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REVIEW

POST-COLUMN REACTORS FOR SENSITIVE AND SELECTIVE DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY CATEGORIZATION AND APPLICATIONS

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LIST OF ABBREVIATIONS

HFM	Hollow-fibre membrane
IMER	Immobilized-enzyme reactor
LC	Liquid chromatography
MDA	Minimum detectable amount
MDC	Minimum detectable concentration
NAD	Nicotinamide adenosine dinucleotide
NP	Normal-phase
RP	Reversed-phase
R S D	Relative standard deviation
SPR	Solid-phase reactor

1 INTRODUCTION

1 1 Trace-level organic analysis

In modern trace-level organic analysis, chromatographic techniques play a predominant role (for a good general review, see ref 1) They are employed to create an efficient separation of the often complex mixtures that have to be analysed, prior to the actual measurement, i e , the detection step Unfortunately, however, even the combined force of an efficient separation plus a sophisticated mode of detection does not always create sufficient selectivity and/or sensitivity for the final goal to be reached – the identification and quantitation of minor sample constituents typically present in the low ppm to low ppt, i e , the 10^{-5}–10^{-11} g/ml, range In such cases, special attention has to be devoted to sample pretreatment (for trace enrichment and clean-up) and pre- or post-separation derivatization or conversion of the analyte(s) of interest (for improved detection selectivity and/or sensitivity)

The challenge which an analytical chemist is likely to meet in an increasing number of cases, viz , the rapid analysis of an often large number of complex sample mixtures, obviously requires the selection of a suitable separation mode or, rather, of a suitable total analytical procedure In this paper, which deals with bioanalytical applications, i e , with a field in which column liquid chromatography (LC) is the preferred separation technique, the potential of on-

line post-column reactors to enhance the sensitivity and selectivity of detection will be critically reviewed Attention will mainly be devoted to the categorization of the existing reactor systems, the (physico-)chemistry currently being used with such reactors and an overview of selected applications Further, the potential of automated on-line pre-column reactions will be briefly discussed and compared with the described post-column systems, as both types of procedures are in use nowadays for improving the potential of the analytical system

1 2 Post-column reaction detection

In recent years, a large number of papers, reviews and books have been published on the use of post-column reactors, i e , the principle of reaction detection (also, but less correctly, called chemical derivatization) in LC A series of selected references, some of which contain exhaustive bibliographies, are included in the list of references to this paper [2–11] In order to understand the growing popularity of reaction detection, one should realize that, although LC has become a powerful separation tool for a wide variety of samples, it is a distinct disadvantage of this technique, especially if compared with gas chromatography, that it often cannot meet the demands of modern organic trace-level analysis with regard to detection performance As already briefly stated above, one way to overcome this problem is to use chemical derivatization or related conversion techniques

In principle, (physico-)chemical conversion of trace-level analytes can be carried out in a pre- or post-column mode The main advantages of post-column reactions are that the analytes are separated in their original form, which often permits the adoption of published separation procedures, artefact formation plays a minor role, which is in distinct contrast with derivatization prior to separation, and the reaction does not need to be complete and the reaction products do not need to be stable, the only requirement is reproducibility

Disadvantages of post-column reaction detection are the need to add reagent(s) or reagent solution(s), which generally will require the use of additional pumps, the extra-column peak broadening caused by the reactor, which may well mar the chromatographic resolution, and the presence of excess of reagent which can interfere with the signal of the reaction products Means for reducing these disadvantages will be included in the pertinent sections below

One should realize that post-column reaction detection is invariably carried out in an on-line mode, which makes the technique well suited for use in automated systems, i e , for the processing of large series of samples Pre-column techniques, on the other hand, are most often employed in an off-line mode For this reason, they are outside the field of reaction detection, as it is usually defined, and will not be considered here For the rest, one should not under-

estimate the pre-column procedures with their versatility with regard to e.g., the time domain, the use of aggressive reagents and the utilization of a multi-step procedure Interesting recent developments in the area of (semi-)on-line pre-column reaction detection will be briefly discussed in Section 5

2 CATEGORIZATION OF POST-COLUMN REACTORS

A typical – although neither the simplest nor the most sophisticated (see below) – schematic diagram of an LC system with a post-column reactor is shown in Fig 1 Pump 1 is used to deliver the eluent After the separation, which will generally be carried out on a chemically bonded phase or an ion exchanger, reagent solution is added by means of the, preferably pulseless (post-column situation!), pump 2 via a low-dead-volume mixing tee The post-column reactor provides the desired hold-up time for the reaction Finally, the combined streams are passed through the detector, either directly, as shown in Fig 1, or after suitable organic–aqueous phase separation

There are three types of conventional reactors, viz , open-tubular reactors, inert or active packed-bed reactors and segmented-stream reactors Such reactors have been discussed in more or less detail in various reviews and books [2,4–11] and therefore only a summary of the most relevant aspects will be presented below. In addition, some attention will be devoted to hollow-fibre reactors, which are starting to gain popularity

2 1 Open-tubular reactors

The open-tubular reactor is the simplest type of reactor It consists of a straight or, more often, a helically coiled or knitted piece of PTFE, glass, quartz or stainless-steel tubing, and its dimensions are primarily determined by the hold-up time required for the selected reaction Under conventional condi-

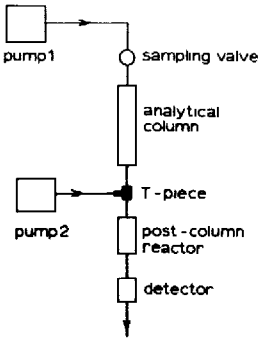

Fig 1 Schematic diagram of LC post-column reactor set-up

tions, laminar flow patterns prevail in open-tubular reactors, and the variance of the peak broadening in time units, σ_t^2, can be described by

$$\sigma_t^2 = t_r d_t^2 / 96 D_m \quad (1)$$

where t_r is the mean residence time, d_t the inner diameter of the tube and D_m the molecular diffusion coefficient of the analyte.

A reduction in peak broadening can be affected by geometrically deforming a straight capillary tube, that is, by coiling or knitting the reactor [12–14]. Owing to centrifugal forces acting on the flow pattern, secondary flows perpendicular to the main flow are produced As a result, better radial mixing and, consequently, a flattening of the laminar flow profile, i e , a reduction in peak broadening, occur The variance of a coiled reactor can be expressed by modifying eqn. 1 to

$$\sigma_{t,coil}^2 = k t_r d_t^2 / 96 D_m \quad (2)$$

with $0 < k < 1$ Under normal experimental conditions, k can easily be as low as 0 14 [15], that is, coiling then reduces peak broadening by ca. 60% The reduction in dispersion resulting from coiling or knitting open tubes is demonstrated in Fig 2

In principle, it is also possible to reduce peak broadening in open-tubular reactors by using smaller sized capillary tubing, i.e., by reducing d_t in eqns. 1

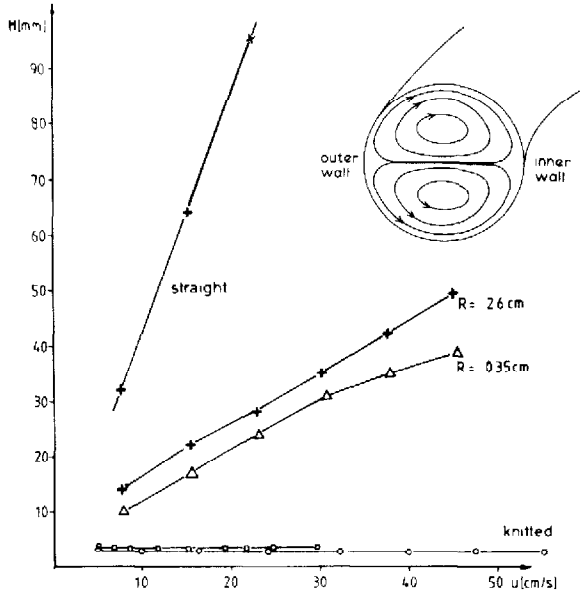

Fig 2 Peak dispersion in ideal, tightly coiled and knitted open tubes Inset secondary flow pattern in the cross-section of a coiled tube

and 2 In that case, the price to be paid is a larger pressure drop, ΔP, which can be calculated from the Poiseuille equation

$$\Delta P = 512\,\eta\,F^2/\pi^2\,d_t^6 \tag{3}$$

where η is the eluent viscosity and F the volumetric flow-rate One immediately sees that a mere halving of the tube diameter causes a 64-fold increase in the pressure drop In other words, this alternative approach is limited in its usefulness

Open-tubular reactors are mainly used for reactions with fast kinetics, i e., for residence times of up to about 1 min The introduction of well designed knitted reactors by Engelhardt and Neue [16] has extended their use to reactions requiring residence times of several minutes

2 2 *Packed-bed reactors*

Initially, packed-bed reactors were stainless-steel columns packed with small (10–15 μm), inert non-porous glass beads. Such reactors have been shown to have favourable characteristics with regard to mixing and peak broadening for reactions with residence times of ca 0 5–4 min [12,17], and also for use with narrow-bore (1 mm I D.) columns [18] According to the LC theory – applied to non-retention conditions – the variance contribution of such reactors to peak broadening can be written as [15]

$$\sigma_t^2 = 2yD_m t_r^3/L^2 + At_r^{5/3} d_P^{4/3}/L^{2/3} D_m^{1/3} \tag{4}$$

where y is the tortuosity factor, L the length of the reactor, A a constant with a value dependent on the bed geometry and d_P the particle size of the packing material Obviously, low σ_t values can be obtained by using long reactors packed with small particles As is to be expected, this must be paid for by an increase in the pressure drop, which can be calculated from the Darcy equation:

$$\Delta P = \eta L^2/k_0 t_r d_P^2 \tag{5}$$

where k_0 is the specific permeability of the packing used A current value for k_0 for non-porous particles is 0 002

Today, packed-bed reactors are more often packed with active than with inert materials These so-called solid-phase reactors (SPRs) are being increasingly used for catalytic (immobilized enzymes, ion-exchange resins) and stoichiometric (redox reagents, finely divided metals) reactions Examples are included in the pertinent sections below Here, we should briefly discuss the specific contribution to peak broadening caused by the use of an SPR In the 'active', as opposed to the earlier 'inert', reactors, some retention of the analytes is required for an efficient reaction, and a separation of reactants and

products can take place Nondek et al [19] developed a quantitative relationship for the so-called reaction peak broadening, $\sigma_{t,r}$, on a solid catalyst which has to be added to the normal peak broadening caused by the packed bed.

$$\sigma_{t,r} = (\sqrt{\ln 2}/8)\ (1-k'_P/k'_R)/k_r \qquad (6)$$

where k'_P and k'_R are the capacity factors of the product and the reactant, respectively, and k_r is the rate constant of the first-order reaction It is obvious that no reaction peak broadening will occur if (i) k'_P is equal to k'_R or (ii) k_r approaches infinity, which implies instantaneous conversion of the pulse of the reactant into that of the product

For enzyme reactors, the kinetics of enzyme reactions should be considered. As discussed by Bowers [20] in a good introductory text, enzyme kinetics are often complicated because of, e.g , the requirement for co-substrates, the need to couple the primary enzyme reaction to an indicator enzyme reaction or the interferences caused by inhibitors For a simple one-substrate system, and for the region of analytical interest, i e , at low substrate concentrations, one can derive that the fraction of substrate converted, X, is represented by

$$X = 1-\exp(-\alpha D_s V_r/F) \qquad (7)$$

where α is the enzyme-loading factor, D_s the diffusion coefficient of the substrate, V_r the volume of the reactor and F the volumetric flow-rate This equation states that if the amount of enzyme per unit amount of support material can be maximized, the residence time in the reactor or, in other words, the reactor volume at constant volumetric (LC eluent) flow-rate, can be decreased without sustaining a net decrease in the fractional conversion The benefits of this are obvious

2 3 *Segmented-stream reactors*

Segmented-stream reactors are widely used in continuous-flow analysers, in 1969, they were introduced as a post-column detection system [21] Segmentation of a flowing liquid stream with air or other gas bubbles is a highly effective means of suppressing dispersion of sample or analyte plugs, allowing extended residence times as segmentation reduces the axial diffusion of the sample zones It is generally accepted that dispersion in a segmented flow is brought about by slow mass transfer between the moving liquid segments and the liquid film at the wall of the reactor Deelder and Hendricks [22] calculated the time-based variance of a peak leaving the reactor to be

$$\sigma_t^2 = 2\ \pi^2 d_f L r^3 l_s/F_1 \qquad (8)$$

where L and r are the length and radius, respectively, of the reactor, d_f is the thickness of the film, l_s is the length of a liquid segment and F_1 its flow-rate If

full wettability of the reactor wall by the liquid is assumed, the film thickness can be calculated from [23]

$$d_f = 1.34\, r(u\,\eta/y)^{2/3} \qquad (9)$$

where r is the radius of the tube, η and y are the viscosity of the liquid and its surface tension on the wall material, respectively, and u is the bulk velocity of the fluid Based on this work, Snyder [24] and Snyder and Adler [25] derived further semi-empirical equations, which permit a more in-depth analysis of the problem It is already evident from eqn 8, however, that the inner diameter of the reactors, the wettability of the tube wall (film thickness!) and the segmentation frequency (liquid segment length) are important parameters

In recent years, solvent-segmented reaction detectors have gained increased popularity. In this instance, the aqueous LC eluent is segmented with a non-miscible organic solvent, or vica versa Segmentation by a liquid has the advantages that the performance of the system is hardly influenced by (small) variations in pressure, temperature or flow, because of the low compressibility of liquids. Although solvent segmentation efficiently reduces axial dispersion [26], so that additional peak broadening is negligible even for reactor residence times of ca 20 min [27], in actual practice solvent-segmented systems are used for their post-column extraction potential rather than to allow one to use reactions with slow kinetics This will be illustrated in some detail below

In solvent-segmented systems, the reactor tubing has to be selected with care, because the wetting characteristics of the tube material have a dramatic influence on peak dispersion This was well demonstrated by Lawrence et al [28] for a model system with post-column fluorescent ion-pair formation and

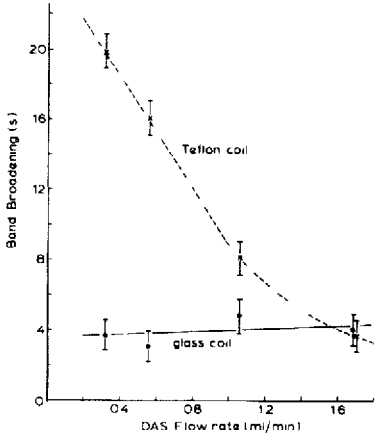

Fig 3 Influence of flow-rate of 9,10-dimethoxyanthracene-2-sulphonate on peak broadening in the determination of hydroxyatrazine using a segmented-stream reactor with PTFE or glass coils [30]

extraction of the ion pair of interest into an organic solvent As can be seen from Fig 3, for glass coils, peak broadening was virtually independent of the flow-rate of the aqueous phase, whereas it strongly increased with decreasing flow-rate in the case of PTFE coils As the ion pairs to be detected reside in the organic phase, dispersion is higher in the hydrophobic PTFE tubing which is strongly wetted by the organic solvent

Working with a segmented-stream system is relatively complicated in that it requires phase separation (or debubbling) prior to the actual detection step In practice, the debubbler or phase separator makes a large contribution to peak broadening [15,29] In one typical example [29], the influence of the length and inner diameter of a PTFE capillary on peak broadening was investigated using water or water–methanol (50 50) as the LC effluent and hexane as the segmentation liquid. Although the capillary length was increased from 1 to 10 m, and diameters of both 0 5 and 0 8 mm were used, which caused the reactor residence time to vary between 9 and 265 s, the peak broadening remained essentially constant ($\sigma_t = 2\,4 \pm 0\,2$ s) In other words, the measured peak broadening is largely due to the phase separator, as was confirmed in a more recent study by Deelder et al [15]

2 3 1 Phase separators

As phase separators are a critical part of post-column reaction detection–extraction systems, much attention has been devoted to their proper construction The major principle used for phase separation is wetting Early designs were based on the insertion of PTFE tubing in a glass tee-piece device [30] In another study, it was found that all-glass tee-pieces show completely satisfactory performance provided that a small plug of PTFE wool is inserted in the proper position [26] In all such instances, the efficient separation of the aqueous and organic segments is created by wetting of the glass and hydrophobic surface, respectively Gravity plays only a minor role, as was demonstrated by the phase separation of an aqueous and organic stream of equal density Good phase separators of the simple wetting type (Fig 4) typically give variance contributions of about 150 μl^2

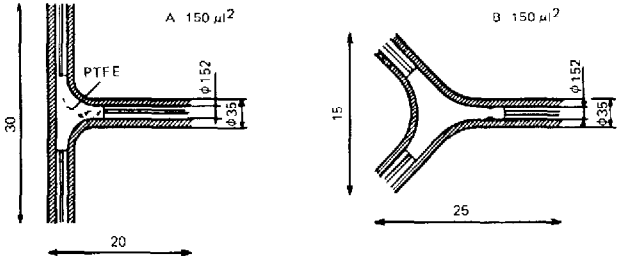

Fig 4 Typical designs of all-glass phase separators [26]

Membrane separators have shown good potential for application in post-column extractions, particularly for miniaturized systems where the dead volume has to be drastically reduced Apffel et al [31] designed such a separator using PTFE membranes with 0 5-μm pores, which had a dead volume of only 0 5 μl Although it worked well for LC with post-column extraction, and subsequently mass spectrometric [31] or electron-capture [32] detection, rapid clogging of the membrane material unfortunately occurred in the analysis of plasma samples.

More recently, De Ruiter et al [33] developed a sandwich-type phase separator Although its design is similar to that of the membrane separator discussed above, it no longer contains a membrane and phase separation is, again, based on wetting Fig 5 shows the scheme of such a separator, which has an internal (groove) volume of only ca 40 μl Phase separation occurs in the groove(s) machined in the upper stainless-steel block and the PTFE disc Further miniaturization to a groove volume of about 8 μl has already been achieved and, in various laboratories, the 40-μl phase separator is being utilized in routine applications

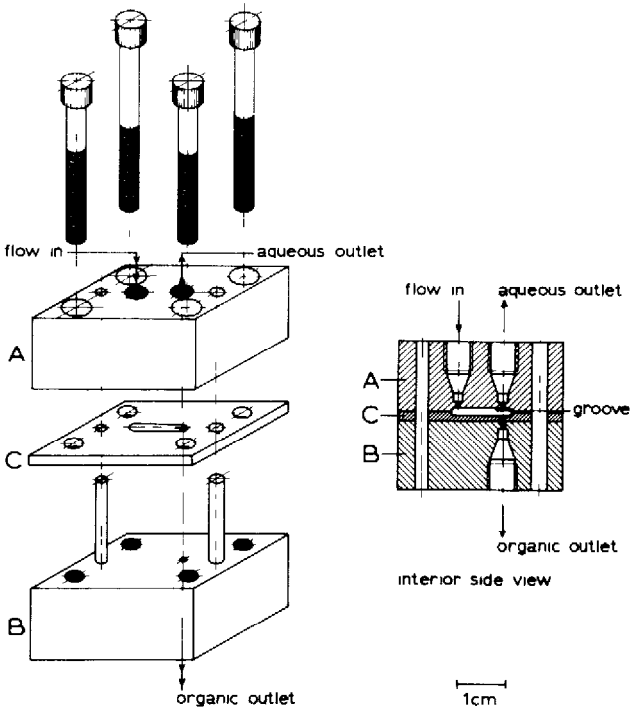

Fig 5 Sandwich-type phase separator Parts A and B constructed of stainless steel, part C constructed of PTFE [33]

2 4 Hollow-fibre membrane reactors

Porous membranes [34,35] and hollow-fibre membranes [36] can also be used for reagent addition and in this way reagent pumps, mixing tees and problems connected with imperfect mixing of solvent streams can be eliminated. This will result in significantly less extra-column peak broadening, an advantage shared with, e g , SPRs and photochemical reactors Cassidy et al [37] used annular membranes, with internal volumes of 1–10 μl, for post-column reactors. It has been shown that a mixing homogeneity of 99 9% of the theoretical value can be obtained

A microporous membrane has been used to separate a reagent reservoir containing glucose oxidase, buffered at pH 5, from a solvent stream containing glucose (as the analyte), potassium hydroxide, luminol and copper(II) bis(1,10-phenanthroline) The membrane limits the enzyme consumption in this reaction, which is an important economic advantage. The precision (relative standard deviation, R S D) for the determination of glucose in serum samples was 2–3% [34]

The principle of hollow-fibre membrane reactors can be illustrated by the determination of carboxylic acids after cation-exchange LC (eluent, 1 m*M* sulphuric acid, flow-rate, 0 8 ml/min) and conductivity detection [38] A significant increase in the detectability can be obtained after post-column pH adaptation Therefore, the eluent was passed through a cation-exchange hollow-fibre membrane placed between the column and the detector The enhancing agents (i e , sodium hydroxide or tetrabutylammonium hydroxide) were delivered outside the membrane (length, 50 cm) at a flow-rate of 1.5 ml/min The minimum detectable amount (MDA) for acetic acid was 40 ng The use of a coiled hollow-fibre membrane reactor with an internal volume of less than 2 μl has been reported; it still showed good performance as a post-column reaction device [37] This suggests the usefulness of such devices for miniaturized LC systems

3 APPLICATION OF POST-COLUMN REACTORS

Post-column on-line reaction detection systems are normally based on relatively fast reactions The reaction principles so far applied in the literature can be classified according to the scheme shown in Table 1 or, alternatively, as labelling and non-labelling reactions Using the latter classification, it is sometimes difficult to draw a proper dividing line, for example, with thermo-initiated, photochemically induced or catalytically accelerated derivatization reactions

Two further aspects should be mentioned (1) Next to reaction, reaction detection of course requires detection itself The high selectivity and sensitivity invariably aimed at in trace-level analysis in complex matrices strongly

TABLE 1

POST-COLUMN REACTION DETECTION PRINCIPLES

Principle	Non-labelling	Labelling
(Physico-) chemical change		
Electrochemical and redox	++	
Hydrolytic	++	
Micellar-enhanced	++	
Photochemical	++	
Physical interaction	+	+
Thermo-initiated	++	+
Derivatization		
Ion-pair formation		++
True chemical derivatization		++
Ligand-exchange and complexation		++
Chemiluminescence	++	+
Solid-phase reaction		
Catalytic	++	+
Stoichiometric	++	++

favour fluorescence as the detection mode It is more broadly applicable and, under real-sample conditions, more reliable than electrochemical detection, and it is more sensitive and selective than UV–visible absorbance detection The latter technique, however, has the advantage of general availability and is a satisfactory alternative when selectivity is relatively unimportant because of efficient sample pretreatment [39] (ii) One of the main drawbacks of many reaction detection systems is that reagent addition requires the use of additional pumps and mixing and demixing units This adversely affects the reliability of the total system, raises the background signal and/or increases the noise level An elegant way to circumvent this problem is the use of pumpless reactor systems such as the SPRs, the photochemical, electrochemical and thermo-initiated reactors Examples will be found in the pertinent sections below

3 1 Reactions based on (physico-)chemical changes

3 1 1 Electrochemical and redox reactions

Electrogenerated reagent addition has the advantage that no additional pumps are needed, that the reagent is extremely pure and that the number of side-reactions is small Redox reactions are often used to convert non-fluorescent compounds into fluorescent products An overview of some bioanalytical applications is presented in Table 2

The determination of vitamin K_1 derivatives is an interesting example of electrogenerated reagent addition Amongst several alternatives for the deter-

mination of these analytes after normal-phase (NP) or reversed-phase (RP) LC [40–42], the preferred procedure involved post-column coulometric reduction to the corresponding hydroquinones, which were detected fluorimetrically The MDA of the resulting vitamin K_1 derivatives in plasma was 25 pg [42]

An elegant approach is the electrochemical on-line post-column generation of bromine or iodine Subsequently, the bromine or iodine reacts with the analytes and the excess of halogen is detected amperometrically [43,44] A specially constructed electrochemical cell should be applied, as the cell contributes to peak broadening An interesting application is the determination of the disulphide and the thiol forms of glutathione, which react with bromine in neutral or acidic media, respectively [45] In this instance, a single-bead-string reactor, with a residence time of 10–20 s, provided more efficient micro mixing and a lower dispersion than an open-tubular reactor The MDA was 0 5 pmol and the linear dynamic range three orders of magnitude The determination of the reduced and oxidized forms of glutathione in rat liver microsomes is shown in Fig 6 [46] Other applications are the bioanalysis of ampicillin and ranitidine in plasma [47]

Another example is the formation of highly fluorescent compounds after the reaction of phenothiazines and aflatoxins with bromine or iodine [44]. The bromine, for instance, was applied as the reagent in order to oxidize the phe-

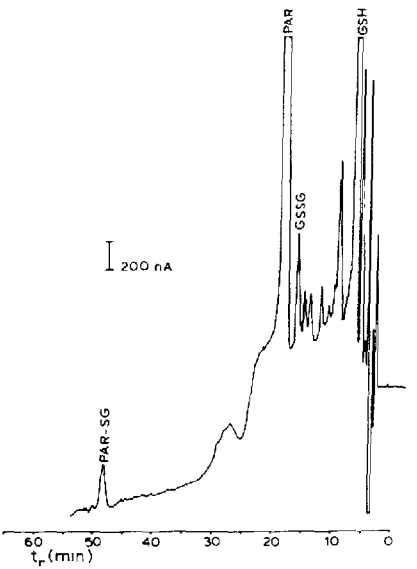

Fig 6 LC of rat microsomal sample using on-line bromine generation and electrochemical detection Peaks GSH = glutathione, GSSG = glutathione disulphide, PAR = paracetamol [46]

TABLE 2

BIOANALYTICAL APPLICATIONS OF ELECTROCHEMICAL AND REDOX REACTIONS

Analyte	Matrix	Reagent	MDC/MDA	Ref
N-Acetylcysteine	Plasma	On-line generation iodine	100 ng/ml	203
Aflatoxins	Feed stuffs	On-line generation iodine	1 μg/kg	204
Ampicillin	Plasma, urine	On-line generation bromine	200 ng/ml	47
Amprolium	Egg yolk, tissue	Electrochemical oxidation	3 μg/kg	205
Carbohydrates	Serum, urine	Cerium(IV) oxidation	100 nmol/ml	56
Catecholamines	Tissue	Hexacyanoferrate(III) oxidation	10 pg	206
Cisplatin	Plasma	Bisulfite + dichromate oxidation	40 ng/ml	207
Digoxin	Plasma	Conc hydrochloric acid, ascorbic acid	1 ng	208
	Serum	Conc hydrochloric acid, hydrogen peroxide	300 pg/ml	209,210
6-Mercaptopurine	Plasma	Chromate oxidation	2 ng/ml	53
Muramic acid	Serum	Cu^{II} bis(1,10-phenanthroline) oxidation	4 pmol	55
Phenothiazines	Plasma	On-line generation bromine	500 pg	48
Ranitidine	Plasma	On-line generation bromine	2 ng/ml	47
Reducing sugars	Serum	Cu^{II} bis(1,10-phenanthroline) oxidation	4 pmol	55
Thiamine, thiamine	Blood	Hexacyanoferrate(III) oxidation	30 fmol	211
phosphate esters	Cerebrospinal fluid	Hexacyanoferrate(III) oxidation	60 fmol	212
Thioridazine	Plasma	Permanganate, hydrogen peroxide oxidation	2 ng/ml	213
Thyroid hormones	Serum	Zn SPR, iodide-catalysed reaction	3 nmol/l	59
Vitamin K_1 derivatives	Plasma	Electrofluorimetric reaction	25 pg	42
Vitamin K_3	Premixes	Sodium borohydride reduction	20 ng/g	54

nothiazines The MDA in plasma samples was 0.5 ng, which was about ten-fold lower compared with a photochemical conversion of the analytes [48]

Actually, for post-column electrochemical reactions homogeneous redox reactions are normally preferred over electrode reactions, because the former usually are more rapid, and in many instances lower potentials can be applied [49] It is, of course, a drawback that reagent addition now requires an additional pumping system (see also, however, Section 3 4)

Post-column oxidation, followed by fluorescence detection, can be used for the determination of a number of biologically important analytes Catecholamines and thiamine were oxidized with potassium hexacyanoferrate(III) [50,51], while phenothiazine derivatives could be analysed after permanganate oxidation [52]. 6-Mercaptopurine was determined in plasma samples after on-line oxidation with potassium chromate An air-segmented reaction coil was used, giving a minimum detectable concentration (MDC) of 2 ng/ml with a within-day precision of 3 8% at the 25 ng/ml level [53]

Post-column reduction has been applied for the determination of menadione sodium hydrogensulphite (vitamin K_3) in animal feed and premixes [54] After aqueous extraction, the vitamin was converted into menadione, which was extracted and subjected to RP-LC Using an open-tubular reactor, menadione was reduced with sodium borohydride and the resulting 2-methyl-1,4-dihydroxynaphthate was detected fluorimetrically. Applying air segmentation and a reaction coil with a length of 1 4 m, the MDC was 20 ng/g

The reducing properties of amino sugars, such as muramic acid and other reducing sugars, can be used for their selective and sensitive detection after ion-exchange LC [55] The sugars are able to reduce copper(II) bis(1,10-phenanthroline) in a post-column system, using an open-tubular reaction coil, in the presence of hydroxyl ions Subsequently, the copper(I) bis(1,10-phenanthroline) formed, was re-oxidized amperometrically at a potential of 40 mV vs Ag/AgCl The MDA for muramic acid in serum was 4 pmol One of the main advantages of the procedure was the low potential of the working electrode, which resulted in high selectivity, a low background current and excellent long-term stability

Another type of redox reaction applied in post-column chemistry is the use of non-fluorescent reagents, which are converted into fluorescent products by the eluting analytes For instance, after anion-exchange LC, carbohydrates, organic acids and acetaminophen can be oxidized by cerium(IV) which is reduced to the highly fluorescent cerium(III) Lactose, mannose, fructose and glucose can be determined in human serum and urine samples [56]

Electrochemical and redox-base reactions can also be performed in an SPR Reduction reactions are popular. Sodium borohydride held on various types of support [57], platinum–rhodium immobilized on alumina [58] and zinc reactors [59,60] have been used

Thyroid hormones could be determined in serum samples by using a zinc

SPR [59] The acidic eluent was pumped through the column and the hydrogen produced split off the iodine from the iodinated thyronines The iodide ion was detected by means of a catalytic principle, based on the iodide-catalysed reaction of chloramine T and N,N′-tetramethyldiaminodiphenylmethane in an air-segmented tubular reactor with detection at 600 nm The reactor could be used continuously for about one day.

SPRs based on oxidation reactions typically use an anion-exchange resin in its permanganate form [61], solid lead(IV) oxide [62] or immobilized manganese(IV) oxide on silica [61] An application is the oxidation of catecholamines to adrenochrome derivatives, followed by homogeneous reduction to the fluorescent trihydroxyindoles, 300 analyses can be performed before depletion of the reagent occurs [63]

All chromium species that exist in a lower oxidation state than the hexavalent form can be oxidized in a lead(IV) oxide SPR The determination is based on complexation of chromate with 1,5-diphenylcarbazide and absorbance detection of the complex at 540 nm [64] The SPR (50 mm×3 mm I.D) was heated at 95 °C and the 1,5-diphenylcarbazide was added on-line using a PTFE reactor The MDC was ca 100 ng/ml and, despite the fact that a double-reactor system was employed, the R S D was less than 10%.

3 1 2 Hydrolytic reactions

Post-column pH changes are used, for instance, for the determination of barbiturates in serum [65], indomethacin in plasma [66], purines and pyrimidines in biological fluids [67], warfarin and its metabolites in plasma and urine [68] and some antimalarial drugs in plasma [69] (Table 3)

With indomethacin, the sample was extracted with dichloromethane, the extract evaporated and the redissolved residue analysed by RP-LC [66] The

TABLE 3

BIOANALYTICAL APPLICATIONS OF HYDROLYTIC REACTIONS (INCLUDING HOLLOW-FIBRE MEMBRANE REACTORS)

Analyte	Matrix	Reagent	MDC/MDA	Ref
Amoxicillin	Plasma, urine	Sodium hypochlorite	10 ng/ml	79
Ampicillin	Bile, plasma, urine	Sodium hypochlorite	25 ng	79
Artesunate	Plasma	Heating at high pH	50 nmol/l	69
Aspirin, salicylic acid	Plasma	Sodium hydroxide	20 ng/ml	214
Barbiturates	Plasma, urine, serum	Hydrolysis at high pH (HFM)	500 pg	76
Catecholamines	Urine	Heating at high pH	250 pg	215
Dihydroartemisinine	Plasma	Heating at high pH	50 nmol/l	69
Indomethacin	Plasma	Hydrolysis at high pH	1 5 ng/ml	66
β-Lactamase inhibitors	Plasma, urine	Hydrolysis at high pH (HFM)	25 ng/ml	77
Warfarin (metabolites)	Plasma, urine	Triethylamine	1 ng	68

eluate was mixed on-line with sodium hydroxide solution to hydrolyse the analyte and the hydrolysate was detected fluorimetrically (MDC, 1 5 ng/ml)

Absorbance detection of the antimalarial drugs artesunate and dihydroartemisinine can also be performed after post-column hydrolysis [69]. Plasma samples were cleaned by liquid–liquid extraction, followed by RP-LC, on-line alkalinization and heating at 70 °C for 2 min. The resulting products were detected at 289 nm (MDC ca. 50 nmol/l). In this work a knitted PTFE reaction coil (4.4 m×0 5 mm I D) was applied

Special solvolytic reactions are the conversion of digitalis glycosides [70] and cortisol [71] into fluorescent products after post-column treatment with concentrated hydrochloric acid and ethanol–sulphuric acid, respectively

Interesting developments in the field of on-line hydrolytic reactions are the use of packed-bed reactors, whether directly on-line (cf. Section 3 4 [72,73]) or using a parallel-column system, and the use of hollow-fibre membrane reactors.

A recent development is the use of parallel-column systems A pronounced disadvantage of non-catalytic SPRs is that some reactors are rapidly depleted and can be used only for a short period before reloading becomes necessary Another drawback is that often the reactor substrate is not available as regular and fine particles, which can result in severe peak broadening In certain instances these problems can be solved by employing a parallel-reactor column approach, as has been demonstrated for both conventional and microbore LC [74,75]

With barbiturates [75], the analytical system consists of an anion-exchange column which is placed parallel to the injection valve and the analytical column. The acetate-containing mobile phase is split· one part flows through the analytical column to achieve separation of the barbiturates and the other part passes through the anion-exchange column and releases hydroxyl ions After the LC separation, the two streams are combined and an alkaline medium is generated which is favourable for 254-nm absorbance detection of the solutes This principle was applied to the analysis of plasma and urine samples.

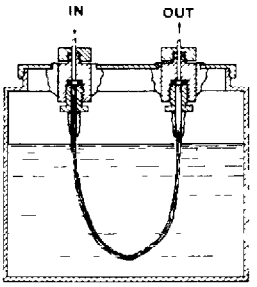

Fig 7 Scheme of hollow-fibre membrane reactor composed of a loop of five (325 μm I D) sulphonated polyethylene hollow fibres (8 m long) in a container with the appropriate reagent [36]

One of the first examples of using a hollow-fibre membrane reactor in bioanalysis was described by Haginaka and co-workers for the determination of barbiturates [76] and clavulanic acid and salbactam [77] in urine and plasma These β-lactamase inhibitors and also the barbiturates are relatively rapidly converted into UV-absorbing products in alkaline solution After ultrafiltration of the samples, the analytes were separated by RP-LC and the eluent was introduced into a sulphonated hollow-fibre (1 2 m $\times$ 0 3 mm I D) post-column reactor suspended in sodium hydroxide solution UV detection at 270–280 nm gave an MDC of 25 ng/ml The hollow-fibre membrane reactor has also been applied to the determination of penicillins [78,79]

For the sensitive detection of ampicillin after RP-ion-pair LC, the analyte should be degraded in alkaline medium with sodium hypochlorite to yield a chromophore with an absorption maximum at 270–280 nm The post-column degradation can be performed in a PTFE open-tubular reactor or using an aminated and sulphonated polyethylene hollow-fibre membrane reactor [79] The aminated and sulphonated reactors, which serve for the selective transport of ions, were connected in series and placed between the column and the detector. Application of the two procedures for the determination of ampicillin in human and rat plasma resulted in an MDA of 25 ng with a precision of ca 6% using the reaction coil and of 2 5 ng with a precision of 4 5% using the hollow-fibre membrane reactor

An elegant reactor was designed by Davis and Peterson [36] Their reactor was made of five parallel 8-in -long, 325-μm I D sulphonated polyethylene hollow fibres, which were suspended in a vessel containing the appropriate reagent (Fig 7) The ion-exchange capacity of the fibre membrane was of the order of 1 mequiv /g The type of membrane should be chosen carefully to ensure a high reagent flux over the membrane in combination with minimum sample losses and it should be compatible with the applied eluent When utilizing multiple-fibre systems, the dimensions of all fibres should be identical in order to minimize extra-column peak broadening.

3 1 3 Micellar-enhanced detection

The application of micelles in combination with LC and several (luminescence) detection principles provides features such as faster analyses and improved selectivity and detectability [80,81] The influence of micelles on the detectability can be explained partly by compartmentalizing effects, changes in the population of the ground state versus the excited states and the ratio of radiative and non-radiative decay processes [82,83].

For a number of fluorescent solutes, the intrinsic fluorescence sensitivity can be increased significantly by transferring the solute to a micellar environment [84] By applying micellar chromatography or by the post-column addition of a micellar solvent, detection limits can be improved fifty-fold [85] For example, the use of a non-ionic micellar system (Brij-35) caused a twenty-

fold increase in the fluorescence sensitivity of the 2-mercaptoethanol–*o*-phthaldialdehyde derivatives of dodecylamine and tyrosinamide [86] About the same gain in detectability was obtained for dansylated amino acids in cationic hexadecyltrimethylammonium chloride micelles [87]

The inclusion of analytes in micelles can also cause a shift in the wavelength of maximum absorbance compared with that in bulk solutions, and may result in improved sensitivity or selectivity [80,88]

3 1 4 Photochemical reactions

Photochemical reactions can be applied for various purposes, for instance, to increase the detectability in absorbance, amperometric or fluorimetric detection, or in photoconductivity detection However, the reactor is mainly used to convert weakly or non-fluorescing analytes into highly fluorescent products [89,90] or to create electroactive products [91,92] (Table 4)

The photochemical reactor is a typical pumpless reactor, as photons are in this instance the only reagent added Photochemical reactors are mainly used in the post-column mode because (i) the photochemical conversions are almost never quantitative and (ii) normally a number of products are formed When using a photochemical reactor, the main variable is the residence time of the analyte in the (open-tubular) reactor The composition of the eluent, the wavelength of UV irradiation and the intensity of the light source play a less prominent role

In a photochemical reactor the analyte is irradiated with a high- or medium-power UV light source, e g , a mercury or a xenon–mercury lamp [93] In addition to the lamp, a photochemical reactor consists of a quartz or, preferably, a PTFE reaction coil (transmissive for >200 nm) and a temperature-controlled (cooled) reactor (Fig. 8) The latter aspect is important, because temperature fluctuations will influence the flow-rate of the chromatographic eluent and the reaction kinetics and, consequently, the accuracy and precision of the analysis Reaction times typically are of the order of 10–60 s In view of these

TABLE 4

BIOANALYTICAL APPLICATIONS OF PHOTOCHEMICAL REACTIONS

Analyte	Matrix	MDC/MDA	Ref
Clobazam	Serum	20 pg	48, 93
Clomiphene	Plasma	60 pg	216
Demoxepam	Serum	100 ng	217
Desmethylclobazam	Serum	50 ng	48, 93
Diethylstilbestrol	Urine, plasma, serum	1 ng/ml	96, 218
Fenbendazole	Serum	1 5 ng	94
Methotrexate	Body fluids	400 pg	219
Phenothiazines	Serum	50 ng	94, 217

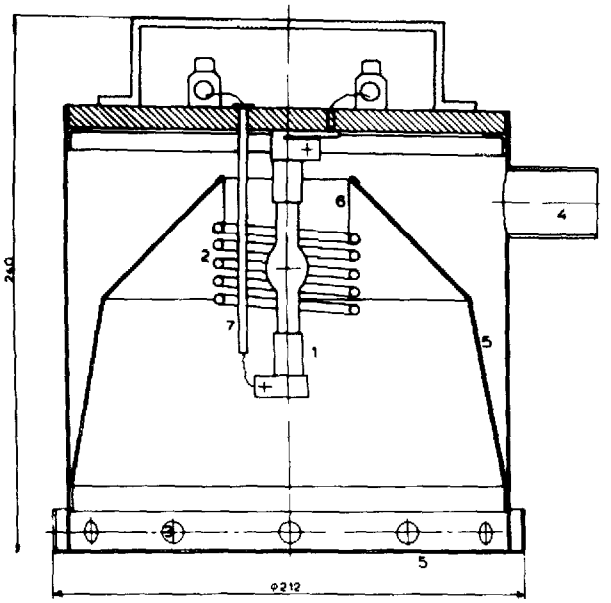

Fig 8 Scheme of photochemical reactor 1=Xe/Hg lamp, 2=quartz capillary, 3=inlet of air cooling, 4=outlet of air cooling, 5=aluminium reflector shield, 6=clip, 7=glass insulator Dimensions in mm [93]

short reaction times, coiled or knitted open-tubular reactors are the best choice [94] For most published applications, the MDAs are below 500 pg

The determination of the non-fluorescent drug clobazam and its main metabolite in serum was possible using RP-LC and fluorescence detection using an irradiation time of only 15 s The MDA was 20 pg [93] Methotrexate and its metabolites are converted into highly fluorescent products, viz., 2,4-diaminopteridine derivatives, after 3 s irradiation at 254 nm in a PTFE capillary, the MDAs were ca 0 4 ng. The solutes were determined in biological fluids (i e , plasma and urine) and the coefficient of variation was less than 2 5% [95] Photochemical conversion of diethylstilbestrol in urine allows fluorimetric analysis down to the 1 ppb level [96]. Another application is the conversion of cannabinol into a fluorescent phenanthrene derivative [97]

Photoconductivity detection is used for the determination of N-nitrosamines, organothiophosphates, sulphonamides and other drugs [98–101] On irradiation the non-ionic analytes are converted into ionic species which increase the conductivity of the aqueous LC eluent

In a number of instances the signal-to-noise ratio and the selectivity of a procedure can be improved by using a dual-reactor approach [72,102] For the determination of ciprofloxacin and its metabolites in urine, serum, plasma, bile, faeces and tissue homogenates [103], the effluent, after RP-LC, was heated in a stainless-steel capillary to achieve hydrolysis and then transported to a

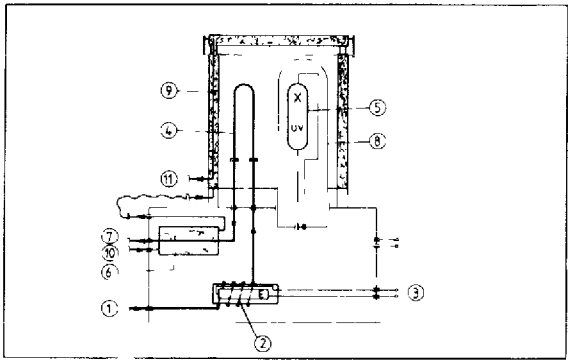

Fig 9 Dual-reactor system for biodetermination of ciprofloxacin 1 = LC effluent, 2 = stainless-steel capillary, 3 = heating unit, 4 = PTFE capillary, 5 = Hg lamp, 6 = cooler, 7 = fluorescence detector, 8 = quartz envelope, 9 = cooled double-wall cylinder [103]

PTFE capillary in which it was irradiated by a mercury lamp (Fig. 9) The MDAs, with fluorescence detection, varied from 1 to 2800 pg, whereas after on-line hydrolysis and photolysis the MDAs were 2 5–25 pg In order to obtain these favourable detection features, relatively short reaction times were utilized. 2 s for the hydrolysis and 0 6 s for the photolysis

3 1 5 Physical interaction reactions

A number of molecules show an increase in their intrinsic fluorescence sensitivity on binding (e g , hydrophobic binding) to proteins or phospholipids Fluorophores that can be applied for this purpose are 8-anilino-1-naphthalenesulphonic acid [104], N-phenyl-1-naphthylamine [105] and 1,6-diphenyl-1,3,5-hexatriene [106]

An interesting application is the determination of the lecithin/sphingomyelin ratio of amniotic fluid using RP-LC and a post-column reaction with 1,6-diphenyl-1,3,5-hexatriene [107] This reagent is a suitable probe for the fluorescence assay of phospholipids because of its low background fluorescence in water Using an air-segmented reaction coil kept at 40 °C and a residence time of 1 min, the MDA for lecithin was 50 nmol A similar procedure was described for the determination of phospholipids in plasma using 8-anilino-1-naphthalenesulphonic acid as the fluorescence probe [108]

3 1 6 Thermo-initiated reactions

With reactions that proceed slowly at ambient temperature, one can add a reagent to the LC mobile phase and initiate the reaction post-column by creating a temperature jump This approach has been used in the LC analysis of amino acids [109] and guanidines [110], the ninhydrin reagent being added to the mobile phase without influencing the separation conditions As the reaction proceeds in alkaline media only, an additional pump was used for al-

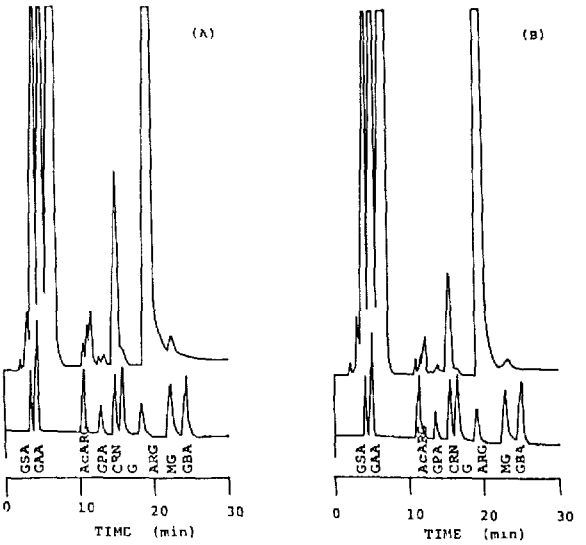

Fig 10 LC of the serum sample from a uraemic patient (A) before and (B) after haemodialysis therapy LC system C_8–15 m*M* octanesulphonate and 5 m*M* ninhydrin in water–acetonitrile–methanol (92 4 5) (pH 4 0) Reaction temperature, 75 °C [110]

kalinization of the eluent; the optimum reaction temperature was 70–80 °C With this method the decrease in the creatinine level after haemodialysis therapy could be determined in uraemic patient serum samples (Fig 10)

3 2 Derivatization reactions

3 2 1 Ion-pair formation

Ion-pair formation with simultaneous liquid–liquid extraction is a useful technique for the analysis of charged compounds [11] Reactions with fast kinetics, such as ion-pair formation, are preferably selected for post-column reactors because, in principle, they allow the use of simple open-tubular reactors Unfortunately, with ion-pair extraction a solvent-segmented system has to be applied because the counter ion added is itself the reagent, i e , the fluorophore, chromophore or electrophore which is present in large excess This excess should, of course, be separated from the analyte ion pair before detection is performed The commonly used approach is relatively straightforward After a suitable LC separation the eluate is mixed with an aqueous solution containing the fluorescent counter ion and a non-miscible organic solvent After reaction and extraction in an reaction coil the aqueous phase, containing the excess of the reagent, is removed and the organic phase, containing the ion pair, is monitored in the detector (Fig 11) If RP-LC is used the concentration of the modifier i e., methanol or acetonitrile, is important, as high concentra-

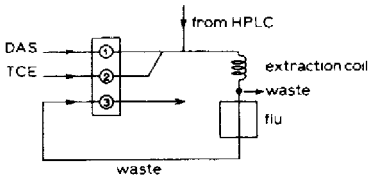

Fig 11 Set-up for on-line post-column ion-pair extraction and fluorescence detection after RP-LC separation DAS = 9,10-dimethoxyanthracene-2-sulphonate in water (1), TCE = tetrachlororethane (2), waste (3), ● = phase separator, flu = fluorescence detector [30]

TABLE 5

BIOANALYTICAL APPLICATIONS OF ION-PAIR EXTRACTIONS WITH 9,10-DIMETHOXYANTHRACENE-2-SULPHONATE AS COUNTER ION

Analyte	Matrix	MDC/MDA	Ref
Brompheniramine	Urine	3 ng	220
Chlorpheniramine	Urine	3 ng	220
Hydroxyatrazine	Urine	30 ng	30
Methadone	Plasma	5 ng/ml	114
Pancuronium-type compounds	Plasma, serum	25 ng	111
Phencyclidine	Plasma	5 ng/ml	114
Remoxipride	Plasma, serum	10 ng/ml	113
Secoverine	Plasma, serum	20 pg	221

tions will increase the co-extraction of the counter ion into the organic phase and, thus, the background signal NP-LC can also be applied. In this instance the organic eluent is segmented with an aqueous stream containing the ion-pairing agent. Again, high concentrations of the polar modifier in the eluent should be avoided as they will negatively affect the signal-to-noise ratio.

A number of fluorescent counter ions have been described such as 9,10-dimethoxyanthracene-2-sulphonate (Table 5) for reaction with amines [111], and the acridine cation for reaction with alkylsulphonates and related sulphates [112] (cf Section 4) Suitable extraction solvents are chloroform, dichloromethane and dichloroethane

Using 9,10-dimethoxyanthracene-2-sulphonate as the counter ion and remoxipride as the analyte, the MDA in plasma samples was about 1 ng/ml The recovery was 88% (200 ng/ml) with an R S D of 3 5% [113] Other examples are the determination of methadone and phencyclidine in plasma [114], the determination of a pancuronium derivative in serum and plasma [111] and the biodetermination of hydroxyatrazine in urine [30] The MDA in the last example was 30 ng

Automated on-line solid-phase isolation combined with post-column ion-pair extraction has been described for some pyrrolidinium bromide agents [115,116] An aliquot of a rat bile, plasma or urine sample, containing the

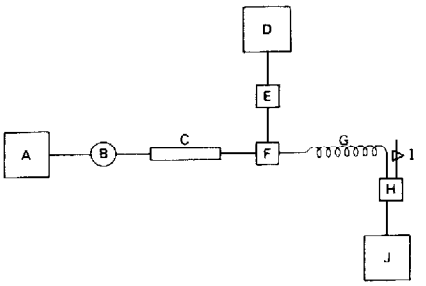

Fig 12 Scheme of automated ion-pair extraction of vencuronium-type derivatives using 9,10-dimethoxyanthracene-2-sulphonate as the counter ion A=pump, B=injector (AASP), C=LC column, D=pump, E=pulse damper, F=T-piece, G=extraction coil, H=phase separator, I=needle valve, J=fluorimetric detector [116]

analyte, was applied to a C_8 cartridge (10 mm×2 mm I D , 40-μm particles) and, after washing with water and a suitable buffer, the analyte was eluted on-line to the C_{18}-bonded silica analytical column and chromatographed using a 9,10-dimethoxyanthracene-2-sulphonate-containing mobile phase of pH 3 (Fig 12) The post-column extraction was performed with 1,2-dichloroethane using a splitting ratio (organic/aqueous) of 35% and a sandwich-type phase separator Approximately 800 samples could be analysed using this set-up.

3 2 2 Ligand-exchange and complexation reactions

Although ligand-exchange reactions are slow, they can also be applied in post-column reactors [117] The biodetermination of penicillamine in serum and urine is an interesting example, which is based on the reaction of the analyte with the non-fluorescent palladium(II)–calcein complex. Complexation between the S-containing analyte and this complex releases an equivalent amount of the highly fluorescent calcein (Fig 13) Because the palladium(II)–calcein complex itself is not fluorescent, separation of the excess of reagent

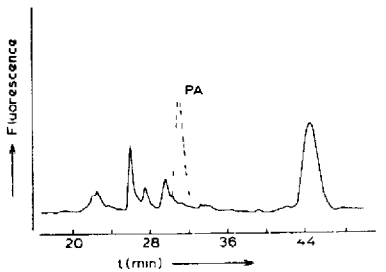

Fig 13 LC of penicillamine-spiked (PA) serum applying a ligand-exchange mechanism based on reaction of the analyte with the Pd^{II}–calcein complex LC system cation exchanger–citrate–phosphate buffer (pH 2 2) Air-segmented reactor, residence time, 20 min at 55 °C [117] Solid line, blank, broken line, 84 ng of PA

and the free calcein via extraction, i e , solvent segmentation, is not required However, in order to suppress extra-column peak broadening (the reaction takes 10–15 min at 50–60°C), air segmentation is a necessity

Biological phosphates such as nucleotides, organodiphosphonates and inorganic phosphates can be separated on a cation-exchange resin [118] Subsequently, an aluminium(III)–morin solution was added and the reaction was performed in a 400-μl reaction coil at 70°C The separated phosphorous acids will diminish the fluorescence of the aluminium(III)–morin complex by a competitive reaction The MDAs were 30–100 ng

Europium(III) and terbium(III) can be used as selective luminophores for analytes such as tetracycline derivatives [119] The detection is based on an intramolecular energy transfer from the analyte molecule to the metal ion Tetracyclines were determined in urine and serum after post-column addition of the europium(III)–ethylenediaminetetraacetic acid complex The detection limit and the selectivity were distinctly better than those with UV detection

The complexation of metal ions with an organic compound can be applied for the determination of metals (e g., Cd, Co, Cu, In, Fe, Mg, Mn, Ni and Zn) using indirect absorbance detection after cation-exchange separation of the analyte ions [120]. In this particular instance Eriochrome Black T was added to the LC effluent Using an open-tubular reactor at 180°C, MDAs of 2–5 ng were obtained As an alternative, organic analytes can be determined after the addition of metals or metal ions as reagents For the determination of disulfiram using a post-column solid-copper-containing reactor, see Section 3 4

Complexation of chiral ligands with a transition-metal ion [121] has been used for the separation of enantiomers The procedure can be applied in both the pre-column [122,123] and post-column modes [124] One post-column procedure is based on the addition of a metal cation–amino acid complex to the eluent of a cation-exchange column [122] For instance, copper(II)–proline complexes were used for the separation of D- and L-amino acids Detection was performed using a post-column derivatization with *o*-phthaldialdehyde

Studebaker [125] was the first to use complexation-based SPRs for the detection of thiols, disulphides and proteolytic enzymes More recently, Irth et al [126] elaborated an application based on the complex formation of dithiocarbamates and related compounds with finely divided metallic copper packed in a 2–4 mm×2 mm I D reactor This short bed length suffices for the quantitative conversion of the analytes into a complex absorbing at the selective wavelength of 435 nm The authors reported the determination of disulfiram in urine. An interesting extension of this principle is reported in Section 4

3 2 3 True chemical derivatizations

In principle, many reactions utilized for pre-column chemical derivatizations can also be applied in the post-column mode The actual number applied is, however, limited (Table 6) One of the most important limitations is the

TABLE 6

BIOANALYTICAL APPLICATIONS OF TRUE CHEMICAL DERIVATIZATION

Analyte	Matrix	Reagent	MDC/MDA	Ref
N-Acetylcysteine	Plasma	Pyrenemaleimide	240 n*M*	138
Amines (secondary)	Serum	Dansyl chloride	100 fg	222
Catecholamines	Urine	Glycylglycine	200 pg	223
Cefatrizine	Serum, urine	Fluorescamine	100 ng/ml	224
Cortisol	Urine	Benzamidine	100 ng	225
Cysteine	Plasma	Pyrenemaleimide	150 n*M*	138
Disulphides	Urine	2-Nitro-5-thiosulphobenzoate	300 pmol	226
Estradiol	Serum	Dansyl chloride	50 pg	227
Glutathione	Plasma	Pyrenemaleimide	150 n*M*	138
Guanidines	Serum	Phenanthrenequinone	50 pmol	228
	Plasma	Ninhydrin	5 pmol	229
Ketosteroids	Plasma	Dansylhydrazine	7 5 pg	230
β-Lactam antibiotics	Fermentation broth	*o*-Phthaldialdehyde	500 ng/ml	231
Phenylpropanolamine	Plasma	*o*-Phthaldialdehyde	2 ng/ml	232
Polyamines	Urine, serum, tissue	*o*-Phthaldialdehyde	500 fmol	233
Salsolinol	Plasma	Glycylglycine	2 pmol/ml	234
Δ^9-Tetrahydrocannabinol	Serum	Fast Blue salt B	50 ng	235

prerequisite that the derivatization reagent itself is not detectable or requires different detection conditions compared with the derivative The use of post-column derivatization plus extraction, of course, is an alternative An overview of post-column true chemical derivatization procedures for important functional groups such as amine, carbonyl, carboxyl, hydroxyl and sulphydryl can be found in the literature [2–6, 8–11] In this paper only some important aspects of true chemical derivatization are discussed

The derivatization of the primary amino functions of amino acids with *o*-phthaldialdehyde and the labelling of thiols with a maleimide are examples of the use of non-fluorescent probes which yield highly fluorescent derivatives Both reagents have been applied in bioanalysis in both the pre-column and post-column modes and the MDAs are in the picomole range [127]

The most widely applied reagent in this area is *o*-phthaldialdehyde In the presence of a reducing agent such as ethanethiol or 2-mercaptoethanol, this probe reacts, at alkaline pH, with primary amines to yield highly fluorescent products Because the probe itself is non-fluorescent, the kinetics are fast and the derivatives are unstable, post-column derivatization is preferred over pre-column derivatization by many workers [128] The *o*-phthaldialdehyde reaction products can also be detected by absorbance [129], amperometric [130,131] and chemiluminescence [132] methods The MDAs of primary amine derivatives are about 50 ng using absorbance detection at 340 nm and 50–150 fmol using amperometric detection at a potential of 750 mV [131] Problems

of labelling with this probe, such as derivative instability, and different intrinsic fluorescence sensitivities when different thiols are used can be avoided by using amperometric detection The thiols have a minor influence on the electrochemical behaviour of the derivative and the isoindole derivatives formed can be very rapidly oxidized irreversibly [131]

The selectivity of the derivatization procedure can be improved by using naphthalene-2,3-dicarboxyaldehyde or anthracene-2,3-dicarboxyaldehyde instead of *o*-phthaldialdehyde [133–135] The use of these probes will result in a red shift of the excitation and emission wavelengths of the derivatives and, thus, in less interferences from the biological matrix A second advantage is the lower background fluorescence at longer wavelengths, resulting in an improved signal-to-noise ratio A disadvantage may be that even stronger nucleophiles than the thiol (e g , cyanide) are needed in combination with these two reagents

The sensitivity of fluorescence detection can be improved significantly by applying laser-induced instead of conventional fluorescence detection For both *o*-phthaldialdehyde and naphthalenedialdehyde derivatives laser-induced fluorescence gives detection limits that are about two orders of magnitude lower than with conventional detection [135] The MDC of norepinephrine in rat brain samples after labelling with *o*-phthaldialdehyde and fluorescence detection applying an argon-ion gas laser (351–363 nm multi-line, 0 015 W) was $2 \cdot 10^{-11}$ *M* [136]

A suitable reagent for the determination of thiols is pyrenemaleimide [137] The derivatization of a thiol with the maleimide is based on a nucleophilic addition of the thiol to the carbon–carbon double bond of the reagent The reaction should be performed in alkaline media, as the thiolate anion is a better nucleophile than the thiol itself Johansson and Lenngren [138] described the determination of cysteine, glutathione and N-acetylcysteine in plasma After protein precipitation with perchloric acid, the analytes were reduced with dithiothreitol and separated by RP-LC The derivatization reaction itself was performed in a 10 m × 0 3 mm I D knitted capillary The MDC for N-acetylcysteine was about 0 15 μM

The bioanalysis of sodium 2-mercaptoethanesulphonate in urine [139] can be performed by post-column derivatization of the sulphydryl function of the analyte with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] followed by absorbance detection at 412 nm Using an inert packed-bed reactor, 75 ng of the solute can be determined The sensitivity was less good than with direct amperometric detection (MDA, 25 ng), but sufficient with respect to the levels found in urine

A selective post-column derivatization procedure for the ketolic function of 17-hydroxycorticosteroids was described by Seki and Yamaguchi [140] After liquid–liquid extraction from urine the analytes were separated by RP-LC Selective labelling, with benzamidine, was performed in an alkaline aqueous

medium using a PTFE (30 m×0 5 mm I D) reaction coil heated at 95°C The MDA for tetrahydrocortisol was 5 ng

In structural studies of carbohydrate chains in glycoproteins, the monosaccharides present, e g , aldoses, hexosamines and sialic acids, can be determined using post-column labelling with 2-cyanoacetamide [141] After cation-exchange LC, the eluate was alkalinized and mixed with a 2-cyanoacetamide solution The reaction was performed in a PTFE reaction coil at 100°C In this particular instance, absorbance detection was employed, because the intrinsic fluorescence of the derivatives was quenched by the acetonitrile in the mobile phase The monosaccharides were determined in, for instance, human serum transferrin with MDAs of 50–400 pmol. A virtually identical system has been described for the determination of uronic acids after anion-exchange LC in plant and animal polyuronides (MDA, 100 pmol) [142]

Pre- and post-column derivatizations can also be applied in the same analytical procedure. An example is the derivatization of primary and secondary amines with 2-methoxy-2,4-diphenyl-3(2*H*)-furanone [143] An aqueous sample of amines was mixed with a solution of the fluorescence probe in acetonitrile and shaken for 30 min at room temperature Subsequently an aliquot was injected on to an RP system and the fluorescent pyrrolinones formed from the present primary amines were separated. The LC effluent was mixed online with an ethanolamine solution and this mixture was passed through a reaction coil at 60°C In this way the non-fluorescent aminodienones formed from secondary amines were converted into fluorescent pyrrolinones

3 3 Peroxyoxalate chemiluminescence

The potential of chemiluminescence for detection in LC has been discussed in several recent papers [11,144,145], and is extensively treated by De Jong and Kwakman [146] in this volume In this paper only a few important aspects of (peroxyoxalate) chemiluminescence will be emphasized

The emission signal of a chemiluminescence reaction displays a growth and an exponential decay curve This means that in a dynamic LC system, the half-life of the chemiluminescence signal is of major importance For given flow-rates of the LC eluent and the reagent streams, the dead volume between the mixing tee and the flow cell, the construction of the flow cell itself and the chemiluminescence half-life determine the percentage of emitted light that can be measured [147] Since chemiluminescence half-lives are often of the order of only 1 s, in actual analyses the decay of the signal can be so fast that even a small dead volume between the mixing of the reagent solution(s) with the LC effluent and the detection cell inlet will result in a considerable loss of the emitted light Therefore, several special mixing devices have been designed [147,148].

Effective light collection is another parameter of major importance in che-

mıluminescence detection In principle, a conventional fluorescence detector cannot be applied for this purpose because its collection efficiency is too small The application of a 2π steradian mirror in front of the flow cell [144,148] or of an integrating sphere enhances the collection efficiency significantly Further, positioning of the flow cell to, or on, the photomultiplier is essential

The three most widely applied chemiluminescence systems are the (iso)luminol, lucigenin and peroxyoxalate systems, the last being the most versatile and efficient [144] During the peroxyoxalate chemiluminescence reaction an energy-rich intermediate is formed by the reaction of hydrogen peroxide and an aryl oxalate The intermediate excites the fluorophore, which returns to the ground state via emission of light [146] In this way the excitation of any fluorophore with an excitation energy of less than 105 kcal/mol is, in principle, possible The system has been applied to the determination of dansylated amino acids [149–151], dansylated amines [152], fluorescamine derivatives of catecholamines [132], *o*-phthaldialdehyde and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole derivatives of aliphatic amines [153], coumarin derivatives of carboxylic acids [154] and estradiol in serum [155] In the last example, the serum samples were pretreated with a C_{18} solid-phase isolation cartridge, derivatized with dansyl chloride and further purified on a C_{18} cartridge The actual analysis was performed on a silica system The overall recovery was 90% and the MDA was 50 pg For the separation of the lipophilic estradiol derivative NP-LC was preferred over RP-LC, an additional advantage was that the chemiluminescence signal was strongly enhanced in the organic LC eluent.

Peroxyoxalate chemiluminescence can also be applied for the determination of trace levels of hydrogen peroxide. Examples are the determination of acetylcholine and choline using an immobilized enzyme reactor (IMER), based

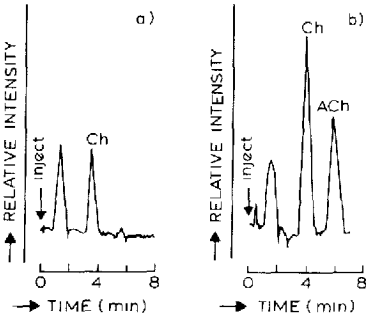

Fig 14 LC of a deproteinized pooled serum sample (a) and a serum sample (b) spiked with 200 pmol of choline (Ch) and acetylcholine (ACh) using an immobilized enzyme reactor and peroxyoxalate chemiluminescence detection LC system cation exchanger-0 05 *M* phosphate (pH 7 4) containing 20 m*M* tetramethylammonium nitrate [158]

on oxidation of choline to hydrogen peroxide (cf. Section 3 4 [156–158]) (Fig 14)

An example of the use of the (iso)luminol system is the determination of eicosapentaenoic acid in serum [159] After pre-column labelling of the analyte with N-(4-aminobutyl)-N-ethylisoluminol and RP-LC, an MDA of 200 fmol was obtained The lucigenin system was applied to the determination of steroids having α-hydroxycarbonyl functions such as cortisol and cortisone [160], and for the detection of carboxylic acids after their derivatization with *p*-nitrophenacyl bromide [161] The MDAs for the latter procedure were about 500 fmol

3 4 Solid-phase reactors

Heterogeneous reactions performed in an SPR can be divided into two categories catalytic and stoichiometric. As discussed before, the main advantage of SPRs is that no additional reagent pumps are required. Detailed information on SPRs is given in some recent reports [18,162]

In catalytic SPRs the substrate in the reactor participates in the chemical reaction as a catalyst which can be used over long periods of time One of the important prerequisites is that no surface poisoning of the catalyst bed occurs Catalytic SPRs are used for acid- or base-catalysed hydrolysis reactions [102,163] (cf Section 4), but nowadays the main application area is the IMER, which will be discussed below

In stoichiometric solid-phase reactions, the substrate in the reactor participates in the reaction as a reagent source Normally, the reagent is held on a suitable support or packed directly into the reactor column It will be obvious that the reactor will have to be repacked periodically and that, in actual practice, the lifetime is an important parameter The stoichiometric SPRs are mainly used for redox-based and complexation reactions, which have been discussed in Sections 3 1 and 3 2

3 4 1 Immobilized enzyme reactors

The popularity of the IMER is largely due to the fact that the catalytic activity of the enzyme is directed towards a single compound or a related group of similar compounds, this means that the selectivity will be high [164] In comparison with other catalytic SPRs there are additional problems such as enzyme bonding problems, enzyme stability and the (often) detrimental effect of the LC eluent on the activity of the immobilized enzyme, which is usually active over only a relatively short pH range and often cannot withstand high percentages of organic modifier A relatively important aspect of the preparation of an IMER is the immobilization step [20,165,166] The support materials normally are agarose, cellulose, polyacrylamide, nylon, glass, silica and ion exchangers However, in combination with LC, glass is to be preferred be-

cause of its pressure resistance and the availability of narrow-graded small particle sizes Covalent attachment of the enzyme is, in principle, preferred as physical adsorption has the disadvantage that loss of enzyme due to desorption processes can take place The most widely applied method for enzyme immobilization uses an aminopropyl coating on the support material and subsequent activation with glutaraldehyde [164]

The principles of IMERs have been discussed extensively in a number of papers [11,20] Therefore, only a series of bioanalytical applications will be reported in this section (Table 7)

An important application of IMERs is the determination of bile acids [167] After RP-LC separation of the bile acids the column effluent was mixed with a nicotinamide adenosine dinucleotide (NAD) solution and led to the packed-bed reactor. The latter contained glass beads holding immobilized 3α-hydroxysteroid dehydrogenase and was kept at 30°C The reactor eluate was mixed with phenazine methosulphate solution in a reactor capillary, with final amperometric monitoring at +0 1 V The MDAs in serum and bile samples were about 20 pmol This enzyme system has been described also for the determination of sulphated 3α- and 3β-hydroxysteroids in human serum The analytes were deprotonated with ethanol, solvolysed with sulphuric acid in ethyl acetate and separated by RP-LC The LC eluate was mixed with NAD and passed through a column containing 3α- and 3β-hydroxysteroid dehydrogenase, subsequently the NADH formed was detected fluorimetrically The MDCs were of the order of 5 ng/ml, which is a considerable gain in detectability in comparison with UV detection [168] It is an advantage over other studies that in this instance the determination of sulphated 3α- and 3β-hydroxysteroids is possible by immobilizing two enzymes on to the same column

Another interesting bioanalytical application of IMERs is the determination of urinary estriol glucuronides using β-glucuronidase immobilized on controlled-pore 37–74 μm glass beads The estriol produced by the IMER was trapped on a pre-column and, after switching of the valves, eluted to the analytical column [169,170]. The combination of an IMER and chemiluminescence detection was described by Klopf and Nieman [171] for the determination of conjugated glucuronic acids Instead of the peroxyoxalate chemiluminescence system, the lucigenin system was used In this semi-automated method the IMER was placed before the analytical column – an anion exchanger – which was used to remove other organic reductants (Fig 15) A four-way valve was inserted in the system, programmed to monitor only a specific fraction of the chromatogram (MDC, 5–10 μM)

Another elegant application is the simultaneous biodetermination of uric acid, hypoxanthine and xanthine utilizing two IMERs in series (Fig 16) [172] The MDCs for hypoxanthine and xanthine were 68 and 76 ng/ml, respectively

A well documented LC–IMER application is the determination of acetylcholine and choline in tissue homogenates (e g , mouse cerebral cortex)

TABLE 7

BIOANALYTICAL APPLICATIONS OF IMMOBILIZED ENZYME REACTORS

Analyte	Matrix	Reagent	MDC/MDA	Ref
Acetylcholine, choline	Brain	Acetylcholine esterase, choline oxidase	100 fmol	236, 237
	Serum, urine	Acetylcholine esterase, choline oxidase	500 fmol	158
	Cerebrospinal fluid	Acetylcholine esterase, choline oxidase	300 fmol	175
Bile acids	Serum	3α-Hydroxysteroid dehydrogenase	200 nM	238
	Bile	3α-Hydroxysteroid dehydrogenase	800 pg	167
	Serum, urine	3α-Hydroxysteroid dehydrogenase	50 pg	239, 240
Glucuronides	Urine	Glucuronidase	5 μM	171
Glycerol	Serum	Glycerol dehydrogenase		241
3α-,3β-Hydroxysteroid sulphates	Serum	3α-,3β-Hydroxysteroid dehydrogenase	5 ng/ml	168
		3β,17β-Hydroxysteroid dehydrogenase	20 ng/ml	242, 243
Phenolic glycosides	Urine	Glycosidase	20 pmol	244
Saccharides (mono-,di-)	Fermentation plant	Glucose dehydrogenase	2 ng	245
Trimethoprim	Urine	Glucuronidase	18 pmol	246
Urea	Urine	Urease	10 nmol/ml	247
(Hypo)xanthine	Serum, urine	Xanthine oxidase	50 pg	248, 249
(Hypo)xanthine+uric acid	Serum, urine	Xanthine oxidase+uricase, catalase	70 ng/ml	172

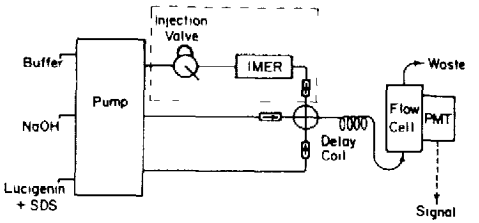

Fig 15 Set-up for the determination of conjugated glucuronic acids by combining an immobilized enzyme reactor and lucigenin chemiluminescence [171]

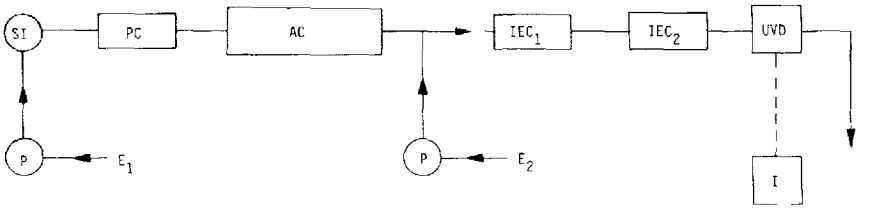

Fig 16 Scheme of LC system coupled with immobilized enzyme reactors for the determination of xanthine and hypoxanthine in serum and urine AC = analytical column, E_1 = eluent, E_2 = 0 01 *M* sodium borate, I = integrator, IEC_1 = immobilized enzyme column (uricase catalase), IEC_2 = immobilized enzyme column (xanthine oxidase), P = pump, PC = pre-column, SI = sample injector, UVD = UV detector [172]

[173,174]. After RP-LC on a PRP-1 polymer column, selective and sensitive detection was performed using the IMER A flow-rate of 2 ml/min was maintained, which resulted in a residence time of less than 10 s The acetylcholine esterase and choline oxidase were immobilized on an ion-exchange resin. First acetylcholine was hydrolysed to choline and, subsequently, choline was oxidized The latter reaction resulted in the production of hydrogen peroxide, which was detected amperometrically The MDA was 5 pmol The same analytes can also be determined in cerebrospinal fluid, which results in an MDA of 0 3 pmol for acetylcholine [175] The procedure yields results comparable to those given by gas chromatography–mass spectrometry, but it is less expensive and faster [176]

Owing to the selectivity of the chemiluminescence system, the pretreatment of the biological samples could be simplified considerably [158,177] Another feature of this reaction detection system is that detection limits down to 50 fmol can be obtained [178]

Regarding the growing interest in enantiomeric separations, the possibility of using an IMER in combination with stereoselective enzymes should be mentioned An example is the determination of L-amino acids using an IMER containing immobilized L-amino acid oxidase [179], which, in another study [180],

was combined with peroxyoxalate chemiluminescence detection During the reaction

$$\text{L-amino acid} + H_2O + O_2 \xrightarrow{\text{L-amino acid oxidase}} \text{2-oxo acid} + NH_3 + H_2O_2 \qquad (10)$$

hydrogen peroxide is formed The residence time of the amino acids in the reactor was about 1 min The peak broadening, which was almost solely due to the reactor, could be reduced significantly by reducing the size of the glass beads [181] Detection of the hydrogen peroxide produced resulted in an MDA of ca 0 7 μmol in urine samples

4 BIOANALYSIS AND ENVIRONMENTAL ANALYSIS

In this review, the use of post-column reaction detection for biomedical and pharmaceutical purposes only is discussed However, it should be realized that similar approaches can be used – and have been used – in other application areas, notably in the field of environmental chemistry Because the principles applied are fundamentally the same in all instances, interesting information can sometimes be obtained by studying results reported for completely different sample types The selected examples given below should suffice to demonstrate the versatility of the post-column reaction detection approach

Photochemical reactors have been utilized for the determination of organic nitro compounds Photolysis releases the nitrite ion, which is subsequently detected on-line under oxidative electrochemical conditions De Ruiter et al. [182] have shown that dansylated chlorophenols, prepared by off-line precolumn derivatization, rapidly release their dansyl moiety on post-column UV irradiation About 5 s is sufficient to achieve detection limits of 200 pg for all analytes tested

Ion-pair formation has been employed successfully for the determination of organic sulphates and sulphonates used as detergents [112] Working at a low pH permits the use of the highly fluorescent acridinium-H^+ ion as the fluorescent counter ion LC separation with an acetone–water gradient did not unduly raise the baseline or increase the noise

True chemical derivatization has elegantly been combined with heterogeneous catalysis in the trace-level determination of N-methylcarbamates [72,183] The analytes were separated by RP-LC and then hydrolysed at 100°C in an SPR packed with an anion-exchange resin The methylamine formed was reacted with *o*-phthaldialdehyde and the fluorescent derivative monitored fluorimetrically The detection limits were between 0 1 and 1 ng

In the field of stoichiometric SPRs, Sigvardson and Birks [184] used zinc reduction combined with fluorescence or chemiluminescence detection for the determination of nitro-containing polyaromatics As an alternative, the reduction was carried out also in the pre-column mode, again on-line; detection lim-

its can be as low as 0.1–10 pg The powdered copper-containing reactor studied by Irth et al. [126] for the determination of the tetraethyl drug disulfiram can also be employed for the trace-level determination of the tetramethyl pesticide thiram in surface water, food and soil

As for chemiluminescence detection, in the presence of an excess of a fluorophore, the peroxyoxalate system has been used successfully to determine traces of hydrogen peroxide either produced in LC–IMER systems (cf Section 3 4) or present in rain water [146]

The parallel-column system was adopted for the determination of aflatoxins [74] In this instance, the parallel column was packed with solid iodine and a knitted open-tubular reactor was mounted between the (post-column) mixing tee and the detector Iodine is only sparingly soluble in the aqueous LC mobile phase; therefore, the parallel column can be used for the delivery of a saturated iodine solution over long periods of time In the reactor (40 s; 60°C), attachment of iodine to the double bond of aflatoxins B_1 and G_1 is effected, which makes them as fluorescent as aflatoxins B_2 and G_2. Analysis of peanut butter gave MDCs of about 1 ppb for all four solutes.

5 AUTOMATED PRE-COLUMN REACTION DETECTION

Another aspect of interest concerns the application of reaction detection techniques in an on-line pre-column rather than post-column mode, which has already been briefly mentioned above [169,171,184]. The appearance on the market of fully automated injection systems for LC has stimulated this development Simple and relatively fast pre-column true chemical derivatization procedures with reaction times of ca. 1 min or less can be automated by using sample processors. These processors are able to perform a limited number of sample manipulations such as addition of solvents and, in the presence of one or more six-port valves, pre-column derivatizations.

Amino acid analysis is a typical example where automated pre- and post-column derivatization compete with each other Commercial instrumentation is available from, e.g., Hewlett-Packard, Varian and Spectra-Physics. The combination of an auto-injector and an autosampler, both fully programmable, allows the pre-column labelling of amino acids The system is able to dilute, to extract, to adjust the pH and to add another solvent or reagent before injection on to the analytical column The derivatization kinetics can be optimized by using the injector's thermostatically controlled micro-oven. Derivatization takes place in the injection needle and sample loop, and mixing is controlled by moving the liquids forward and backward in the loop The method has been applied to the determination of amino acids in biological, pharmaceutical, plant and food samples and also protein hydrolysates [185] The primary amines are first derivatized with *o*-phthaldialdehyde in the presence of 3-mercapto-

propionic acid, while the secondary amino groups are derivatized in a consecutive step with 9-fluorenylmethyl chloroformate [186] The reaction time in this two-step derivatization procedure was 1 5 min, resulting in MDAs of 20 fmol The total analysis time, including the reaction, separation and reconditioning, ranged from 20 min for the hydrolysates to 60 min for the physiological fluids A similar approach was described by Liang [187] In this system a mixer (e.g , a short coiled packed-bed reactor) can be installed between the column and the injector to increase the reaction time

As an alternative, 9-fluorenylmethyl chloroformate can be applied in combination with 1-aminoadamantane [188] or as the only derivatizing agent [189]. Application of this reagent for analysis was previously hampered by the formation of a fluorescent side-product, fluorenylmethanol The generated excess of the side-product is now removed via on-line extraction with, for instance, pentane (Fig 17) Automated pre-column derivatization of secondary amines can also be performed with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole The labelling was performed in a reaction coil for 11 5 min at 60 °C [190]

An interesting extension of the use of the *o*-phthaldialdehyde derivatization principle has been published by Buck and Krummen [191] A laboratory-made automated pre-column derivatization unit coupled on-line with the LC system was employed for the conversion of amino acid enantiomers into their fluorescent diastereomers by rapid reaction with *o*-phthaldialdehyde and N-*tert* -butyloxycarbonyl(Boc)-L-cysteine instead of 2-mercaptoethanol (Fig 18) Gradient elution on a conventional C_{18}-bonded phase yields the required separation for about twenty D,L-amino acid pairs with low-picomole detection limits Application of the pre-column mode was possible because the isoindoles formed are relatively stable, with a loss in fluorescence intensity of 37% in 12 h The labelling reaction was performed in an inert packed-bed reactor at ambient

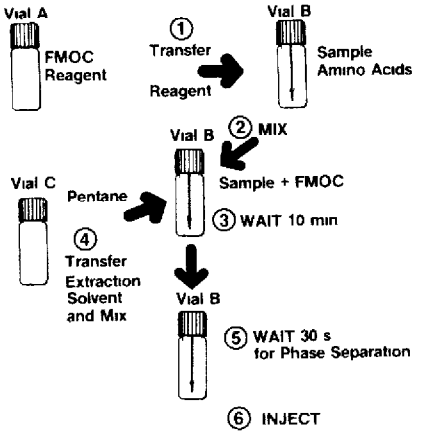

Fig 17 Automated pre-column derivatization sequence of amino acids with 9-fluorenylmethyl chloroformate (FMOC), marketed by Varian (Sunnyvale, CA, U S A) [188]

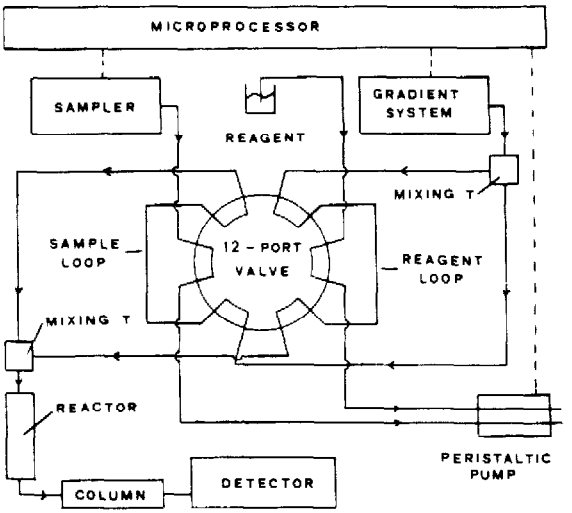

Fig 18 Automated pre-column derivatization scheme for labelling of amino acids with *o*-phthaldialdehyde [191]

temperature and using a reaction time of 75 s Similar on-line chiral probing has been studied by Nimura and Kinoshita [192]

Automated derivatizations can also be performed on-column [193] With amino acids the analyte solution and the premixed reagent solutions are automatically transferred from two different vials, by an injection system, on to the column Mixing is performed at a flow-rate of 0.02 ml/min. After a reaction time of 1 min the flow-rate is increased to 0 35 ml/min and the derivatives are separated by a gradient programme on a C_{18} column. Over 100 samples can be analysed unattended with this system Another device for in situ pre-column derivatization allows the direct application of samples and the introduction of the whole derivatization mixture on to the analytical column [194] This device is applied for the derivatization of primary amines with fluorescamine.

Recently, phase-transfer catalysis has become an interesting method to improve the kinetics of derivatization reactions. Generally such work is done in an off-line mode, but two papers have appeared in which derivatization is performed on-line, viz , using an autosampler. De Ruiter and co-workers [195,196] reported on the rapid (5 min) water–dichloromethane two-phase dansylation of phenolic steroids with tetrabutylammonium bromide as phase-transfer catalyst After reaction, an aliquot of the organic phase was transferred on-line to the NP-LC system Fluorescence monitoring of the derivatives allowed the detection of low-nanogram amounts of the studied steroids (Fig. 19).

Van der Horst and Holthuis [197] used micellar transfer catalysis for the rapid labelling of, for instance, valproic acid and plasma free fatty acids with the highly fluorescent label 9-bromomethylacridine. With Arkopal N-130 as

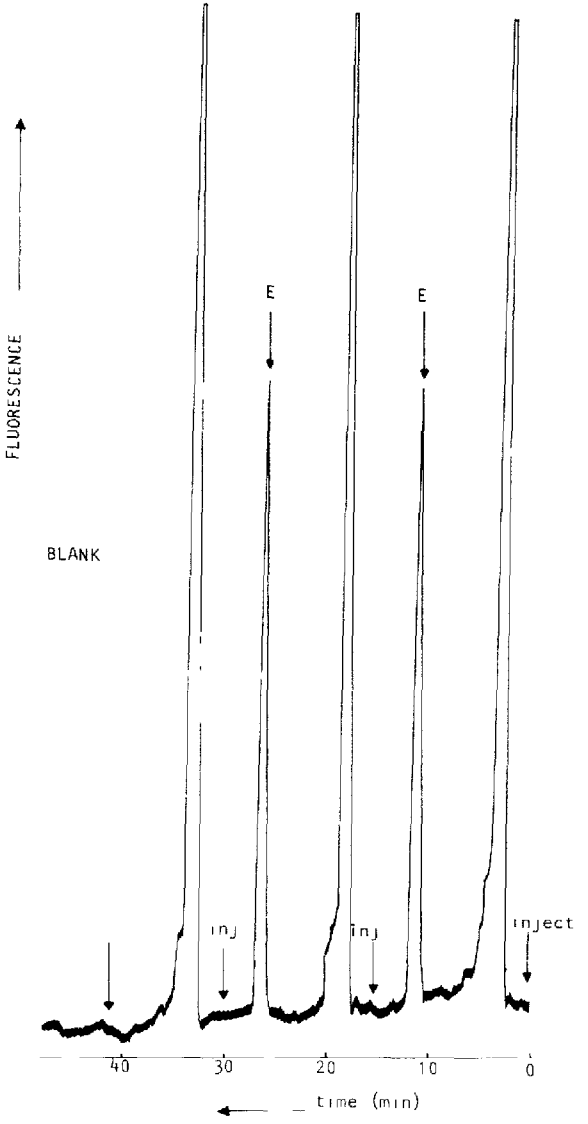

Fig 19 LC of dansylated estradiol (E) using on-line phase-transfer catalysis Blank urine sample (left) and 50-ml urine samples spiked with 10 ng/ml E (centre and right) LC system C_{18}-acetonitrile-water (35 65) [196]

the surfactant and tetradecylammonium bromide as the catalyst, 10 min at 50 °C were sufficient for conversion of the analytes Even with non-deproteinized plasma samples, an aliquot of the micelle-containing reaction mixture could be introduced directly on-line into the RP-LC system

5 1 Pre- and post-column sample treatment

An efficient chromatographic separation combined with a powerful mode of detection is often not enough to allow the determination of trace-level constituents In this paper, the potential of post-column reaction detection as a means of remedying this deficiency is highlighted One should be aware of the fact, however, that on-line pre-column sample manipulation – other than the reaction detection systems discussed above – is a powerful alternative for achieving the desired sensitivity and/or selectivity, while maintaining the automation potential For an exhaustive treatment of on-line pre-column technology for sample clean-up and the enrichment of trace-level analytes, readers should consult the pertinent chapters in a book by Frei and Zech [198] The recent introduction, next to the conventional non-selective C_8- and C_{18}-bonded phases, of selective pre-column packing materials such as metal-loaded phases [199] and supports loaded with immobilized antibodies [200] will often allow the analytical chemist a choice between sophisticated pre- and post-column sample manipulation This novel development is, perhaps, reflected in the fact that LC with direct UV–visible absorption detection is becoming more popular again for trace-level analysis For the rest, it should be evident that the combined use of on-line pre- and post-column manipulations is an option open when solving complicated problems. Several examples utilizing this approach are summarized in Table 8.

TABLE 8

EXAMPLES OF COMBINED ON-LINE PRE- AND POST-COLUMN BIOANALYTICAL MANIPULATION IN LIQUID CHROMATOGRAPHY

Analyte(s)	Procedure[a]	Ref
Amprolium	Dialysis, precolumn, LC, oxidation, fluorescence detection	205
Bile acids	Dual precolumn, LC, IMER, chemiluminescence detection	250
Chloramphenicol	Dialysis, precolumn, LC, derivatization, detection	251
Peptides	Dialysis, precolumn, LC, derivatization, fluorescence detection	252
Remoxipride	AASP, LC, ion-pair formation, fluorescence detection	125
Secoverine	Precolumn, LC, ion-pair formation, fluorescence detection	221
Vencuronium-type drug	PROSPEKT, LC, ion-pair formation, fluorescence detection	115

[a]On-line pre-column manipulation by means of the AASP (Varian, Sunnyvale, CA, U S A) or the PROSPEKT (Spark Holland, Emmen, The Netherlands)

6 CONCLUSIONS

In the last decade, the potential of on-line post-column reaction detection in LC has become increasingly popular in the biomedical and other research areas Commercial equipment is starting to become available although, surprisingly, much less readily than in the area of on-line pre-column technology Additional hardware, such as mixing tees, phase separators and reactor construction materials, today are of such quality that extra-column peak broadening can be kept to a minimum, for both conventional-size and microbore LC applications

It is obvious that open-tubular reactors will always remain popular because of their easy construction and simplicity Reactor residence times typically should not be over about 1 min but, in view of the many applications, this obviously is not a stringent demand For reactions with slower kinetics and, more often, when an excess of reagent has to be removed on-line post-column, segmented-stream reactors serve a useful purpose

Most early reaction detection studies require the use of one, or more, additional LC pumps for reagent introduction This distinctly increases the cost per analysis and often also affects the sensitivity because of increased noise One possibility for circumventing the above problem is the use of, e.g , electrochemical, photochemical or thermo-initiated reactor systems, where electrons, photons and heat are the only 'reagents' used In actual practice, the first two alternatives are often found to have excellent selectivity Another option is the utilization of hollow-fibre membrane reactors, which possess a wide range of possibilities for simple reagent introduction They certainly should be studied in more detail in the future

The on-line introduction of an SPR is another way to set up a pumpless reaction detection system, with the parallel-column approach as an imaginative alternative Stoichiometric solid-phase reactions certainly are useful, but systems based on heterogeneous catalysis and, notably, IMERs attract much more attention, and rightly so The inherent sensitivity and selectivity of the latter type of reactors, especially if combined with chemiluminescence or laser-induced fluorescence detection [201], should not be underestimated It should be a favoured research area in years to come

On-line and semi-on-line pre-column derivatization or analytical conversion is gradually becoming a worthwhile alternative to post-column techniques. So far, attention has been virtually restricted to the determination of amino acids in various matrices, but the potential of the approach is, of course, much wider In this field, pre-column derivatization with chiral agents to allow the separation of the (labelled) enantiomers on the conventional LC column is an area of much interest

As regards the detection step proper, in most instances this is done by means of fluorescence monitoring In the near future, the emphasis on luminescence

detection will probably remain unaltered, especially if laser-induced fluorescence becomes a more reliable and less expensive tool. This does not imply that other modes of detection will become superfluous On the contrary, and to name only a few, UV–visible absorbance because of its simplicity, electrochemical detection because of its selectivity and mass spectrometry because of its identification power will all remain of interest. As a final demonstration of this statement and of the great versatility of post-column reaction detection, even radioactivity monitoring has recently been applied in a reaction detection system. One example is the determination of ^{14}C-labelled urapidil and its metabolites in rat plasma [202]. After RP-LC, a water-immiscible liquid scintillator was mixed with the LC effluent and, subsequently, the radiolabelled analytes were extracted into the scintillator plugs and led on-line through the radioactivity detector

Finally, one may state that, in principle, the main advantage of post-column reactors over pre-column systems is the fact that they can be easily automated. Automation as such is an important parameter as the accuracy and precision can be improved significantly in this way However, nowadays various derivatization and related reaction detection techniques can also be introduced as automated pre-column procedures using programmable autosamplers and autoinjectors For the rest, the power inherent in systems combining automated pre- and post-column manipulation has already been discussed in Section 5 1.

7 SUMMARY

The increasing interest in the rapid trace analysis of large series of biomedical samples using column liquid chromatographic techniques requires the use of well balanced combinations of sample pretreatment, separation and detection techniques In such work, selectivity, sensitivity and reproducibility are the key parameters. The application of automated or semi-automated on-line pre-column technology and/or post-column reaction detection are excellent ways to meet these requirements

A critical review is presented of the theoretical background of on-line post-column reactors with emphasis on their categorization, viz , open-tubular, packed-bed, segmented-stream and hollow-fibre membrane reactors The evaluation of these reactor systems is performed by discussing selected applications of, for instance, systems based on electrochemical and redox, hydrolytic, photochemical, ion-pairing, true chemical derivatization, peroxyoxalate chemiluminescence and solid-phase reactions

As automation is becoming even more important, a number of labelling procedures, which can be performed in an on-line pre-column mode, are briefly discussed and a comparison is made between the potential of on-line pre- and post-column procedures.

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