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Flow-Injection Methods: A New Tool for Instrumental Analysis [and Discussion]

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Flow-injection methods: a new tool for instrumental analysis

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Gradient flow-injection techniques are reviewed and their use for serial assays and research in instrumental methods of analysis is outlined. The increasing importance of flow injection analysis as a new solution-handling technique in a laboratory is demonstrated with several examples.

Introduction

It is now well recognized that flow-injection analysis (f.i.a.) is a new technique for the rapid serial analysis of a variety of sample solutions. For this reason alone the method has recently attracted a wide attention, which manifested itself by the appearance of several review articles and in a number of research papers. The overwhelming majority of these works deals with the simplest form of f.i.a., while the more advanced so-called gradient f.i.a. techniques still did not attract wide attention. It is the purpose of this work to review briefly all these new approaches, some of which are here described for the first time, and to relate them to each other. Thus it will be shown that f.i.a. is about to extend its role from being an efficient analytical technique to becoming a general concept of solution handling in the laboratory, thus becoming a tool of research in its own right.

The principle of flow injection analysis is deceptively 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 while it changes as a result of the passage of the dispersed sample zone in the flow cell (Růžička & Hansen 1981). In its simplest form the f.i.a. analyser (figure 1*a*) consists of a propulsion 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. Microlitre 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 (table 1) will undoubtedly lead to a widespread use of f.i.a. within the next few years. It would, however, be a pity if f.i.a. were regarded only as a means of rapid, economical handling of samples with the aim of performing the analysis of a large series of samples. Such a superficial view could be adopted if the concept of controlled dispersion of the sample zone were not understood and appreciated.

In contrast to any other technique utilizing continuous flow, the f.i.a. 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 assay 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.

Thus 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 f.i.a. techniques. The principles of these techniques will be reviewed below and are graphically presented and summarized in figure 2.

TABLE 1. DETECTORS AND TECHNIQUES USED IN COMBINATION WITH F.I.A.

spectrophotometry	gas diffusion
atomic absorption	dialysis
atomic emission (i.c.p.)	solvent extraction
nephelometry	titration
fluorescence	reaction rate measurement by stopped flow
chemiluminiscence	stopped flow
potentiometry (i.s.e.)	merging zones
voltammetry, polarography	scanning methods
conductimetry	process control and monitoring
coulometry	

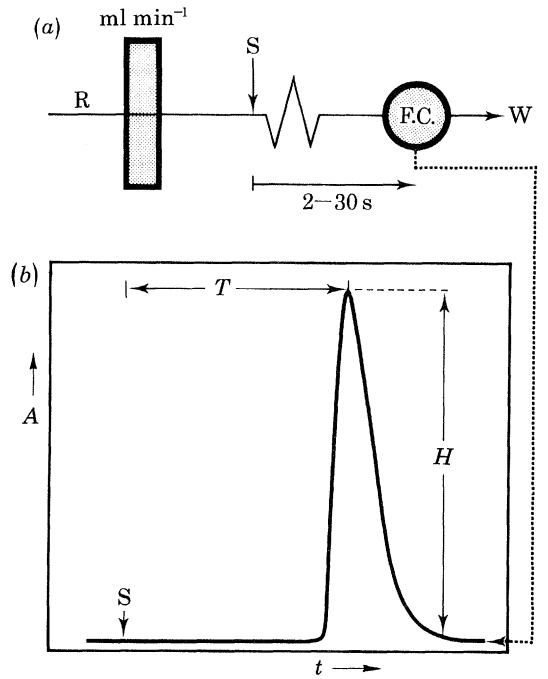

FIGURE 1. Schematic diagram of a single line flow injection analyser (*a*) and of a typical recorder output (*b*). S, sample injection; *T*, residence time; H, peak height; W, waste; F.C., flow cell. For details see text. Reproduced with permission from Růžička & Hansen (1981).

It follows from the theory of f.i.a. (Růžička & Hansen 1981) that a dispersed sample zone consists of a continuum of concentration profiles in both axial and radial directions. Depending on the characteristics of the detector used, these concentration profiles are reflected in the shape of the recorded 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 (figure 2*a*). Thus dispersion is defined as

$$D = C_0/C,$$

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

$$D = C_0/C_{\max},$$

which means that for, say, $D = 2$ the sample solution has been diluted with carrier stream 1:1.

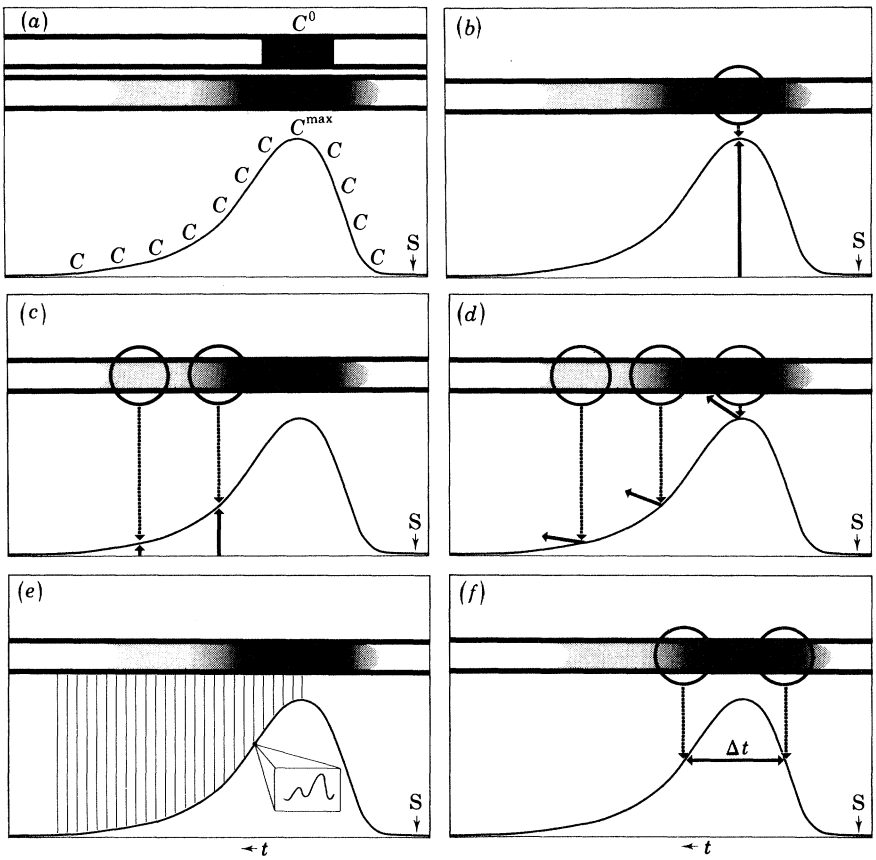

FIGURE 2. Dispersion of injected sample zone (*a*) and principles of gradient f.i.a. techniques (*b–f*). S, point of injection; *t*, time.

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 the f.i.a. system serves only as a means of transport and the sample material should not become mixed with the carrier stream (pH, atomic absorption or conductivity measurements). Medium dispersion ($D = 3$ to 10) has the widest range of applications as it allows chemical reactions to take place after the mixing of reagent, contained in the carrier stream, with the sample components contained in the dispersed sample zone. Large dispersion ($D > 10$) is nowadays used only when the sample material to be analysed is too concentrated to be accommodated within the detector range.

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Gradient dilution

An overwhelming majority of f.i.a. methods designed so far are based on peak height measurement (cf. figures 1 and 2*b*), mainly because the peak top and its distance from the baseline are the easiest to identify. However, by choosing the peak top we use only one out of an infinite number of elements of fluid from which the dispersed sample zone is composed. One may very well use any other section of the response curve to get the analytical readout, provided that it is always the same section, in other words an element of fluid that has always exactly the same D and t values, the latter being the time between the moment of injection and the readout. This can be explained best with reference to figure 2*c* where two consecutive readouts are shown on

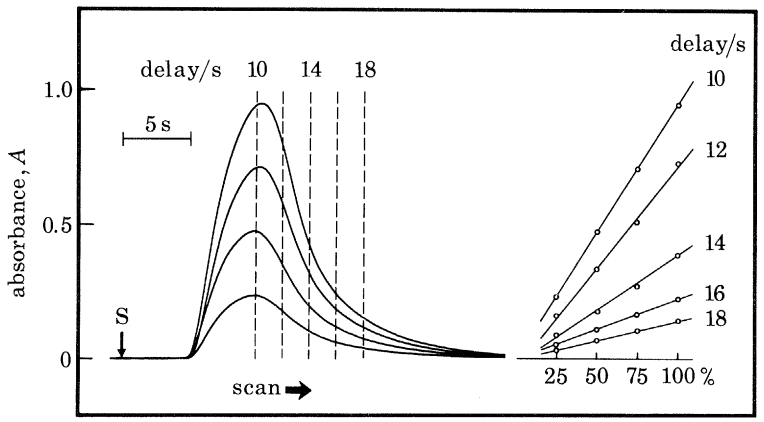

Figure 3. The principle of electronic dilution. Samples of bromothymol blue (BTB) designed as 25, 50 and 100 % dye concentrations were injected into a colourless carrier stream (0 % dye) and peaks were recorded repeatedly from the same starting point (S). As well as at the peak maximum (delay 10 s) readouts were collected electronically at 12, 14, 16 and 18 s delay, yielding a family of calibration curves with decreasing slopes. For further details consult Olsen *et al.* (1982), from which this figure is reproduced with permission from Elsevier.

the tailing section of the peak. Obviously these signals are lower than the corresponding peak height values (figure 2*b*) because they correspond to elements of the dispersed zone with higher D values. Therefore, readouts obtained with longer (or shorter) delay times (t) than the peak maximum appearance time (T) will yield calibration curves with lower slope (figure 3) that would accommodate a wide range of concentrations, and would thus be useful when the sample material is too concentrated to be accommodated within the detector range (Olsen *et al.* 1982). In other words, by obtaining the readout electronically after a certain delay has elapsed since the moment of injection, one can avoid manual pre-dilution of the sample material before injection into the f.i.a. apparatus, and thus spare one manual operation per sample. Expressed in yet another way, one may choose limited, medium or even large dispersion within the *same* dispersed sample zone by electronic means. It is of interest to mention in this connection that the peak top measurement is in fact the most susceptible to errors because in the element of fluid that corresponds to peak maximum the reagent is always likely to be in the least excess; deviation from linearity is therefore most likely to occur just there. Thus by choosing a longer delay a nonlinear calibration curve may well be 'straightened out'. Closely related to the concept of gradient dilution is the concept of gradient calibration, which uses only one

standard solution from which a number of data points are collected and are subsequently reconstructed into a conventional calibration curve (Olsen *et al.* 1982). It is outside the scope of this short review to discuss this technique in detail.

Stopped-flow rate reaction measurement

Though in principle enzymatic assays could be performed as simple peak height measurements, because in f.i.a. the time between the point of injection and the peak height maximum is well defined, it is much more useful to perform these assays as reaction rate measurements,

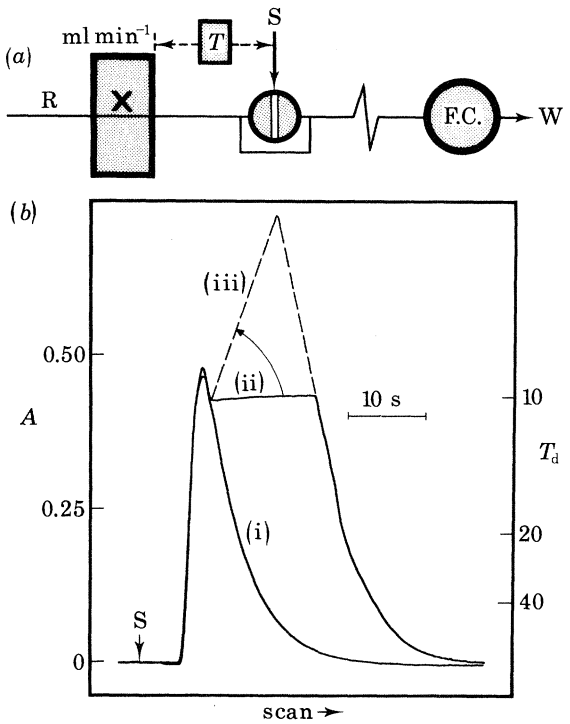

Figure 4. Stopped-flow f.i.a. system (*a*) and corresponding recorder output (*b*). All curves were recorded from the same starting point (S). (i) A continuous recording; (ii) a stopped-flow recording (10 s delay, 15 s stop period and then washout); (iii) the superimposed reaction rate curve. Reproduced from Růžička & Hansen (1981), with permission.

where the rate of formation (or consumption) of a certain species is measured on a larger number of data points. This not only improves the reproducibility of the assay but also secures its reliability, because interfering phenomena such as blank values, existence of lag phases and nonlinearity of rate reaction curves may be identified and eliminated. F.i.a. is ideally suited for this purpose because the non-segmented stream can be manipulated exactly in space and time so reproducibly that the same section of the dispersed sample zone can always be held still in the detector while the measurements of reaction rate take place. The methodology of this approach has been explained in detail elsewhere, and therefore a short reference to figure 4 should be sufficient for the present purpose. A simple f.i.a. system (figure 4*a*) is supplemented with a timer (T) that allows the pump – and the forward movement of the carrier stream – to be stopped after a predetermined delay. If no chemical reaction takes place, the recorded curve

(figure 4*b*(ii)) will be horizontal because no species will be formed within the detector cell and the absorbance will remain unchanged with time. Should, however, a chemical reaction take place in the flow cell, then a reaction rate curve will be recorded (figure 4*b*(iii)), the slope of which will increase with the reaction rate.

The gradient principle allows in this case an optimum choice of the conditions for the reaction rate measurements: by simple choice of delay a section of the dispersed sample zone may be chosen (figure 2*d*), yielding (*a*) a slope that can be accommodated best within the detector readout, and (*b*) a linear response curve, due to the optimized reagent:sample ratio.

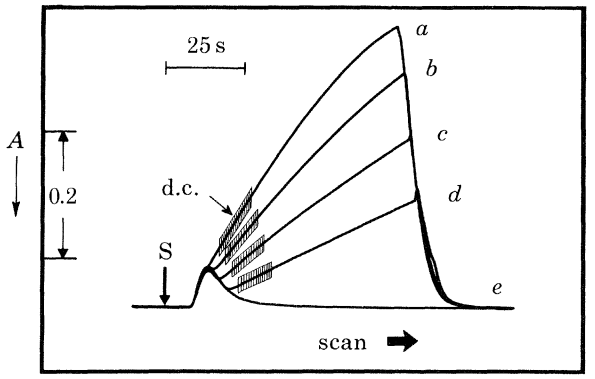

FIGURE 5. Reaction rate measurement of the enzymatic activity of lactate dehydrogenase (LDH) by stopped-flow f.i.a. Curves recorded on top of each other from the same starting point (S) with increasing delay times (*a*, 12 s; *b*, 14 s; *c*, 16 s; *d*, 18 s; *e*, continuous pumping), by injecting repeatedly 25 µl of LDH solution containing 1155 U/l. Reproduced from Olsen *et al.* (1982), with permission from Elsevier.

An actual example of this approach is the assay of lactate dehydrogenase, which catalyses the redox conversion of pyruvate to lactate in the presence of NADH, the decreasing absorbance of which is measured spectrophotometrically (figure 5). By choosing different time delays (curves *a*–*d*) the reaction rate curve becomes more linear, while its slope decreases. This approach (Olsen *et al.* 1982), which can be used both for substrate and enzyme assays, has the advantage of avoiding tedious manual predilution as well as optimizing the substrate:enzyme ratio by simple adjustment of an electronic timer.

HIGH-SPEED TITRATIONS

While previously described gradient f.i.a. techniques used only one element of fluid from each dispersed sample zone to obtain the analytical readout, f.i.a. titrations (Růžička *et al.* 1977; Ramsing *et al.* 1981) use the relation between a pair of elements of liquid that have the same dispersion (figure 2*f*).

High-speed f.i.a. titrations (Ramsing *et al.* 1981) are thus an example of an advanced f.i.a. gradient technique. If, say, a sample of an acid to be titrated is injected into a carrier stream of a strong base containing a suitable indicator, then the concentration gradients formed at the interfaces at the front and tailing edges of the dispersed sample zone will consist of two titration curves, starting at the front edge with the excess of base, passing through the first equivalence point, where the base has been neutralized by the acid contained in the sample, then towards the peak top, where the acid is in excess and finally down the peak tail, passing through the

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second equivalence point and into the region with the base excess, where the titration cycle is concluded. The time between the two equivalence points will increase with the increase of the concentration of the injected acid, not only because the respective D values of these two liquid sections are equal but also because the concentration of base is *equivalent* to that of the acid within each of these elements of fluid. A simple f.i.a. manifold used for this purpose is shown in figure 6*a*, while five titration curves obtained when titrating HCl with NaOH using bromothymol blue (BTB) as an indicator (the basic form of BTB is blue, the acid form is yellow) are

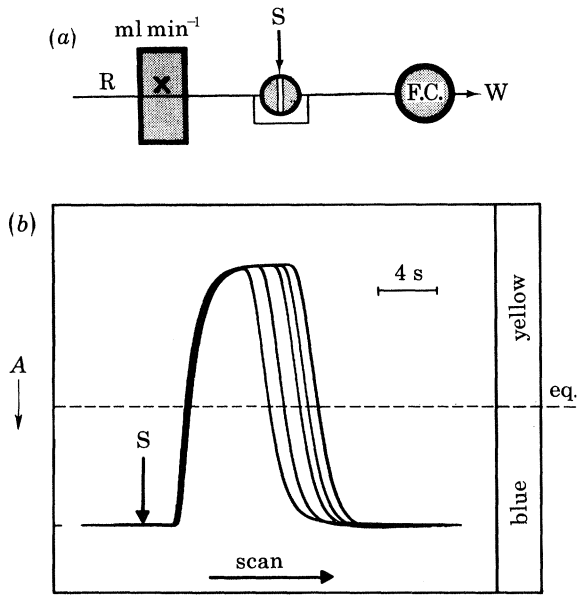

FIGURE 6. High-speed f.i.a. titration system (*a*) and a recorder output (*b*) showing the titration of hydrochloric acid by sodium hydroxide, with bromothymol blue as indicator. All five titration curves were recorded from the same starting point, while 25 µl of sample solutions of increasing concentrations of HCl were sequentially injected. For details see Ramsing *et al.* (1981), from which this figure has been reproduced with permission from Elsevier.

shown in figure 6*b*. The details of the theory and experimental conditions for high-speed titrations are described in the original paper (Ramsing *et al.* 1981). At this stage it is, however, worth mentioning that the titration cycle takes a maximum of 12 s to complete, that the r.s.d. of this titration is better than 1% and that the method requires as little as 30 µl of sample material per titration. The method is suitable not only for acidobasic, but also redox, complexometric and precipitation titrations, using colorimetric, potentiometric or amperometric detection.

SCANNING METHODS AND 'THREE-DIMENSIONAL' F.I.A. DIAGRAMS

Until now all f.i.a. techniques used detectors that can be described as 'static': they were tuned to a certain wavelength (spectrophotometry), or operated at a fixed potential (voltammetric), while – due to the very nature of f.i.a. – the solution was passed through these detectors in a dynamic mode, so that a continuum of concentration profiles was continuously monitored. An exciting combination, however, exists of operating the f.i.a. system in a *double dynamic mode*, by using a rapidly scanning detector so that each of a number of elements of fluid will be

scanned within the whole operational range of a dynamic detector, such as a spectrophotometer or a polarograph (Janata & Růžička 1982). In other words the concentration profile formed at the tailing section of the dispersed zone will yield at numerous points, not one single value of readout but an entire curve (figure 2*e*). To facilitate the graphical presentation of this type of multiple information the scanning can be unfolded through computer control into a three-dimensional form, shown in figure 7. This multiple recording of a single-sweep voltammogram was obtained with a stationary micropool mercury electrode situated in a one-channel Fiastar system. The injected sample contained Cu^{II} in 0.1 M sodium acetate buffer

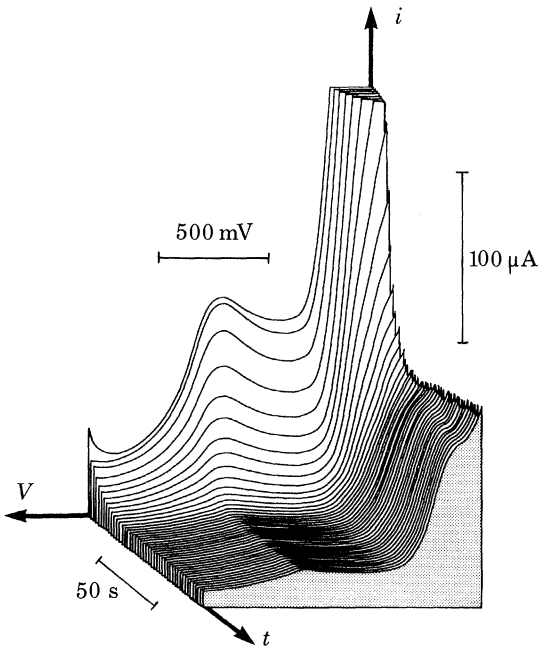

FIGURE 7. F.i.a. scanning diagram showing a single-sweep voltammetric scan repeated 45 times while an injected sample zone was moving through a detector operating with a micropool mercury electrode. The depolarizer concentration decreased along the dispersed sample zone; the peak height therefore decreases along the time axis (towards the reader). Reproduced from Janata & Růžička (1982), with permission of Elsevier. Axes: *i*, current; *t*, time or concentration within dispersed zone; *V*, potential imposed during 1.95 s scan. All 45 scans were recorded on a single zone starting at the peak top and continuing along the dispersed zone.

supporting electrolyte, while the carrier stream consisted of the same 0.1 M sodium acetate buffer supporting electrolyte alone. The reduction of Cu^{II} to Cu^{0} yielded a single peak as known in single-sweep voltammetry while the decreasing concentration of copper complexes due to dispersion of the sample zone caused a decrease in the peak height along the time axis. All sweeps were recorded in real time while the dispersed sample zone was in motion, and the whole experiment was completed within less than 2 min.

The above experiment should serve only as an example of the new approach, which is currently at an early stage of development. Single-sweep voltammetry was chosen because the equipment was simply at hand. Obviously, cyclic voltammetry would be even more suitable for this purpose (Janata & Růžička 1982). Spectrophotometry will, of course, have an even wider range of applications for the rapid study of chemical reactions of light-absorbing species in solution. It should be clearly understood that for such types of study one would not create a concentration gradient only from the dispersed sample material, but would also form pH

gradients as described earlier (Betteridge & Fields 1978; Klinghoffer *et al.* 1980), or by using concentration gradients of various ligands. The kinetic implications of such approaches are far-reaching, but the instrumental approach will remain simple: to distinguish between the kinetics of chemical reactions and the physical kinetics of mixing or of the speed of the detector response, one would use a combination of continuous flow technique in one scanning experiment, and then use the stopped-flow methodology to resolve the kinetic contribution of chemistry and detector by stopping the zone before it reaches the detector, or within the detector itself.

Conclusion

The gradient f.i.a. techniques are a logical extension of the original concept of the method within which the idea of controlled dispersion of the sample zone plays the key role. Obviously, the homogeneous mixing sought in the design of air-segmented continuous-flow systems, as well as throughout all the early attempts of making the non-segmented flow systems practicable, caused not only a loss of sampling frequency but also a loss of much valuable information, contained in the dispersed sample zone, by reducing it to this single point.

Though the concept of homogeneous mixing is an integral part of the philosophy of the manual handling of solutions in the analytical laboratory, and though this approach has also been correctly applied when designing automated analysers of the batch type, its transplantation into continuous-flow methodology is a mistake. This is why the concept of 'steady state' was adopted – and maintained – for so long in air-segmented continuous-flow analysis. For the same reason, mixing chambers or pulsers were incorporated into the early designs of non-segmented continuous flow analysers. The continuing tendency to integrate the peak area (Renoe *et al.* 1980) has the same background as it reduces all accessible data points to a single one, through the process of 'electronic homogenization'.

It is characteristic that those papers on f.i.a. theory and methodology that are based on the concept of controlled dispersion are among the most innovative. It was Betteridge & Fields (1978) who constructed an f.i.a. system with a large dispersion and let the pH change along the length of the sample zone. This allowed them to exploit the differences in the conditional stability constants between metal ions and the colour-forming reagent (PAR) and obtain a series of peaks, each corresponding to a certain metal ion, along the front and tail of the dispersed sample zone. This multi-component f.i.a. was performed with a detector operating at fixed wavelength. It is left to the imagination what the 'landscape' of information would look like if the gradient technique of Betteridge & Fields were used in combination with a scanning detector yielding the three-dimensional f.i.a. diagram similar to that shown in figure 7.

With far less sophisticated instrumentation, a Brazilian group (Reis *et al.* 1981) designed a sampling system that selected well defined sections of a dispersed sample zone and reinjected them into another carrier stream for further treatment. This ingenious approach, which has a practical application as a pre-dilution technique for atomic absorption, is based on a good understanding of the key role of the dispersion process, and may be considered the predecessor of the gradient dilution technique. Most recently Tyson & Idris (1981) conceived an original approach to the use of f.i.a. as a vehicle for sample introduction to atomic absorption spectrometry. While previously only f.i.a. systems with limited dispersion were thought to be useful for flame methods, the work of Tyson & Idris shows that each of the three modes of dispersion has its own merit. Thus high sampling frequency may be obtained with limited dispersion,

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interference effects may be eliminated by using a medium dispersion – standard addition method, and single-standard calibration may be effected by creating a purely exponential profile in a mixing chamber. The applicability of the medium dispersion – standard addition f.i.a. technique extends beyond the domain of flame methods because it is applicable to all kinds of detectors. A similar work recently published by Greenfield (1981) for i.c.p. detectors confirms the feasibility of this ingenious approach, which could never be conceived without a thorough understanding of the concept of dispersion.

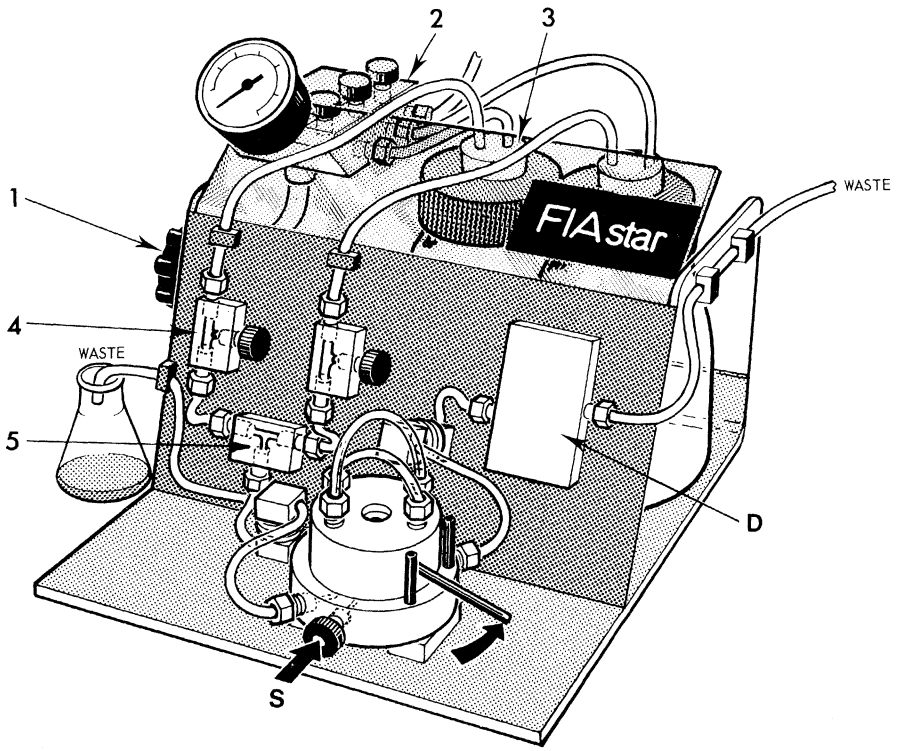

FIGURE 8. The Fiastar system. For details see text and Růžička *et al.* (1981), from which this figure is reproduced with permission from Elsevier.

It is unfortunate that the development of f.i.a. instrumentation lags somewhat behind the progress achieved in the research of f.i.a., as this makes the technique inaccessible to a wider community. The difficult task of constructing f.i.a. apparatus from first principles without prior experience is not helped by some misconceptions that were unfortunately introduced in the past. An apparent similarity between f.i.a. and h.p.l.c. led to the use of high pressure for propelling the carrier stream and the uncritical use of chromatographic components (valves, narrow long tubes and narrow beam detectors) that are unnecessary, expensive and often unsuitable for use in an f.i.a. system, which can be operated well at pressures below 10^5 Pa. Equally unfortunate is the design of a commercial instrument where a complex array of computer-controlled magnetic valves is used to inject the sample solution.

To present an alternative construction, an opposite attitude was chosen to design a flow-injection analyser with the lowest possible level of complexity. This so-called Fiastar system (figure 8) has been designed for *s*tudent *t*eaching *a*nd *r*esearch (Růžička *et al.* 1982). As the acronym implies, the system has been designed to allow access to exploration of various facets

of f.i.a. to the widest analytical community. It is beyond the scope of this paper to give a detailed description of the instrument. It should be mentioned, however, that an ultra-low pressure of gas (20–60 kPa) is used to propel carrier streams. The gas from a primary source is deregulated and its pressure maintained at fixed level by means of a regulator (1) and then distributed (2) into reagent reservoirs (3). From these containers the carrier streams proceed via shut-off valves (4) into mixing T (5) and further into the injection valve, which allows injection of sample solution (S) via a sample loop. Reaction coil and detector (D) are situated downstream. It is characteristic of the Fiastar construction that all components may be moved freely on the board (by being attached by means of Lego components) and that the system is so miniaturized that it requires only microvolumes of sample and reagent solutions for each assay. Though many may regard the simplicity of the system exaggerated it should be kept in mind that Fiastar is meant to offer an opportunity to get experience at first hand with a well functioning f.i.a. design. It is hoped that experiments even with such simple f.i.a. apparatus will lead to a better understanding of what is happening when the sample zone is injected and forced to flow. Thus many may become inspired to design new f.i.a. techniques and new practical applications of f.i.a. so that, in future, solutions will be handled in a dynamic fashion, truly compatible with the contemporary high technological level of data processing.

I wish to express my gratitude to Elo H. Hansen for his friendship, help and contributions throughout all stages of the development of f.i.a.

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Discussion

D. Betteridge (*Department of Chemistry, University College of Swansea, U.K.*). Professor Růžička is right to focus attention on the possibilities of exploiting the concentration gradients over the interface between sample and carrier that necessarily exists in flow-injection analysis. Since a visit to his laboratory 6 years ago, when we learnt of his novel method, we have devoted our efforts towards obtaining reliable, reproducible and useful analytical results from the interfacial region.

Some procedures have been relatively easy, such as the improvement in the f.i.a. determination of sulphate, by using a pH gradient first to ensure precipitation of barium sulphate and then to dissolve the precipitate before it clogs the flow system (Baban *et al.* 1980). This system,

like most f.i.a. systems published so far, operates with 1–2 % precision and is remarkably robust. Changes in pumps, tube parameters and concentrations of reactants have some effect, but satisfactory results can be obtained with almost any combination of variables. It is this robustness that was early perceived by Professor Růžička to be a major advantage of the method.

However, for other applications, such as the measurement of viscosity and the application of pH gradients to the determination of several elements in a mixture with just one injection, progress has been slower. Results have been given at conferences (Betteridge *et al.* 1981) and in short communications (Betteridge & Fields 1978), but only recently have more detailed procedures been published (Betteridge & Fields 1982). This is because, in our view, it is desirable to aim at a repeatability of 0.1–0.2 %. The improvement of an order of magnitude presents numerous technical problems: no pump or sample valve operates to this degree of precision and, given the length of a sample peak in f.i.a., data readings should be taken every 1–10 ms, well beyond the capacity of today's home computer. We think that solutions to these problems have now been found. The sample plug in f.i.a. may be viewed as a mobile three-dimensional chemical reactor whose dimensions may be varied with ease, and vary during the course of the experiment, as do the concentrations of reactants. The chemical problems associated with making full use of the information within the system are difficult but solvable, and the chemical and analytical information is rich because in all of the work that stemmed from that visit to Copenhagen, it has been very evident that flow-injection analysis is a means of acquiring a wide range of chemical information rapidly, reproducibly and economically. It is also a technique that allows the practitioner full scope for imagination and inventiveness.

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