FROM BEAKER CHEMISTRY TO PROGRAMMABLE MICROFLUIDICS

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"Chemists have Solutions"

(American Chemical Society)

Solution handling is one of the most frequently performed laboratory tasks. In an analytical laboratory, it is also the most exacting and laborious one, as solutions have to be precisely metered, well-mixed, incubated, heated or separated, and finally monitored in a reproducible way, using spectroscopy, electrochemistry or other means for quantification of target analytes. This review with 65 references follows the development of solution handling techniques from manual to mechanized, and into a microfluidic format. It documents the superior versatility of programmable flow, as it is applied to a wide variety of instrumental assays ranging from biochemical to trace analysis of elements. All types of flow injection analysis (FIA) are reviewed, including flow injection (FI), sequential injection (SI), bead injection (BI), and sequential injection chromatography (SIC).

Keywords: Instrumental analysis; Automated solution handling; Flow injection analysis; Bead injection; Sequential injection; Microfluidics; Sequential injection chromatography; Programmable flow.

Analytical chemistry is the oldest branch of chemistry[1] and also one of the most important. Prior to the development of quantitative analysis, chemical experimentation remained within the realm of alchemy. Since success of any systematic investigation critically depends on the reliability of available tools, the development of instruments for weighing and fluid metering had the same impact on chemistry as the development of the microscope had on biology. It is in Lavoisier's book[2] that we find the first drawing of volumetric glassware, which, during later centuries, developed into pipettes, volumetric flasks, and measuring cylinders. Only at the beginning of the 19th century did solution handling and quantitative analysis become refined to encompass titrations. By 1806 volumetric glassware was perfected (Fig. 1) and has remained in its form almost unchanged to this day[3].

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Thus originated the solution handling method, often referred to as "beaker chemistry". As time went by, this approach became refined, miniaturized, and mechanized, ultimately evolving into current microwell plate formats that have been designed to meet the needs of high throughput pharmaceutical assays. The characteristic feature of this approach, also known as "*batch analysis*" is that each sample solution to be analyzed is processed within a container (test tube, beaker, microwell), where it is *homogenously* mixed with auxiliary reagents and the readouts (end point, absorbance, etc.) are being taken (Fig. 2, top). When mechanized for serial assays, the individual containers are moved on a conveyor belt (Fig. 2, below) through stations where samples are pipetted, reagents added, solutions are mixed, etc., as required by assay protocol. While precise, reproducible, and well suited for parallel processing of end-point-based assays, batch analysis is labor-intensive and it becomes less reliable when microminiaturized down to microlitre volumes, as it suffers from the adverse effects of evaporation, differences in solution viscosities, and the inability to carry out separations.

It was Tsvett, a botanist, who unwittingly became the father of *continuous-flow analysis*. In 1906, he published work on the separation of components of chlorophyll on a column of calcium carbonate, eluted continuously with

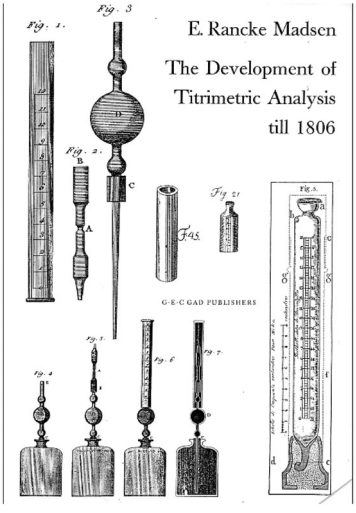

FIG. 1
Analyst's tools at the beginning of 19th century. (Reproduced from front page of Madsen's Doctorate thesis[3].)

petroleum ether[4]. For almost 50 years, chromatography, which Tsvett discovered and named (Fig. 3), was the only analytical technique where samples were analyzed while being carried in flow-through tubing towards a detector. This all changed in 1957, when Skeggs, a clinical chemist, designed an *air-segmented, continuous-flow analyzer* (Fig. 4), where sample solutions were drawn into a system of flow channels by a peristaltic pump, metered and mixed with reagents on the way to detector, while being heated, filtered, extracted etc., as required by an assay protocol[5,6]. The essential feature of Skegg's design was air segmentation, which divided the moving car-

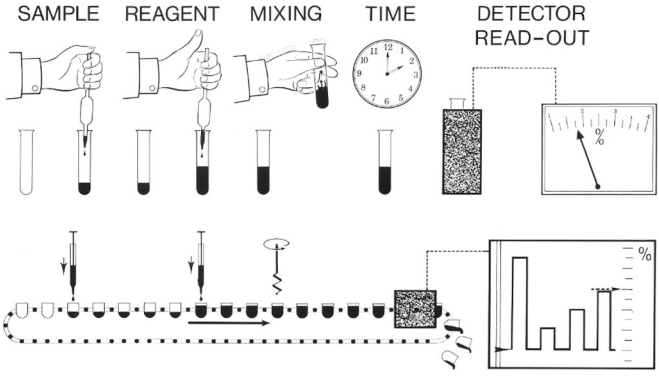

FIG. 2
Manual (top) and mechanized version (bottom) of batch type reagent based assay showing individual steps of assay protocol: sample metering, reagent addition, mixing, incubating, and measurement. (From ref.[55] with authors permission.)

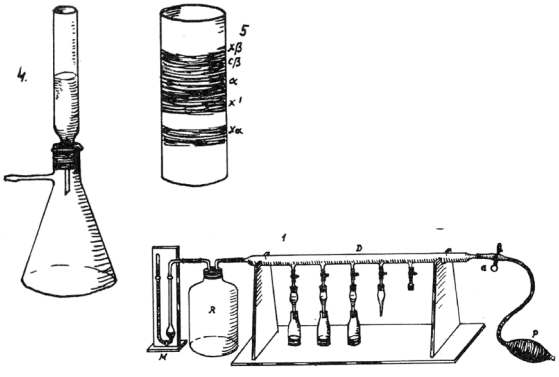

FIG. 3
Chromatographic apparatus of Tsvett. (From ref.[4])

rier stream into many separate segments using large number of air bubbles. Air segmentation facilitated *homogenous mixing* of sample with reagents because the liquid circulated within each segment, and hindering their intermingling preserved the identities of adjacent samples. Skeggs' invention was an undisputed success, largely in the field of clinical assays, as almost all clinical laboratories in technically advanced countries used the Technicon Autoanalyzer® for serial assays of multiple analytes. Interestingly, academic research, textbooks, and university teachings mostly ignored this revolutionary approach to solution handling, which dominated the field of real-life assays for the next 20 years.

It was in 1974, almost by accident, that the paradigm changed once again. While investigating the response of an electrode, it was realized that air segmentation was not necessary for performing reagent-based assays[7]. The study of dispersion of solutes in tubular conduits using *nonsegmented, continuous flow*, led to the realization of a new technique, termed flow injection analysis (FIA), which operated on three principles: sample injection, controlled dispersion, and reproducible timing[8]. *Sample injection* defined the volume of analyte and its initial geometry in the tubular channel, while the *controlled dispersion* was achieved by holding the flow rate of the carrier

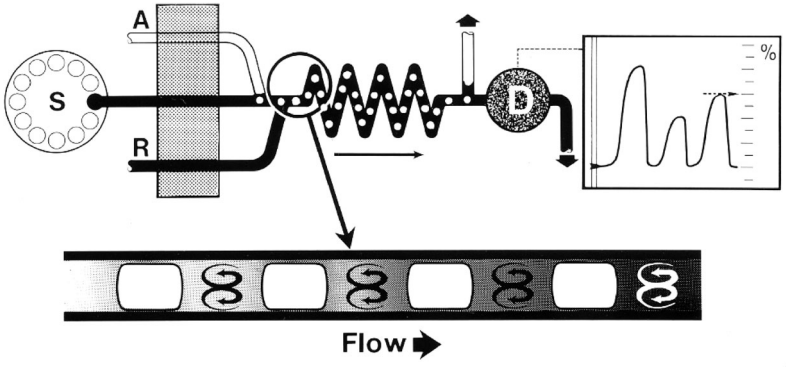

FIG. 4

Skegg's continuous flow analyzer. Top: samples were drawn from autosampler carousel (S) by a peristaltic pump that also aspirated air (A) and reagent (R). Air-segmented stream was pumped through a reaction coil, air bubbles were removed and reacted mixture was pumped into a flow-through cell, where absorbance was measured. The readout was obtained at a "steady state" flat portion of a peak. Below: air bubbles separated individual aqueous segments, where the circular movement of liquid promoted mixing. (From ref.[55] with authors permission.)

stream constant and by maintaining a fixed geometry of the flow path. The precise *timing* of the start of the assay cycle and of the residence time of the analyte zone in the system was controlled by flow rate and by the volume of the flow path (Fig. 5B).

Initially, the method was not well received, since the idea of using controlled dispersion rather than homogenous mixing of sample with reagent solutions was entirely at odds with the way in which reagent-based assays had been carried out until that time. Indeed, all FIA techniques rely on a *concentration gradient*, formed by mutual dispersion *of analyte and reagent solutions* within each other (Fig. 5A). Therefore monitoring of a resulting reaction product yields a peak that is used to construct a calibration graph. It took several years before experimental evidence finally prevailed, documenting that the dispersion process and timing of all events could be controlled with such repeatability, that flow injection (FI) based assays, based on monitoring of *concentration gradients*, are as precise and reproducible, as those obtained in the traditional batch format, where sample and reagent solutions are homogenously mixed.

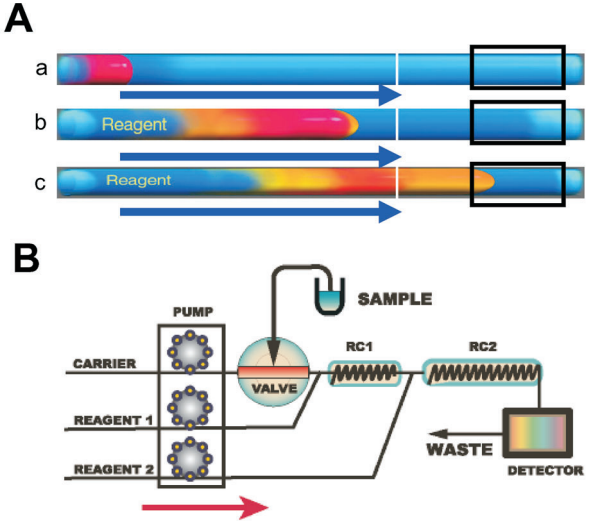

FIG. 5

A Principle of flow injection operated by continuous forward flow: sample is injected into carrier stream of reagent (a), dispersion and reaction takes place on the way to detector (b), reaction mixture reaches flow cell where is monitored (c). **B** A typical FI apparatus comprises a multichannel peristaltic pump, two-position injection valve, reactor coils (RC1, RC2) and flow-through detector

In the following years, FI became a workhorse in numerous laboratories, where real-life assays of soil, water, and pharmaceutical and biochemical materials are still carried out in large series. FI also became a research topic discussed in numerous papers and monographs[9]. By the end of last century, the continuous-flow methods of chromatography and flow injection analysis were firmly established in research and routine laboratories.

Programmable Flow

However, there is yet another way in which solution-based chemical assays can be automated and miniaturized. While chromatography, air-segmented continuous-flow analysis, and flow injection are based on *continuous forward* flow, automated solution processing is far more flexible if it is based on *programmable flow*. The first step in this direction was made in 1990 [10], when the principle of sequential injection (SI) analysis was proposed and experimentally verified. SI is based on the same principles as FI: sample (and reagent) injection, controlled dispersion, and reproducible timing. Consequently, SI also relies on repeatability of the two kinetic processes that take place simultaneously: the *physical process of dispersion* of injected zones and the *chemical process* of formation of chemical species to be detected. The difference is in the way solutions are manipulated. In SI mode, sample and reagent zones are sequentially stacked upstream from the injection valve (Fig. 6A) and subsequently mixed by reversing the flow. Dispersion is controlled by means of flow programming. Flow reversal and sudden acceleration are used to promote mixing and to speed up flushing of the system, while stopping the flow is used to control reaction time. Also, in SI mode, solutions are pumped *only* when a sample is being processed and, for that reason, smaller volumes of reagents are used and smaller volumes of waste are produced, as compared to continuous-flow-based FI. The research community rapidly recognized these advantages and SI has been increasingly applied to a variety of assays, using spectroscopic, electrochemical, radiochemical, turbidimetric and chemiluminescent detection. An excellent review published by Barnett[11] has summarized the development of SI in 300 references up to 2002. Since then, SI has expanded into separation techniques, of which sequential injection chromatography (SIC, see below) and SI solvent extraction (SISE)[12] are innovative techniques with potential for many practical applications.

Miniaturization

Since SI was initially designed for automated monitoring of industrial processes[10,11], the instrument size was of little or no importance, so no attempts were made to miniaturize the apparatus. Also, SI systems designed for laboratory use were assembled in the same fashion as FI instruments, by connecting the pump, valve, and detector with tubing that, when coiled, served as a reactor. What prompted the development of the microSI technique was the impact of a novel trend in analytical instrumentation, emphasizing integration and miniaturization of system components. The use of fiber optics, microfabrication, and novel materials was being highlighted at μTAS meetings[13–15], where the platform for these new technologies was referred to as "lab-on-chip". The undisputable advantages of microminiaturization: low sample and reagent consumption, minimized production of chemical waste, small instrument size, and portability, could no longer be

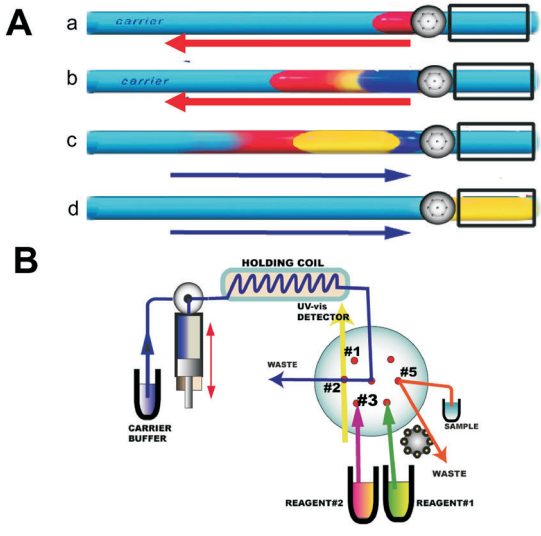

FIG. 6

A Principle of sequential injection operated by programmable flow: sample solution is aspirated (a), in a next sequence a reagent solution is aspirated (b), the stacked zones are mixed by rapid flow reversal (c), and transported into a detector for stopped-flow measurement (d). **B** MicroSI-LOV apparatus comprises a precision syringe pump that moves carrier stream through the holding coil and through a multiposition valve into a flow cell that is integrated along with flow-through port and reagent ports within a mezzofabricated unit mounted atop a multiposition valve. (From ref.[21] with author's permission.)

ignored. Downscaling of SI was achieved by integrating the flow cell, sample introduction channels and access to reagent reservoirs within a mezzo-fabricated monolithic structure, mounted atop a multiposition valve[16] (Fig. 6B). The resulting device, named in jest "lab-on-valve" (LOV), became a platform for microSI techniques, using microlitre volumes of samples and reagents per assay. Rather unexpectedly, LOV has become a vehicle for new techniques such as bead injection[17] and sequential injection affinity chromatography[18]. It has also served as a "front end" solution-processing module for mass spectrometry[19], capillary electrophoresis[20], and has been applied to biomolecular assays[21]. For trace analysis by atomic spectroscopy, microSI in the LOV format was adopted as a sample processing "front end" to atomic absorption and ICP [22–29]. Virtually all reagent-based assays can be automated in the microSI-LOV mode. An enzymatic assay is outlined below as an example of a typical sequence of operational steps in a microSI protocol.

Reagent-based assays comprise an ever growing group of analytical techniques, ranging from classical, reagent-based colorimetric procedures to reaction rate-based enzymatic assays, immunoassays, fluorescence tagged assays, and radiochemical assays, applied to soil, water, environmental, agricultural, biochemical, and pharmaceutical research and in routine laboratories.

Protocols for reagent-based assays in microSI format fall into two categories[21]: *flow-through measurement*, monitored as the sample zone passes through a detector where the readout has form of a *peak*, or reaction *rate measurement* monitored while the sample zone is arrested within a detector. For reaction rate measurement, enzymatic activity and auxiliary regent concentrations are selected in such a way that pseudo-zero-order reaction conditions are maintained, assuring that the concentration of the analyte, c_A, is proportional to the slope dA/dt of the reaction rate curve.

The principle of stopped-flow microSI is shown in the following example of an enzymatic assay of glucose based on the following reactions, where hexokinase is the primary enzyme and glucose 6-phosphate (G6P) participates in the indicator reaction:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{G6P} + \text{ADP}$$

$$\text{G6P} + \text{NADP} \rightarrow \text{6-phosphoglyceric acid} + \text{NADPH} + \text{H}^+$$

where NADPH is the species monitored at 340 nm (Fig. 7). Note that increasing glucose concentration is correlated with increasing reaction rate,

while the blank value with elevated absorbance value has a zero slope. Since the assay result is based on the slope of the reaction rate and not on the initial absorbance value, colored samples or colored reagents that yield a background absorbance at the same wavelength as reaction products do not elevate the result of an assay. The assay cycle, lasting 60 s, was comprised of the following steps (Fig. 6B): (i) glucose sample (25 µl) was aspirated from the flow-through port (#5) into the holding coil at a flow rate of 25 µl/s, (ii) reagent (50 µl) was aspirated from reagent port (#3) at a flow rate of 25 µl/s into the holding coil, (iii) sequentially stacked zones of sample and reagent were mixed by a rapid flow reversal at 100 µl/s and positioned within the flow cell at port #2 for reaction rate monitoring, (iv) the flow was stopped for 25 s to collect data, (v) the flow cell was flushed with 250 µl of a carrier buffer solution using an accelerated flow rate of (250 µl/s). In principle, all regent-based assays can be automated in a similar way, provided that the reaction rates of these reactions are neither much slower (which will affect sampling frequency since the stopped time would have to be increased to collect sufficient data) nor much faster (in which case the reaction would be completed before reaching the detector). In the latter case, the reacted mixture is moved from the holding coil to port #2 and through the flow cell at a flow rate of 5 µl/s, yielding a peak, the height of which is proportional to the analyte concentration.

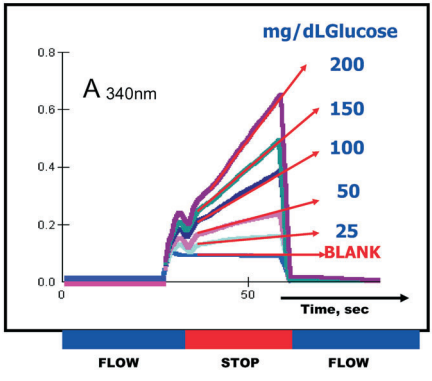

FIG. 7

A series of reaction rate curves are superimposed on this recording, showing increasing slope, due to increasing reaction rate, as correlated with increasing concentration of glucose. Reaction rate curves were recorded by means of microSI-LOV technique using spectrophotometric detection. Sample volume was 20 µl, reagent volume 50 µl, carrier solution of 250 µl per assay. (For details, see text and ref.[21])

Bead injection[17,21,30] can be viewed as a reagent-based assay carried out in SI mode, whereby the reagent is immobilized on the surface of a solid phase, which is in the form of micropspheres (typical bead size: 30 to 200 μm in diameter). The assay protocol (Fig. 8A) comprises the following steps: (i) injection of precisely metered volume of beads into a flow channel, where they are captured in a well defined geometry, (ii) injection of a metered volume of sample, (iii) perfusion of the bead column by the sample zone, while analyte molecules are retained or a reaction occurs at the surface of the beads, (iv) beads are discarded from the conduit. BI assays can be carried out in numerous, not yet fully exploited ways that fall into three broad categories. The first (A) involves monitoring analytes as they are captured on the beads. This approach is shown in Fig. 8B, where the bead column is trapped within a flow cell and monitored by UV-VIS spectroscopy. The second approach (B) involves monitoring analytes after elution from the beads. While the first approach can be viewed as "on-column spectroscopy", the second can be viewed as miniaturized chromatography carried out on a renewable micro column. The third approach (C) is based on the incineration of beads flushed from the column into a graphite furnace and subsequent AA detection.

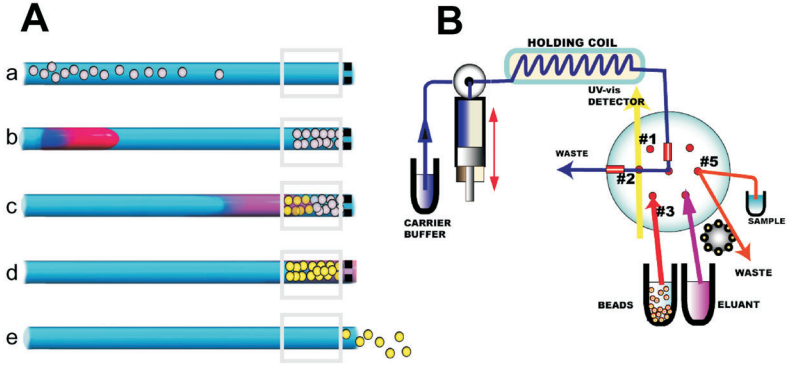

FIG. 8

A Principle of bead injection, operated by programmable flow: beads are injected into the flow channel where they will be retained in a form of microcolumn (a), sample is injected (b), and perfused through microcolumn (c), while target analytes are captured on bead surfaces (d). Finally, beads are automatically discarded (e). (From ref.[21] with author's permission.) **B** MicroSI-LOV apparatus adopted to bioligand interaction assay, where beads are captured within a flow cell and monitored by UV-VIS spectroscopy. (For details, see text and ref.[21])

Bead injection spectroscopy was originally proposed for the study of stimulant–receptor interactions aimed at drug discovery. Chinese hamster ovary cells with an appropriate receptor were grown on Cytodex® beads that were held within the observation field of a fluorescence microscope and stimulated in SI mode by various stimulants, while the release of calcium within the cells was monitored by fluorescence microscopy[31]. Later, in LOV format, Sepharose® beads furnished with protein A or protein G as immobilized ligand were used to capture antibodies, while continuously monitoring the bead layer by UV-VIS fiber optics spectroscopy in order to study the binding of IgG on the beads[16,32]. Termed, bioligand interaction assay (BIA), this approach serves as a tool for immunoassays and for ranking of affinity of proteins by capturing them with ligands attached to the solid phase. The central issue of the BI technique for BIA applications is a reliable manipulation of bead suspension, as the beads need to be precisely metered and transported through a system of channels and into a flow cell, where binding takes place. Bead transport and metering are accomplished by means of flow acceleration, at flow rates up to 200 μl/s, moving the beads at high linear velocities[21].

Bead injection and atomic spectroscopies. Enhancement of the performance of atomic spectroscopies by BI was proposed by Hansen and Wang and documented in a series of innovative research articles[22–29]. The need for matrix removal and analyte preconcentration prior to spectroscopic detection was elegantly resolved through automated solution handling. In their seminal paper, the authors selected Ni as a model element and demonstrated two ways in which BI can be used as a "front end" to AA and ICP instrumentation. In the first approach, the target element was captured on a microcolumn of beads that were subsequently transferred into a graphite furnace and incinerated. The second approach was based on elution of the target element, followed by atomization of the eluate. In both cases the microcolumn, comprising Sephadex® C-25 cation-exchange beads, was automatically renewed for each assay by BI technique carried out in a "lab-on-valve" manifold. The transfer of Ni from the LOV manifold to the graphite furnace was facilitated by sandwiching the beads (or the eluate) between two air bubbles, which prevented dispersion of the transported material. Selectivity of the assay in the presence of Pb(II), Zn(II), Co(II) and Mn(II) was improved up to 10–50-fold and the linear calibration range was extended to cover the range of 0.02–1.20 μg Ni/l. The authors expanded their method by using hydrophilic and chelating beads for preconcentration of heavy metals for subsequent detection by AA or ICP. A similar method, based on bead incineration in ETAS instrument, was developed by

Ampan et al.[33] and applied in trace analysis of lead, using Sephadex® beads impregnated with dithizone.

Sequential injection chromatography (SIC) was conceived by Šatínský et al.[34], and demonstrated with the assay of pharmaceutical compounds separated on monolithic reversed phase columns. In contrast to HPLC, which employs conventional particle columns and uses high-pressure pumps to force mobile phase through the column, the first work on SIC used porous silica rods termed "monoliths". Monolithic columns, made by the sol-gel process, exhibit low flow resistance and a sufficient number of theoretical plates to provide separation efficiency suitable for resolution of closely related compounds. The SIC experimental setup (Fig. 9) comprised a syringe pump, multiposition valve, and a reverse-phase monolithic column, placed between the valve and the flow cell connected to a UV-VIS detector. Unlike conventional chromatographic techniques, SIC is based on *programmable flow of the mobile phase*, which allows convenient selection of injected volumes of sample and eluant. As eluant disperses into a carrier stream, it forms a concentration gradient that elutes analytes sequentially from the stationary phase. The assay protocol comprises six stages: 1) column conditioning, 2) sample injection, 3) retention of analytes and elution of matrix materials, 4) gradient formation, 5) analyte elution and detection, and 6) column purification. The advantage of SIC format is that flow rates can be individually tailored to accommodate needs of each stage of the assay

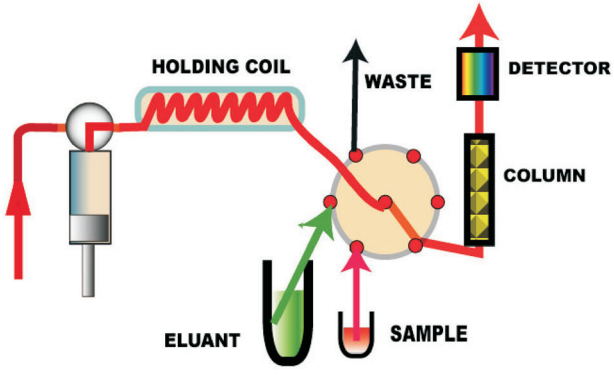

FIG. 9

Apparatus for sequential injection chromatography. Syringe pump propels mobile phase through holding coil, multiposition valve, column and into a UV-VIS detector cell. Flow reversals are used to meter sample volume and to aspirate eluent that forms elution gradient within the holding coil. (For details, see text and refs[31–37], reproduced from ref.[21] with author's permission.)

protocol. Thus steps 1 and 6 can be carried out at high flow rates, while steps 3 and 4 are carried at low flow rates. In their seminal work[34], the authors used an isocratic gradient for separation of methylparaben, propylparaben, sodium diclofenac and butylparaben from a pharmaceutical preparation using Chromolith® SpeedRod Column, equivalent to a C-18 column filled with 5 μm particles (Fig. 9). In their ensuing work, yet another type of stationary phase, called restricted access material (RAM), was used to remove interfering proteins from biological samples for subsequent assay of drugs[35]. Both RAM and classical SPE sorbents are compatible with SIC technique[36]. This approach is destined to fill the gap between HPLC suitable for resolution of complex mixtures and SPE separations carried out manually, since the SIC-SPE technique provides quantitative information in an expedient and automated fashion. For more sophisticated separations, such as separation of paracetamol, caffeine, and acetylsalicylic acid, with benzoic acid as internal standard, Solich's group recently used 4.6 mm Chromolith® column, operated in SIC mode, with acetonitrile as the eluent and UV detection at 210 and 230 nm[25]. The assay time was less than 6 min and the parameters for separation of paracetamol were: 3302 theoretical plates, peak symmetry 2.75 and retention time of 112.3 s. Reagent consumption and cost of the apparatus was a fraction of conventional HPLC [37].

Recent Developments

MicroSI affinity chromatography. Immunoaffinity chromatography, used for separation of immunoglobulins, is based on molecular recognition between a site fixed on stationary phase and a target immunoglobulin that is being captured, while unwanted matrix components, such as salts and proteins, are washed out by the mobile phase. Conventional affinity chromatography is carried out in a continuous-flow format on a stationary phase of Sephadex® or Sepharose® beads, furnished with protein A or protein G as a selective bioligand. Protein A, isolated from *Staphylococcus aureus*, has the form of a cylinder and has multiple antibody binding domains that are recognized by the Fc portion of IgG molecules. At pH of 6 or higher histidine residues become charged and thus selectively capture mammalian IgG, while at pH lower than 3, captured IgG are released. In microSIC format[18], the bead column is formed *within lab-on-valve* module, in a conduit situated between the central port of the multiposition valve and the flow cell (Fig. 10). The bead column volume is 10 μl, the flow cell volume 8 μl, and the dead volume between the flow cell and the end of the column is 0.1 μl. By using a 5 μl pulse of 0.1 M HCl, almost all IgG molecules retained on the column

are instantaneously and simultaneously eluted into the flow cell, where they are detected spectrophotometrically at 280 nm. The use of programmable flow, tailored to perform IgG on-column adsorption at a flow rate of 5 µl/s, IgG elution from the column at 2 µl/s, and column regeneration at 50 µl/s, allows completion of the entire assay within 120 s, using only 2 ml of mobile phase[18]. In contrast, traditional affinity chromatography of the same IgG, performed on the same stationary phase packed in a 1 ml column, used 30 ml of mobile phase and required 30 min to complete[38]. Future applications of microSI affinity chromatography will focus on the assay of purity of commercially available IgG and on process control of production of monoclonal antibodies by cellular cultures. The use of microSIC for DNA and RNA separations and assays, by monitoring native (at 280 nm) and/or tagged molecules (using UV-VIS spectrophotometry or fluorescence) has yet to be explored.

MicroSI titrations with spectrophotometric detection are based on measuring residual alkalinity within sequentially stacked zones of sodium hydroxide, indigo carmine indicator, and acid sample. The reaction mixture is bracketed by two air bubbles that prevent the stacked zones from dispersing into the carrier stream during mixing, which was accomplished by a single

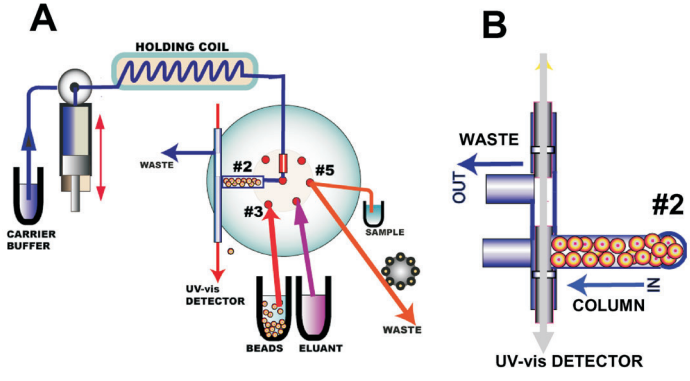

FIG. 10

A MicroSI apparatus adopted for sequential injection chromatography. A renewable microcolumn is formed within the LOV module, by injecting bead suspension at the beginning of assay cycle. Sample is aspirated via port #5 into holding coil, and perfused through microcolumn by flow reversal via port #2. Next eluant is aspirated and passed through the column, eluting target analyte into the flow cell. (Reproduced from ref.[21] with author's permission.) **B** Microcolumn is held in place by optical fiber that allows mobile phase to pass, but retaines packed beads in place. Column volume is 10 µl, flow cell volume 8 µl. (For details, see text and ref.[41], reproduced from ref.[21] with author's permission.)

flow reversal. In order to obtain a smooth response, air segments were discarded prior to absorbance measurement (at 609 nm). The method was applied to the assay of acidity in citrus juices. This innovative approach, designed by Jakmunee et al.[39] can be, in principle, extended to all acid-base titrations and possibly to redox and complexometric titrations as well, using optical or electrochemical detectors.

MicroSI hydride generation. Atomic absorption spectroscopy (AAS), inductively coupled plasma spectroscopy (ICP), and inductively coupled plasma mass spectrometry (ICP-MS) are routinely coupled with the hydride generation technique that allows traces of Hg, As, Bi, Ge, Pb, Se, Sn, and Te to be separated as volatile hydrides from interfering matrix components. Hydride generation, based on the reaction of sodium tetrahydroborate in acidic solution with a sample containing the target element, was originally designed and practiced as a batch procedure. It was Astrom[40] who proposed and demonstrated the feasibility of using FI for automation of hydride generation for AA of bismuth. His pioneering work rapidly expanded to all hydride-forming elements and a full range of atomic spectroscopies. Although reliable and widely used, continuous-flow operation has a drawback of using reagent flow rates of 10 ml/min or more. Thus, downscaling into the microSI format would have the advantages of reducing reagent and waste volumes as well as the size of the apparatus. Downscaling was accomplished by furnishing a microSI-LOV system with a gas liquid separator that was purged by a stream of air[41]. Mercury-containing samples were sandwiched between two zones of sodium tetrahydroborate solution in a holding coil, mixed by flow reversal, and transported into the separator. The stream of air carried Hg vapors into the flow cell where absorbance was measured at 254 nm[40]. The use of microSI mode and programmable flow reduced reagent consumption to 100 µl per assay. The system is, in principle, applicable to the assay of other hydride-forming elements by detection with AA or ICP-MS.

CONCLUSION

Solution handling in analytical laboratory has undergone, within last 100 years, profound change, both conceptually and technologically. Following the concept of gravimetry, the development of volumetric and colorimetric techniques adopted the same principle, i.e. quantitative conversion of a target analyte into a measurable product. While technological advances gradually produced sophisticated tools, such as glassware, electrochemical and spectroscopic instrumentation, and, during 1920's, the first generation of

electronic laboratory instrumentation (such as the Beckmann pH meter), all analytical techniques have continued to operate on the principle of quantitative conversion, which inevitably necessitated that reactions reach chemical equilibrium. It was Heyrovský, a physical chemist, who was the first one to base a quantitative assay on *dynamic equilibrium*, controlled by diffusion, whereby only a miniscule quantity of analyte was converted to a measurable species and sensed by electrode[42,43]. During the following years, other methods of instrumental analysis gradually accepted the principle of dynamic equilibrium as a basis for a readout that was, through calibration, converted to a quantitative result.

In contrast, the concept of solution handling remained anchored in the principle of *homogenous* mixing of samples with reagents. This "batch-mode" concept was, surprisingly, also the centerpiece of Skeggs continuous-flow analyzer, in which air segmentation was used to separate liquid segments, each of them being homogenously mixed by liquid circulation promoted by wall friction[5,6] and where the readout was obtained at "steady state", i.e. at equilibrium conditions. It was the advent of FIA that introduced the concept of a *concentration gradient* of analyte, *dispersed* within a carrier stream of reagents, that exploited *nonequilibrium* conditions as a basis for a calibration process. And it is sequential injection technique that, by expanding this principle to complex gradients composed of sample and reagent zones, allows automated sample processing in microscale. The result is miniaturization of apparatus, time and reagent savings, and applicability of programmable flow to a wide variety of assay protocols that open the way for yet unforeseen innovations of instrumental analysis.

What we learn from a brief overview of inventions related to analytical chemistry is how the time gap between invention, proof of a concept, and its acceptance by analytical community has decreased. It took 25 years before Tswett's invention of chromatography (in 1902) was used again in 1931 by Kuhn for separation of carotenoids[44], and yet another 10 years until Martin and Synge proposed and developed partition chromatography[45]. In a similar way, Heyrovský's discovery of polarography, first reported in 1922, and his subsequent invention of the polarograph with Shikata, in 1925, did not attract much interest internationally until 1933 when Heyrovský traveled to the United States for the first time and the trademark "Polarograph" was registered in U.S. Patent Office[46]. Even more recently, when Walsh invented atomic absorption in 1952, to use his words, there was "almost complete lack of interest in our work over the next few years". Four years later, "the method was scientific curiosity rather than a practical ana-

lytical method" and by 1958 "there was no sign of any instrument manufacturer willing to produce the type of instrument we thought desirable"[47].

Flow injection analysis suffered a similar fate, although the lag time from its invention to acceptance was less than 5 years[9,27]. Sequential injection and its miniaturized version were accepted even faster. The first conclusion that can be drawn is that the flow of information, accelerated by e-technology, assists in faster dissemination of new ideas and in gathering the tools and materials needed to bring new concepts to fruition more quickly. The second is that one should not be discouraged, by initial lack of interest or skepticism by others, from pursuing an idea. The last, rather sobering conclusion, is that unless a new idea is not widely accepted within, say, ten years, it is unlikely to succeed and its funding becomes questionable[48].

It is important to emphasize that flow-based techniques, including programmable flow highlighted in this review, are not "better" than batch-mode handling of solutions. Obviously, each approach has its advantages and drawbacks. What is remarkable is the impact of FIA and its modifications (SI, BI, SIC) on the academic community, which has contributed enormously to their development and widespread use. According to recent reviews by Hansen at end of 2003 [9,27], about 14,000 articles on flow injection techniques have been published in many different journals, mostly in English, and at an increasing rate in a multitude of languages, especially Chinese[9,49]. Last year, in Japan, the Journal of Flow Injection Analysis celebrated its 20th year in existence, during which presidents of the Japanese Association for Flow Injection Analysis, Professors Ishibashi, Ohkura, Motomizu and Sakai also served as the journal's Chief Editors[50]. While the number of FIA publications initially showed an exponential growth, in recent years the publication rate has stabilized at a level of 1000–1100 papers per year. The Ph.D. theses and book chapters have yet to be counted. To date, over 10 monographs dealing with various FIA applications have been published[51–65]. Without a doubt, FIA has stimulated interest in the ways solutions can be handled in a chemical laboratory, and it is my hope that examples of applications of programmable flow highlighted in this review will inspire many to carry out research in this exciting field of instrumental analysis, where is much more has yet to be discovered.

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