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Post-column reaction detection and flow injection analysis

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ABSTRACT

Post-column derivatization with chemical reaction detection (CRD) and flow injection analysis (FIA) use the same principle, *viz.* reaction in a flow system. For CRD, dispersion must be minimized so as to maintain the separation achieved. In FIA, dispersion is required to mix the reagent and solute. It has been demonstrated that by using low-dispersion knitted capillaries and various modifications of the FIA principle (forced mixing in a branched system; inverse FIA; plug injection of solute and reagent in an inert carrier), the detection limit can be improved. In plug injection, one can also use coloured reagents and solutes to reduce reagent consumption.

INTRODUCTION

Continuous-flow analysis in open tubes is of increasing interest for process monitoring and control and for the colorimetric identification of solutes. Starting from identical fundamentals, two very similar analytical systems have been developed in parallel, varying only in their purpose:

(1) Chemical reaction detection (CRD) [1] for the derivatization of chromatographically separated solutes is intended to improve detection selectivity, specifity and sensitivity while preserving the chromatographic separation achieved. The axial dispersion (mixing), which causes additional peak broadening (impairing the separation) and hence a reduction in peak height (detection sensitivity), must be minimized during derivatization in the reaction coil.

(2) In flow injection analysis (FIA) [2], the sample is injected into a stream of reagent. A certain dispersion (axial mixing, band broadening) is required to mix sample and reagent so as to start the derivatization reaction.

Although the two methods have been developed separately and independent of each other, the basic principle of both methods is the same, namely the use of open tubes holding the sample and reagent during derivatization. The main difference is that in CRD axial dispersion is undesirable, whereas it is required in FIA. In both instances, quantitative reaction is unnecessary, because the reaction time is determined by both the flow-rate and the length of the reaction tubes. The aim of this paper is to compare the principles of CRD and FIA and to point out synergistic possibilities for both methods.

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CHEMICAL REACTION DETECTOR

CRD is applied in high-performance liquid chromatography (HPLC) for the selective and sensitive detection of solutes in complex matrices or for the sensitive detection of solutes without a chromophore or fluorophore, such as sugars and amino acids. The advantage of this method, especially for multi-functional solutes, where complete and quantitative precolumn derivatization is difficult to achieve, is that they can be separated as such.

In a typical experimental set-up for post-column detection, the reagent is added continuously to the effluent from the chromatographic column. An additional pump is required, which should be able to deliver the sometimes very corrosive reagents continuously and free from pulsations with great precision. The use of a cyclone-type mixer [3,4] with an extremely small dead volume (10 nl) is advantageous. Cyclone mixers have the additional advantage that a relatively small reagent flow (as little as 1/25th of the eluent flow) can be applied, in contrast to the 1:1 flow-rate required with conventional mixers with T- or Y-connections. This helps to improve the detection sensitivity, because the inherent dilution of the solute peak with reagent is minimized.

The most important part of the experimental set-up is the reactor, where the effluent and reagent must be held for the time required for the reaction to proceed. Principally three different types of reactors have been applied: (a) open tubes with large diameters (>1 mm) and segmentation of the flow, either by air or by an immiscible liquid; (b) packed columns; and (c) narrow, open tubes (<0.4 mm), geometrically deformed [3].

The advantages and disadvantages of these three types of reactors have been discussed in detail [5], so only a brief summary will be given here. With increasing column efficiency the peak volumes in HPLC have been drastically reduced (10–100 μl). Consequently, reactors with flow segmentation cannot be used in HPLC without vitiating the separation achieved in the column. Only for extremely long reaction times (>5 min) may their application have some advantage.

Packed reactors are inert chromatographic columns. The efficiency of both should be identical. Consequently, the reactors must be packed with particles of a diameter similar to that of the chromatographic column. The additional back-pressure may also be a limitation and particulate matter precipitating from the reagent may plug the reactor. The use of packed-bed reactors for heterogeneous catalysis is advantageous.

When open tubes are used, the formation of a parabolic stream profile, causing peak broadening, must be prevented. This can be achieved by segmentation, but more efficiently by deforming the capillaries geometrically either by coiling, knitting or stitching. With straight, open tubes, the peak broadening, measured as the H value, increases linearely with linear eluent velocity. This curve can be described by the Aris–Taylor equation [6,7], correlating effective dispersion in open tubes with tube diameter and flow velocity. By coiling the tube, the effective dispersion becomes smaller the tighter the tube is coiled. The most efficient reduction of dispersion is obtained with "knitted" tubes [3–5]. Here, tight coiling and a change of flow direction after each half circle introduces artificial radial mixing, thus preventing the formation of a parabolic flow profile. The induced secondary radial flow increases the back-pressure with a knitted tube by a factor of 2–3, compared with the theoretical value for

straight tubes, calculated via the Hagen–Poisseuille equation. Of course, if turbulent flow were reached here, a much higher pressure would have to be applied.

Comparison of the efficiency of packed reactors and knitted open tubes (KOT) showed that a packed-bed reactor with 5–10-μm particles gives the same peak broadening (measured as volume variance per unit reactor volume) as a KOT with 0.34 mm I.D. [5]. Both reactors could be coupled to a 5-μm column without noticeable destroying the separation, as can be seen by comparing the chromatograms in Fig. 1.

KOT reactors of I.D. 0.25–0.35 mm and standard length of 10 m permit reaction times of up to 90 s at the usual chromatographic flow-rates of 0.5–1 ml/min, and are compatible with the efficiency achievable with chromatographic columns packed with 5–10-μm particles. KOT reactors are also good heat exchangers. Short (less than 50

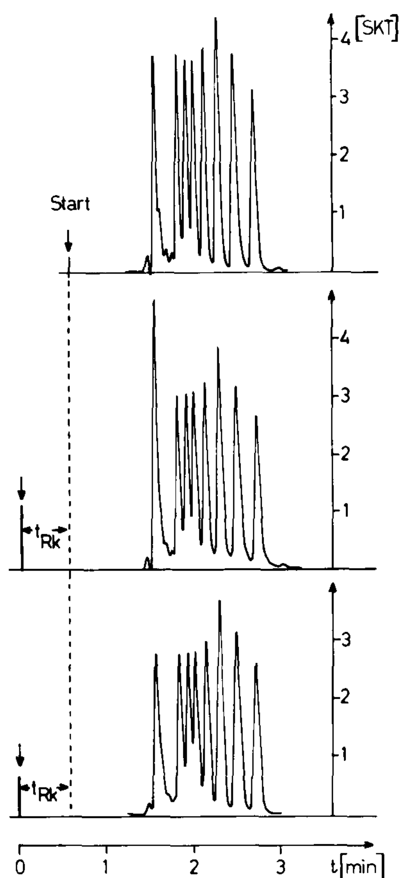

Fig. 1. Demonstration of the effect of various reactors on separation efficiency. Top chromatogram: 120 × 4 mm I.D. column packed with LiChrospher Si 60 RP-8; d_p, 4 μm; eluent, 95% aqueous methanol; flow-rate, 1 ml/min; pressure, 87 bar. Samples: inert = nitromethane; phenylalkanes (C_1–C_6) (k' range 0–1.2). Middle chromatogram: identical conditions, plus a packed reactor (100 × 4 mm I.D., d_p 5 μm). Reaction time, 35 s; additional pressure, 35 bar. Bottom chromatogram: identical conditions, but with KOT reactor (1070 × 0.33 mm I.D.). Reaction time, 40 s; additional pressure, 9 bar.

cm) pieces are sufficient to cool the liquid to the detector temperature necessary for optimum fluorescence detection.

Applications of CRD in chromatography are manifold, starting with the classical amino acid analysis by Spackman *et al.* [9] involving ninhydrin reaction detection. By applying HPLC technology, it was possible to improve the detection sensitivity drastically. The relatively slow ninhydrin reaction was replaced with *o*-phthalaldehyde (OPA) derivatization and fluorescence detection. Nowadays, amino acid analysis in the picogram range is possible [4]. As it is not our purpose here to review the literature on CRD, only two examples will be given to demonstrate the two main advantages: the selective detection of solutes in complex matrices and the sensitive detection of solutes without chromophores.

The determination of carbamate pesticides in vegetables and water is of increasing importance. They can be detected sensitively and selectively by gas chromatography with a nitrogen-specific detector. However, sample pretreatment and carbamate extraction are tedious and time consuming and constitute inherent sources of errors. For HPLC with the CRD, sample pretreatment can be reduced to an extraction with dichloromethane, solvent evaporation and redissolution in methanol. For selective detection, two-step CRD is required [8], hydrolysis with sodium hydroxide and detection of the methylamine, the common structural group of all carbamates, which is produced, with OPA.

Sugars cannot be detected sensitively by photometry. However, they can be detected with pulsed amperometric detectors. Although matrix solutes may block the electrode surface, reducing sugars can be detected by CRD, whereas non-reducing sugars and sugar acids can only be detected with reagents such as thymol and concentrated sulphuric acid. It has been shown [10] that sensitive detection of trace amounts of sugars and sugar acids in wine can thus be achieved.

FLOW INJECTION ANALYSIS

With FIA, solute derivatization also takes place in a stream of reagent. The instrumentation resembles that for CRD. Typically, peristaltic pumps are used in FIA. Because the pressure drop is more limited, wider capillaries must be used, usually of 1 mm I.D. The solute is injected into the streaming reagent. In contrast to stationary colorimetric reactions, but common to CRD, the reaction does not have to go to completion. Because the solute is injected as a plug into the reagent, mixing must occur during transport through the tube. Consequently, the reaction does not start immediately with the whole sample. The laminar flow in the capillary leads to the parabolic Hagen–Poiseuille profile. Mixing of reagent and sample is caused by concentration differences at the front and rear ends of the solute plug. This axial dispersion is essential in FIA to start the derivatization reaction. Mixing of sample and solute occurs in the capillary. Consequently, there is a limitation to the applicable sample volume. With large sample volumes in the middle of the solute plug no reaction may occur. This is demonstrated in Fig. 2, where various solute volumes were injected. Only up to solute volumes of 20 μl was a strict linearity of peak height and peak area observed. At sample volumes of 50 μl and above mixing is incomplete. This leads to peak splitting at sample volumes above 100 μl. Here, reaction takes place only at the two ends of the solute plug.

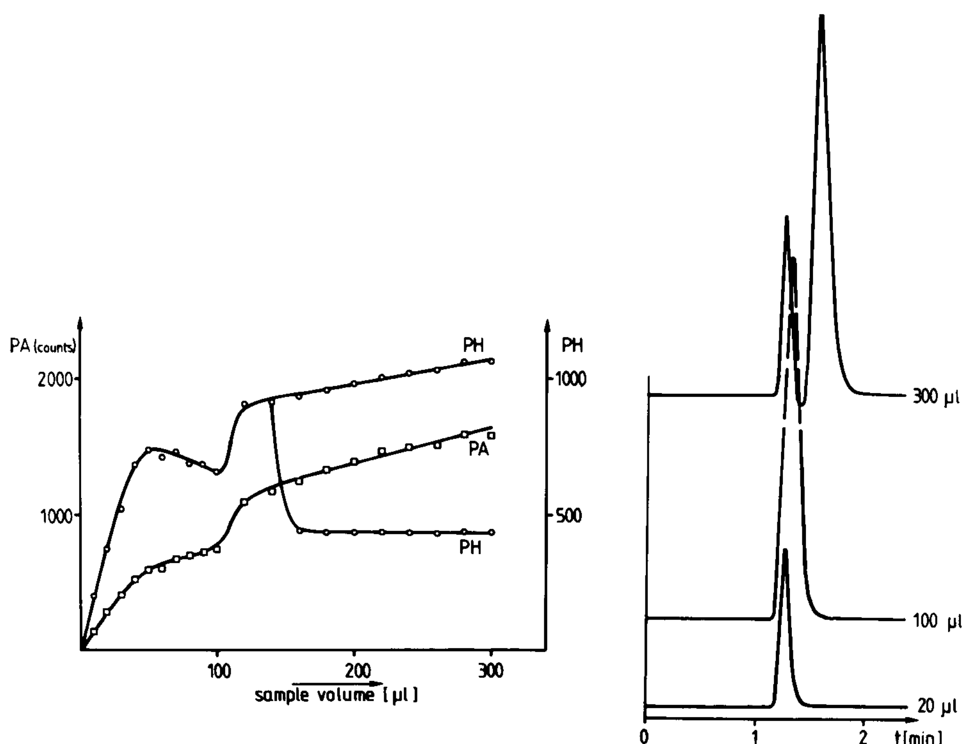

Fig. 2. Effect of sample volume on quantification by conventional FIA. Determination of formaldehyde with acetylacetone and ammonia forming dihydrolutidine [12]. PA = peak area; PH = peak height.

RESULTS AND DISCUSSION

Impact of CRD on FIA

The problems discussed here can be prevented if a FIA system identical with a CRD system is used. The solute is introduced into a carrier stream (in the simplest case, pure water) and the reagent is added by a mixing device. The principle of this set-up is compared in Fig. 3 with a classical FIA system. With this branched FIA system (BFIA), complete reaction with the whole sample volume can be achieved even if it is large, and a linear calibration graph of peak area could be obtained, as shown in Fig. 4. It should be mentioned that much larger areas have been measured for sample volumes above 50 μl than with the classical FIA set-up. With this system, low-dispersion capillaries, such as KOT, with smaller diameters can be used, because dispersion is no longer required to mix the solute and reagent. Hence better detection sensitivity can be achieved.

Another advantage of BFIA is the possibility of displacing the inevitable refractive index peak caused by the injection pulse from the signal, which usually causes problems in quantification at low detection levels. With the set-up shown in Fig. 3, the injection pulse is separated from the sample peak by the short capillary between

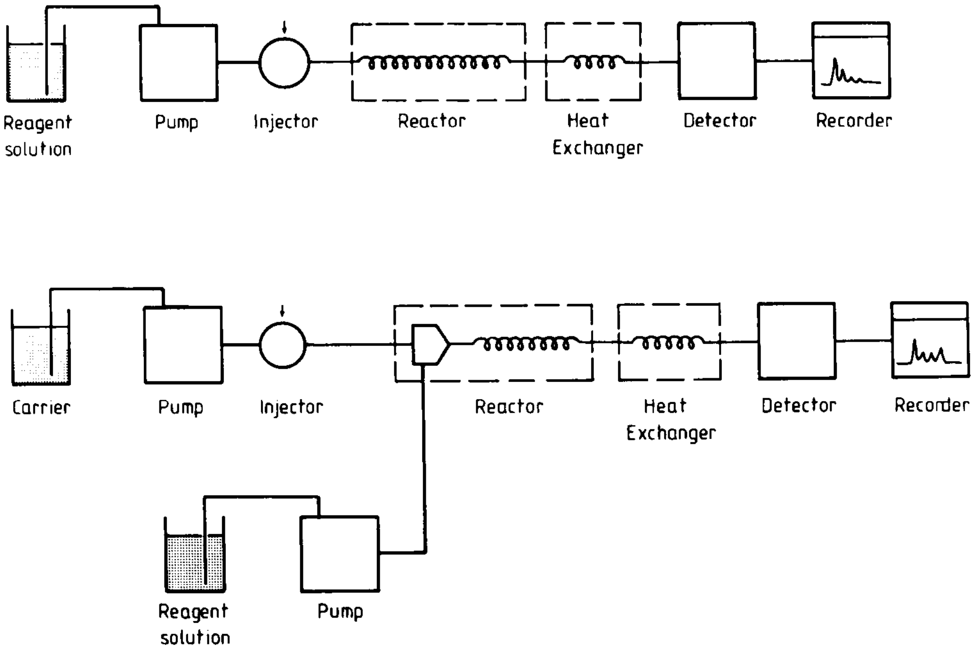

Fig. 3. Schematic diagram of the set-up for FIA. Top, conventional FIA; bottom, branched FIA.

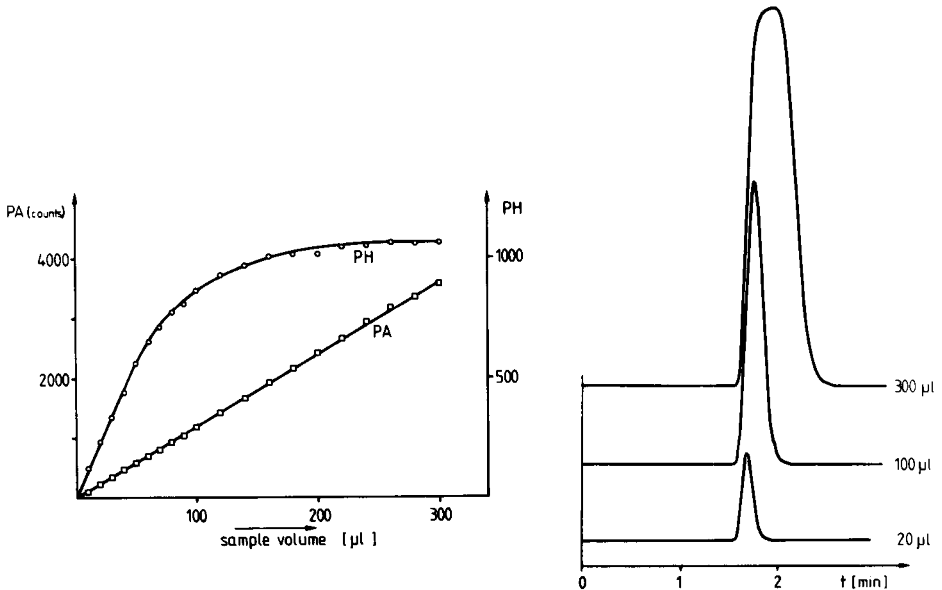

Fig. 4. Effect of sample volume on quantification with branched FIA. Conditions as in Fig. 2.

the injector and mixer. The improvement in detection sensitivity which is thus achievable has been demonstrated earlier [11].

It should be added that the use of low-dispersion capillaries common to CRD, such as KOT, and effective mixers will be most advantageous only with branched systems. High-speed analysis without limitations on solute volume can be attained without a reduction in detection sensitivity caused by inefficient mixing of solute and reagent.

Table I shows the advantages of BFIA with and without a spacer capillary. The introduction of KOT instead of conventional coiled FIA capillaries (in both instances the tube diameter was identical) into a normal FIA system improves the detection sensitivity of the determination of formaldehyde [12] by a factor of 4 and increases the number of determinations from 100 to 430. The use of a BFIA set-up improves the detection sensitivity again by a factor of 4. Compared with standard FIA, the improvement is 16-fold. The spacer capillary reduces the influence of the refractive index peak, and a slight improvement in detection sensitivity can be obtained. An additional advantage of BFIA is that reagent absorption does not cause any problems.

Inverse- and switched-flow injection analysis

FIA offers the possibility of performing many quantitative determinations of the same kind per unit time. More than 200 analyses per hour have been reported. Such fast analysis cycles are not often required. Because in FIA the reagents are pumped continuously, they are wasted when no analysis is performed. With the standard set-up, changes in the determination reaction by changing the reagents is time consuming. In this instance, two different approaches can be used to save reagents and to perform different determinations without completely changing the equipment.

In the simplest case, when several different determinations must be carried out in a continuous stream, the system can simply be inverted. In inversed flow injection analysis (IFIA) the sample is pumped continuously and the reagent is injected. Consequently, IFIA can advantageously be applied in process control or in the determination of various components in waste water. The use of automatic HPLC injectors, where the different reagents can be stored, permits the direct and automatic determination of many solutes in the system. The required change in detection

TABLE I

COMPARISON OF FIA AND BFIA IN FORMALDEHYDE DETERMINATION

Parameter	FIA		BFIA	
	Standard tubes	KOT	KOT	KOT and spacer
Peak volumes (flow-rate = 1.1 ml/min; tube, 0.3 mm I.D.) (μl)	430	100	150	>150[a]
Samples per hour	100	430	430	<430[a]
Detection limit (ppb)	80	20	5	3
Problems with RI switching peaks	No	No	Yes	No
Influence of absorption of reagent	Yes	Yes	No	No

[a] Depending on length of spacer capilary.

wavelength can be initialized by the automatic sample injector. The second generation of HPLC injectors can be programmed to take samples from various vials and to inject them in one step. In HPLC, this has already been used for automatic precolumn derivatizations. The advantage of this type of injector in IFIA is that reagents can also be used which have only limited stability when all the components are mixed together. This simplest approach is demonstrated in Fig. 5 for the determination of cyanide in waste water. Pump I feeds waste water continuously into the system. In the mixing device one part of the reagent (barbituric acid in pyridine) is mixed with the waste water. For the determination of cyanide, a plug of chloramine T in a buffer solution is added to this mixture ahead of the mixer. Only when this plug is present in the water will the reaction take place. The detection limit for cyanide with this set-up is 2 ppb. Surprisingly, in the classical set-up, where both reagent components are pumped and water is injected, the detection limit is inferior by a factor of 10 [12]. The calibration graph for cyanide determinations with normal FIA and IFIA is presented in Fig. 6. With the same set-up it was also possible to determine nitrite in water with a detection limit of 1 ppb by diazotization of sulphanilamide and coupling with N-(1-naphthyl)-ethylenediamine to an azo dye.

Table II summarizes the results of the determination of various anions and of zinc. The reagent compositions are also given. Again, with IFIA the detection limit is always better by a factor of at least 5. Nitrate is reduced to nitrite in an additionally installed solid-phase reactor, packed with cadmium beads, prior to the addition of the reagents. Only with rhodanide could no difference be observed between both types of set-up. The reason for this may be the small molar absorption coefficient of the iron–rhodanide complex.

Some of the reagents used in the determination of inorganic ions are extremely aggressive and can ruin pumps and other metal parts owing to complexation reactions. Also, at least one reagent component must be pumped continuously with the set-up shown in Fig. 5. Therefore, a different set-up has been developed where an inert carrier stream, in most instances pure water, can be used. The reagent components and/or the sample are introduced simultaneously in the form of plugs into the carrier and mixed, and the reaction products are detected photometrically. The equipment used for this type of FIA with reagent switching (SFIA) is shown in Fig. 7.

The inert carrier is displaced by the two pumps, P_1 and P_2. The use of two pumps is superior to that of one pump and a splitting device. With a single-pump system and reagent solutions differing in viscosity, the flow through the two branches will differ.

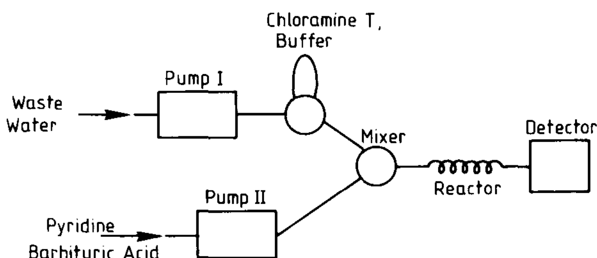

Fig. 5. Schematic diagram of the set-up for inverse FIA. Determination of cyanide in waste water. For experimental conditions, see Appendix.

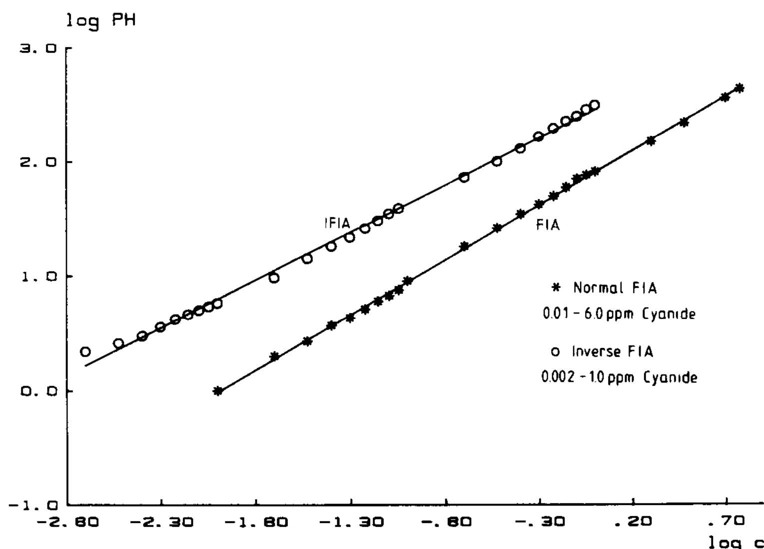

Fig. 6. Calibration graphs for cyanide determination in waste water. For experimental conditions, see Appendix. (×) Normal FIA (0.01–6.0 ppm cyanide); (○) inverse FIA (0.002–1.0 ppm cyanide).

When the reagents or reagent and sample are injected with the two valves (S_1 and S_2), both plugs are mixed and, after reaction in the knitted tube, the reaction products can be determined photometrically. The volume in both branches between sampling device and mixer must be identical. Relatively large sample volumes (300–600 μl) must be injected to ensure overlap. This approach also permits the use of coloured reagents and samples where the absorption is in the same region as where the reaction products are determined. In this instance, it is advantageous to delay one partner for a certain time. This is demonstrated schematically in Fig. 8. With this set-up it is possible to determine first the absorption of the solute or of reagent I (A1 in Fig. 8). When both plugs overlap, it is possible to determine the absorption of the reaction product together with that of the reagent and solute (A2). In the last part it is possible to determine the absorption of the second injected component (A3), and the absorption of the reaction product can be obtained by subtracting the absorption values of the two components

TABLE II

DETECTION LIMITS FOR INORGANIC IONS

Ion	RC I	RC II	FIA	IFIA
CN^-	Chloramine T	Pyridine-barbituric acid	10 ppb	2 ppb
SCN^-	$Fe(NH_4)(SO_4)_2$	–	1 ppm	1 ppm
NO_2^-	Sulphanilamide	N-(1-Naphthyl)ethylenediamine	50 ppb	1 ppb
NO_3^-	Cd-sulphanilamide	N-(1-Naphthyl)ethylendiamine	100 ppb	10 ppb
$[Fe(CN)_6]^{4-}$	$FeCl_3$	4,7-Diphenyl-1,10-phenanthroline	1 ppm	–
Zn^{2+}	Xylenol orange	–	1 ppm	0.1 ppm

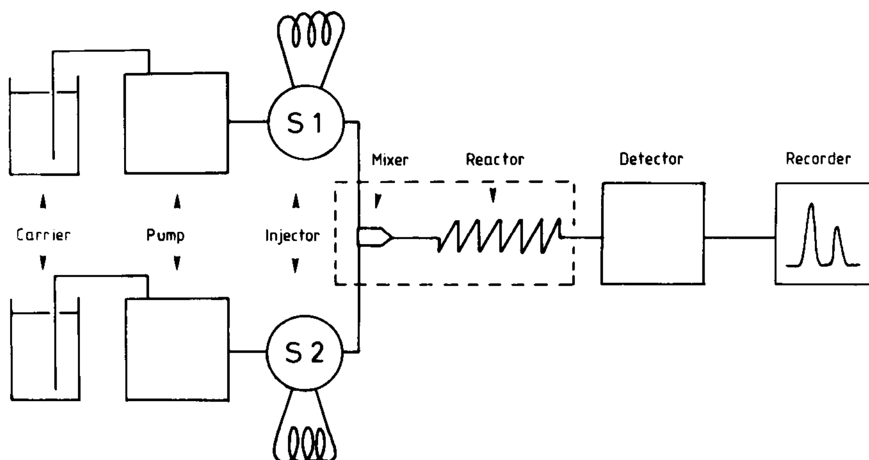

Fig. 7. Schematic diagram of the set-up for switched FIA.

(A2–A1–A3). The applicability of this set-up is demonstrated in Fig. 9 for the determination of formaldehyde in a yellow shampoo. The usual reagent [12] was additionally dyed with riboflavin. The experimental curves show, first, the signal obtained for the reagent and the injected shampoo solution. In a second step, the blank reagent was injected. The UV detector used permitted directly the subtraction of this reagent blank from the absorption peak. The peak thus obtained after the reagent absorption has been subtracted is also shown. The absorption caused by the colour of

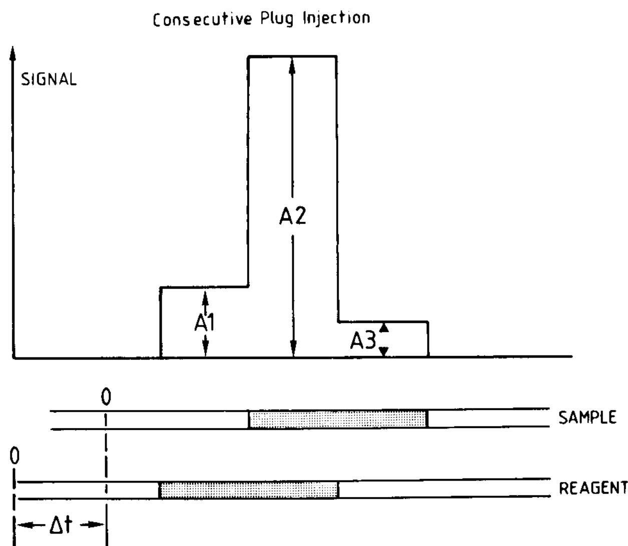

Fig. 8. Schematic presentation of plug injection with switched FIA for reactions with coloured samples and reagents.

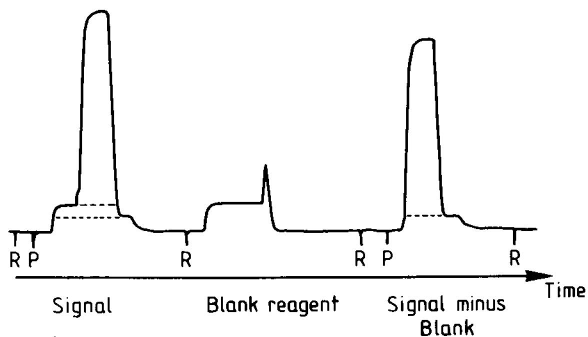

Fig. 9. Determination of formaldehyde in a coloured shampoo. Experimental conditions as published [12]. R = reagent; P = sample.

the shampoo is below the dashed line and can easily be subtracted from the main absorption.

For these measurements, the plug volumes are chosen to be large enough to reach a plateau and to make the plugs overlap at least for 50% of their width. Consequently, additional precautions must be taken to minimize zone dispersion in the sample loops and to reduce the so-called "log bottle" effect when the sample loop is

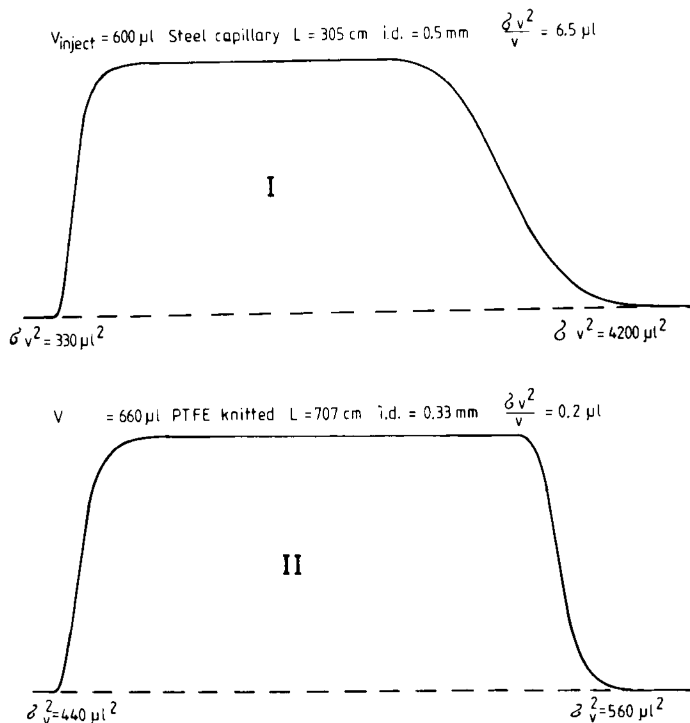

Fig. 10. Plug forms with large sample loops. For explanations, see text.

flushed with carrier. Here, the use of knitted Teflon capillaries in the sample loops is also advantageous. Fig. 10 shows the plug profile obtained when a 600-μl sample loop (coiled stainless-steel capillary, 305 × 0.5 mm I.D.) is emptied (I). The broadening of the tailing part of the plug is unacceptibly large. The volume variance of this part is 4200 μl^2, compared with 330 μl^2 for the leading part of the plug. The volume variance per unit volume ($\sigma_v^2 v^{-1}$), corresponding to the H value ($\sigma_L^2 L^{-1}$) of this loop, is 6.5 μl. The plug form with a knitted Teflon capillary (707 cm × 0.33 mm I.D.) as the sample loop is shown as curve II in Fig. 10. The variance of the tail end is only 560 μl^2 and that of the leading portion is 400 μl^2. The volume variance per unit volume is in this instance only 0.2 μl. This shows clearly that geometrically deformed open tubes are also advantageous as sample loops when large sample volumes must be injected. In preparative chromatography this is the case, when dilute sample solutions must be applied, owing to limitations in sample solubility.

CONCLUCIONS

The synergistic effects of using both CRD and FIA have been demonstrated. By applying forced sample reagent mixing and hence low-dispersive reaction capillaries, the limitations of FIA in sample volume can be obviated. The low dispersion in KOT improves the detection sensitivity by a factor of at least 10–50. Continuous determination of products in process control is possible when the reagent is injected into the sample stream. Various forms of switching techniques permit the use of aggressive reagents, even at an absorption wavelength where the reaction product is measured. An additional advantage is that in this instance the amount of reagents required can be reduced drastically.

ACKNOWLEDGEMENTS

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APPENDIX

Experimental conditions for the carbamate and formaldehyde determination have been described elsewhere [8,12]. For the equipment set-up, various HPLC components were used: pumps (Waters Assoc. M6000, 590; LKB or Dionex); detectors (Waters Assoc. 490; Milton Roy); Teflon tubing (0.3 mm I.D.) was knitted as described in Ref. 7.

Reagent compositions for anion determinations in Table II were as follows:
Cyanide: Solution I:

(1) CN^-:

RC I: 1 g of chloramine T in 100 ml of water.

RC II: 15 g of barbituric acid, 75 ml of pyridine and 15 ml of concentrated HCl, diluted with water to 250 ml.

(2) SCN^-:
RC I: 0.25 g of $Fe(NH_4)(SO_4)_2 \cdot 12\ H_2O$ in 100 ml of 0.5 *M* HNO_3.

(3) NO_2^-, NO_3^-:
RC I: 2.5 g of sulphanilamide in 13 ml of concentrated HCl, diluted with water to 250 ml.
RC II: 0.25 g of N-(1-naphthyl)ethylendiamine in 250 ml of water.

(4) $Fe(CN)_6^{4-}$:
RC I: 0.0033 *M* iron(III) chloride in 0.1 *M* acetic acid.
RC II: 10 mg of 4,7-diphenyl-1,10-phenanthrolinedisulphonic acid in 30 ml of water.

(5) Zn^{2+}:
RC I: 0.012 g of xylenol orange in 100 ml of water.

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