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New approaches to coupling flow-injection analysis and high-performance liquid chromatography

M. D. Luque de Castro* and M. Valcárcel

Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, 14004 Córdoba (Spain)

ABSTRACT

An overview of the advantages gained in coupling a flow-injection manifold to a liquid chromatograph is presented. Improvements in the analytical features arising from this association and the peculiar pre- and postcolumn arrangements are discussed, as are the promising prospects of arrangements to be developed for avoiding the preliminary steps of the analytical process.

INTRODUCTION

Flow-injection analysis (FIA) [1,2] and high-performance liquid chromatography (HPLC) are the two most promising hydrodynamic techniques. The instrumentation used in both is remarkably similar; in fact, both use analogous liquid reservoirs, propulsion systems, injectors and continuous detection systems (whether optical, electroanalytical or otherwise). The greatest difference between the modules or units required for each lies in the presence of the separation column, which is essential to HPLC and dispensable in FIA. Other operational differences between these techniques are the working pressure, which is high in HPLC and low in FIA (this accounts for the high cost of HPLC compared with FIA systems) and the presence of a liquid–solid interface, which is always used in HPLC but only occasionaly in FIA [3]. However, the most significant difference perhaps lies in the main analytical goal of each technique, namely the discrimination and/or determination of several components in the same sample in HPLC, and the determination of one of a few analytes in many samples in FIA.

In relation to batch and segmented flow methods, FIA methods are more selective as a result of the kinetic nature of FIA measurements. On the other hand, FIA methods are less sensitive as a result of both the dilution or dispersion of the sample into the carrier stream and the lack of completion of the chemical derivatization reaction involved. Hence FIA manifolds are frequently coupled on-line to a separation or preconcentration system to improve the sensitivity of a given method when low analyte concentrations are to be determined. On the other hand, HPLC methods also require their sensitivity and selectivity to be improved in many instances. This can be accomplished by developing (bio)chemical derivatization reactions in pre- or postcolumn arrangements [4,5] and/or by coupling the HPLC system on-line to a non-chromatographic separation system (liquid–liquid extraction, ion-exchange, etc.) in a precolumn arrangement [6]. Both approaches can be implemented by using a flow-injection manifold which can be coupled on-line to the chromatograph in a pre- or postcolumn position, thus increasing the potential of the derivatization, preconcentration or separation steps. Coupling these two techniques therefore increases their individual potentials through a synergistic effect.

Coupled FIA–HPLC systems are usually intended to improve on such basic features of the analytical process as sensitivity, selectivity, precision, human participation, rapidity, cost, etc.

The features of a given FIA–HPLC system depend on whether the two techniques are coupled in a pre- or postcolumn arrangement. Thus, in precolumn couplings, the FI system is placed before the

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liquid chromatograph, which includes the detection module. Strictly, this is not an FI system proper as it lacks its own detection module. In postcolumn couplings, the chromatographic process takes place before the FIA step. In this instance, the detector is inserted into the FI manifold; hence, strictly again, the chromatograph is not used as an instrument as it has no detection system. Different arrangements offer specific advantages that warrant selection for specific applications.

In this paper we discuss the advantages of FIA–HPLC coupled systems as used in some recently developed methods using pre- and postcolumn arrangements. Unexplored possibilities which could be of interest for special applications are also commented on.

PRECOLUMN FIA–HPLC ARRANGEMENTS

The specific objectives of precolumn arrangements are automation of sample clean-up and/or preconcentration steps, automatic implementation of derivatization reactions, saving of reagents and direct introduction of troublesome samples (solid, viscous, heterogeneous) into FI systems.

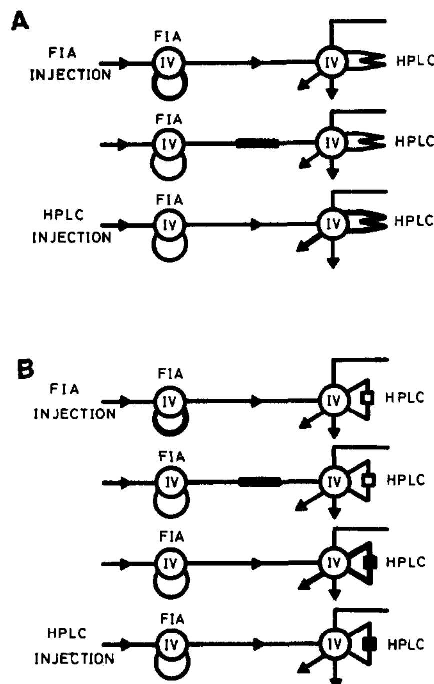

Fig. 1. Two different ways of synchronizing the functioning of the FI and HPLC systems: (A) by trapping the injected sample plug in the loop of the HPLC injection valve, and (B) by using a precolumn in the loop of the HPLC valve. IV = Injection valve.

Precise synchronization of the functioning of the FI manifold and the chromatograph is of paramount importance in precolumn arrangements, which can be accomplished in the two ways shown in Fig. 1. In mode A, the sample plug injected through the FI valve passes through the loop of the HPLC valve, which is then switched to introduce the plug into the column. The second mode involves retaining the analyte injected into the FI system in a precolumn placed in the loop of the high-pressure injection valve of the chromatograph. Large sample volumes can also be continuously aspirated.

There are several possible precolumn FIA–HPLC arrangements and those most frequently used are depicted in Fig. 2.

The merging-zones approach (Fig. 2A) can be very useful for saving expensive or scarce reagents as in chiral separations, which call for expensive derivatization reagents. The basic objective in this instance is to avoid the use of such reagents in the mobile phase. The FI manifold can include a solid reactor (Fig. 2B), whether enzymatic, redox, ion exchanger, etc., depending on the particular purpose. This kind of arrangement has rarely been used in precolumn FI arrangements. An example is the determination of zinc based on its activating effect on immobilized metal-free carboxypeptidase A. The FI valve is used to insert the analyte sequentially into a water stream in order to activate the enzyme first (immobilized on controlled-pore glass and filling a reactor placed between the FIA and the HPLC injection valves) and then the substrate (hippuryl-L-phenylanaline) in order to assess the enzyme activity as its decomposition product is determined spectrophotometrically at 228 nm after the chromatographic separation. The metal-free enzyme is regenerated by pumping 1,10-phenanthroline through the reactor between successively processed samples. This step is performed with the aid of a selecting valve placed before the injection valve which allows the water carrier to be replaced with the regenerating solution [7].

The on-line coupling of a non-chromatographic separation technique (dialysis, gas diffusion, liquid–liquid extraction, etc.) to an FI manifold (Fig. 2C) results in indirectly enhanced sensitivity and selec-

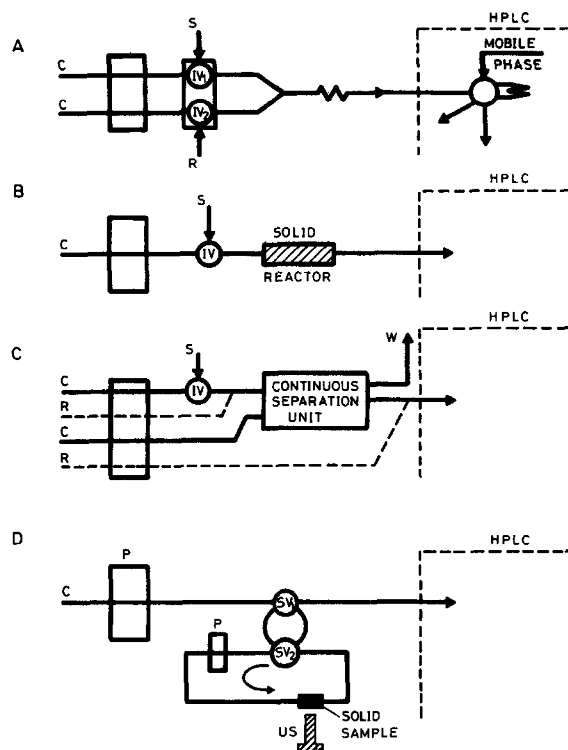

Fig. 2. Generic precolumn FIA–HPLC arrangements. (A) Merging-zones approach; (B) use of a manifold including a solid reactor; (C) on-line coupling of a non-chromatographic continuous separation technique to an FI configuration; (D) use of ultrasonic radiation on a solid sample placed in a special cell in the FI manifold. C = Carrier; R = reagent; S = sample; IV = injection valve; SV = selecting valve; US = ultrasonic probe; W = waste.

tivity and hence in substantial improvements in the subsequent chromatographic separation. An example is the coupling of a liquid–liquid extractor to a normal-phase chromatograph for the determination of caffeine. The sample is injected into an aqueous carrier and then segmented with methylene chloride. The separated organic phase is fed to the normal-phase chromatograph via a precolumn in order to preconcentrate the sample. This method has been successfully applied to real samples such as beverages and urine [8].

A special cell can be used to hold a solid sample for irradiation with ultrasound in the FI manifold (Fig. 2D) in order to enact a solid–liquid extraction process known as "lixiviation" or "leaching". This is a promising application of precolumn coupling aimed at automating the first step of the analytical process [9]. A manifold such as that shown in Fig. 2D has been used for the determination of boron in soils. An amount of 5 mg of sample is placed in a small reaction cell (1.5 mm × 1 mm I.D.). In the leaching step, 300 μl of 0.1 *M* HCl leaching carrier held in the closed circuit is passed through the sample for 5 min. As switching valves SV_1 and SV_2 are simultaneously actuated, the carrier drives the contents of this circuit to the FI manifold, where they are merged with a buffer–masking solution containing the reagent, namely azomethine H. A sharp peak is obtained as a result, the height of which is proportional to the concentration of boron in the solid sample. The results obtained are consistent with those provided by the conventional manual method, which is tedious and time consuming; about 2 h are necessary to perform the manual leaching step, which involves an ammonium acetate solution as leaching agent, whereas up to 30 samples per hour can be processed by the FI method [10].

POSTCOLUMN HPLC–FIA ARRANGEMENTS

Some HPLC workers consider any postcolumn derivatization system to be an FIA system, which is incorrect when the only valve included in the overall system is the high-pressure injection valve of the chromatograph. In fact, only those systems which include two valves should be considered to be true HPLC–FIA configurations. Examples are two of the applications recently developed by our group, namely the twofold use of an FI system and the coupling of a liquid chromatograph to an open–closed circuit. The chief objectives of these approaches were enhanced sensitivity, avoidance of the permanent use of a liquid chromatograph in routine analyses and implementation of continuous monitoring of reaction rates of separated analytes.

The use of the same FI configuration for two different purposes allows the implementation of new, promising methods aimed at avoiding permanent usage of HPLC for monitoring large numbers of samples. The principles behind this approach are illustrated in Fig. 3. Routine monitoring of the total analyte content (*e.g.*, toxic substances) is achieved with the FI manifold. A large number of samples are injected and, within a few hours, it is possible to

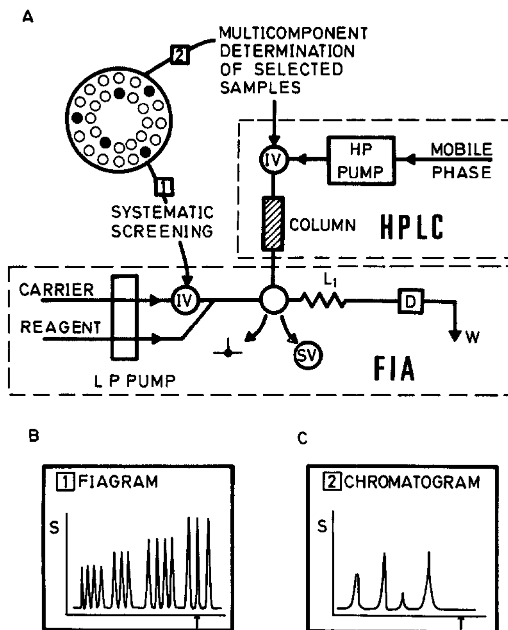

Fig. 3. (A) Twofold use of an FI manifold as a screening system and a continuous postcolumn reaction–detection system. (B) Recordings obtained in routine monitoring. (C) Chromatogram of the discriminated analytes in the selected samples. HP = High pressure; LP = low pressure; IV = injection valve; SV = selecting valve; L = reactor; D = detector; W = waste; S = instrumental signal; T = time.

select those with potentially high contents of toxic substances among 200–500 of them. When this overall determination is finished, only the selected samples are injected into the HPLC system, which uses the same FI configuration as a postcolumn reaction–detection system. In this way, the HPLC system is only used for a small number of samples, which is of practical relevance from an economic point of view. The recordings obtained in the routine monitoring (fiagrams) and in the discrimination of the analytes in the selected samples (chromatograms) are also shown in Fig. 3. This approach has been tested on several analytical problems.

One such problem is the determination of aflatoxins based on enhancing their fluorescence by means of a redox reaction with bromine. In the overall determination, all the samples are injected through the FI valve while the chromatograph remains at rest. Discrimination between the analytes in contaminated samples is accomplished by injecting the selected samples via the chromatograph, the FI system thus acting as a continuous postcolumn reaction–detection system. The results obtained by applying this procedure to foodstuffs (peanut, maize, etc.) show the usefulness of this HPLC–FIA approach [11].

One clinical use of this type of arrangement is the determination of bile acids in serum. Healthy individuals feature total bile acid levels below 1 mg/ml, so higher levels are indicative of liver complaints. The ratios between the concentrations of different bile acids allow specific diseases such as cirrhosis, jaundice and cholesterosis to be diagnosed. Thus, samples from a large hospital are first screened through the FI system in order to select those containing bile acid levels higher than 1–2 mg/l, and only those need be analysed by HPLC, by injecting them through the high-pressure injection valve of the chromatograph, which uses the same FI configuration as a postcolumn reaction detection system. The analytical indicator reaction employed for this purpose is the oxidation of the 3-hydroxy group in the acids by NAD^+, catalysed by 3-α-hydroxy-steroid-dehydrogenase which is used immobilized on controlled-pore glass. The NADH formed in this reaction is monitored fluorimetrically. In this instance, the switching valve (SV) allows the FI system to be isolated from the HPLC system. The former is thus a conventional flow-injection system furnished with a packed enzyme reactor located between the injection valve and the flow cell (L_1 in Fig. 3). In the discriminating determination, the selected samples are inserted through the HPLC valve. The switching valve allows the continuous introduction of the chromatographic eluate into the FI derivatization–detection system. In this way, it is possible to discriminate between bile acids in less than 12 min by careful optimization of the experimental conditions of the combined system. This approach has been successfully applied to serum samples from healthy and sick individuals with excellent results [12].

Coupling of a liquid chromatograph to an open–closed circuit is another example of the use of a true FI system coupled on-line to a liquid chromatograph in a postcolumn arrangement. The operational scheme of this combined system [13] is shown in Fig. 4. The switching valve (SV) allows the first-eluted analyte to be trapped in the circuit (2),

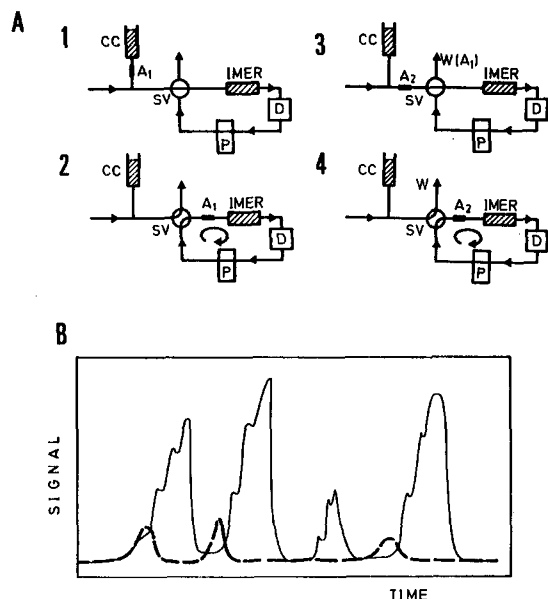

Fig. 4. (A) Operational scheme of a combined HPLC–open–closed FIA arrangement. (B) Normal chromatogram obtained with a conventional continuous postcolumn derivatizing system [valve SV is always kept in the open position (dashed line), and atypical multi-peak chromatogram obtained by using the open–closed postcolumn arrangement. CC = Chromatographic column; SV = selecting valve; P = low-pressure pump; A = analyte; D = detector; IMER = immobilized enzyme reactor; W = waste.

which includes a detector and an enzyme reactor if required. The iterative passage of the sample through the detector is repeated as many times as required; then, the valve is switched to flush the circuit (3) and introduce the next-eluted analyte, which is also trapped in the circuit (4), and so on. A multi-peak recording per analyte rather than a single HPLC peak is obtained (Fig. 4B), which can be compared with the ordinary chromatogram obtained under the same working conditions. Two practical conclusions can be drawn from these recordings: reaction-rate measurements on each analyte can be performed in a continuous fashion by using the distance between maxima or minima whether consecutive or not; and the sensitivity is clearly enhanced. Note that some species yield no peak in the ordinary chromatogram. Good results in this respect are only obtained if the delay time between successively eluted analytes is long enough (at least 1 min) and a microcomputer is used to synchronize the operation of the HPLC injection valve and the switching valve.

This association has been used for the individual determination of creatine isoenzyme activities based on three consecutive reactions. In a first step, the phosphate group in creatine is transferred to ADP in a reaction catalysed by the analyte enzyme. The other two steps involve auxiliary enzymes (hexokinase and glucose-6-phosphate dehydrogenase) immobilized on controlled-pore glass and make up the indicator reaction, which yields the monitored product, NADPH, the reduced form of the coenzyme, which is monitored spectrophotometrically at 340 nm. This methodology has been successfully applied to the determination of these isoenzymes in biological fluids with excellent results [14].

CONCLUSIONS

The development of automatic analytical procedures is one of the main objectives of today's analytical chemistry to respond to the increasing demands for chemical information by a society in frantic evolution [15]. Combined FIA–HPLC arrangements can be regarded as a way of solving real analytical problems in routine laboratories handling large numbers of samples and experiencing problems with conventional HPLC approaches, and interested in the reduction of human participation in analytical processes. Direct introduction of solid or heterogeneous samples, the twofold use of an FIA manifold as a screening system and a postcolumn reactor–detector are the most promising alternatives in this context. The use of postcolumn (bio)chemical FIA sensors based on integrated detection–reaction and/or separation in the flowcell is an interesting trend, as inferred from the first few attempts performed in our laboratories.

REFERENCES

1 M. Valcárcel and M. D. Luque de Castro, *Flow Injection Analysis: Principles and Applications*, Ellis Horwood, Chichester, 1987.
2 J. Ruzicka and E. H. Hansen, *Flow Injection Analysis*, Wiley, New York, 1988.
3 M. Valcárcel and M. D. Luque de Castro, *Non-Chromatographic Continuous Separation Techniques*, Royal Society of Chemistry, Cambridge, 1991.
4 I. S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986.

5 H. Lingeman and W. J. M. Underberg (Editors), *Detection-Oriented Derivatization Techniques in Liquid Chromatography*, Marcel Dekker, New York, 1990.
6 K. Zech and R. W. Frei (Editors), *Selective Sample Handling and Detection in High-Performance Liquid Chromatography*, Parts A and B, Elsevier, Amsterdam, 1988 (Part A) and 1989 (Part B).
7 J. L. Burguera, M. Burguera and A. Townshend, *Anal. Chim. Acta*, 127 (1981) 199.
8 J. J. Halvax, G. Wiese, W. P. van Bennekom and A. Bult, *Anal. Chim. Acta*, 239 (1990) 171.
9 M. Valcárcel and M. D. Luque de Castro, *Automatic Methods of Analysis*, Elsevier, Amsterdam, 1988.
10 D. Chen, F. Lázaro, M. D. Luque de Castro and M. Valcárcel, *Anal. Chim. Acta*, 226 (1989) 221.
11. F. Lázaro, M. D. Luque de Castro and M. Valcárcel, *J. Chromatogr.*, 448 (1988) 173.
12 A. Membiela, F. Lázaro, M. D. Luque de Castro and M. Valcárcel, *Anal. Chim. Acta*, 249 (1991) 461.
13 A. Ríos, M. D. Luque de Castro and M. Varcárcel, *Anal. Chem.*, 57 (1985) 1803.
14 M. D. Luque de Castro and J. M. Fernández-Romero, *Anal. Chim. Acta*, in press.
15 H. M. Kingstom, *Anal. Chem.*, 61 (1980) 1381A.