETs) have been interfaced with FIA to determine pH and potassium and calcium ions [71]. Although the sampling rate and analytical precision are comparable to those obtained with ion selective electrodes, their very small size minimize reagent consumption.

The combination of FIA with atomic absorption spectrometry (AAS) and inductively coupled plasma-atomic emission spectroscopy (1CP-AES) is a major step forward in the applicability of FIA. The advantages are good

precision (2% r.s.d), low detection limits, rapid analysis (up to 300 samples/hr.) and improvement in nebulizer performance achieved when the flow rate of the carrier stream is controlled by a suitable pump rather than by the oxidant flow rate [72]. Nord and Karlberg [73] applied a FIA extraction procedure to AAS. A 15-20 fold increase in sensitivity was reported for copper, nickel, lead and zinc. Kamson and Townshend [74] used an anion-exchange column to remove anionic interferences in the determination of calcium by FIA-AAS. Johanssen et al. [75] developed an on-line FIA trace metal enrichment procedure utilizing a column containing 8-quinolinol immobilized on porous glass. Metal speciation in solution has been performed with FIA, utilizing spectrophotometric as well as atomic absorption detectors in series. The usefulness of the approach was demonstrated in two different determinations. Iron (II) and total iron in mineral process solution and chromium (III) and total chromium in corrosion test solutions [76,77] have been determined. A number of investigators applied FIA to metal determinations in blood serum. Attiyat and Christian [78] applied FIA-AAS to the determination of serum zinc and copper. Mcleod et al. [79] reported simultaneous multielement analysis by FIA/ICP-AES.

Because of speed, economy and simplicity, flow injection analysis has been adapted for many clinical chemistry analyses. Riley et al. have summarized the subject in their recent reviews [80,81].

Yet another latest development in FIA is the application of integrated microconduits [82]. The manifold is situated in a permanent, rigid, planar structure. The grooves forming the flow channels are engraved into a transparent plate and then closed by a flat layer, thus forming a structure of conduits with a hemicircular cross section. The small dimensions of the integrated microconduits allow further miniaturization of the FIA system and reduction of sample and reagent consumption to the microlitre level.

Advantages of FIA over Air-Segmented Continuous Flow Analysis (SFA):

Although FIA and SFA both can be described as continuous flow systems, used to automate nearly similar chemical analyses, because of its higher sampling rate, accuracy and economy, FIA offers the following clear advantages over SFA.

a. The FIA system has very short start up and close down time in comparison to SFA, which requires several minutes of operation before its base-line is established.

b. The analytical readout in FIA will be available usually within 10-30s of sample injection, as against some minutes for SFA, thus there is a higher sample throughput in FIA system.

c. FIA system is more economic, consumes less reagent and sample (even much lesser in the case of merging zones).

d. FIA system is more versatile, with numerous additional modes developed for it, and it can be linked to a wide range of detection systems.

e. Kinetic analyses with stopped flow-FIA can easily be carried out, which in contrast cannot be achieved in SFA, due to the presence of air-bubbles, the elastic nature of which will cause the movement to continue even after the pump is stopped.

f. FIA system is very simple and easy to operate. The air line and several mixing coils are eliminated and certain reagent lines proven to be unnecessary have been totally excluded from the FIA system, but which are essential in an SFA system.

In the light of these attractions, it is hoped that FIA will soon find the place in teaching, research and industry, which it deserves.

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