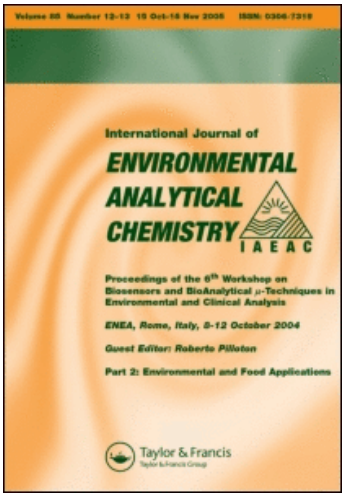

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Handling of Environmental and Biological Samples via Pre-Column Technologies

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Handling of Environmental and Biological Samples via Pre-Column Technologies†

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Sample handling is still a weak point in chromatography and in analytical chemisty in general. One consideration is the automation potential of new procedures. Solid–liquid extraction techniques in combination with pre-column technology are particularly promising in this regards. The construction and geometry of pre-columns both for conventional and narrow-bore HPLC are of major importance, since band broadening should be kept at a minimum for an optimal functioning of the analytical system. The various operations that can be carried out with such a pre-column are trace-enrichment, clean-up of the sample which depends on the type of adsorbents used in the precolumn, i.e., polar or apolar materials, ion exchangers or metal covered surfaces, etc., protection of the analytical column, field sampling and storage of samples and as a substrate for on-column chemical derivatizations. These various operations are demonstrated with practical examples from the fields of environmental and biological analysis. The selectivity can be further enhanced by coupling pre-column technology with selective detection modes such as diode array UV, electrochemical or fluorescence detection. This enables the construction of optimal and integrated analysis sytems which are fully automated and microprocessor controlled. They can also be made compatible with miniaturized LC-technology.

INTRODUCTION

The major short comings in modern HPLC technologies can be found in the area of sample handling; availability of stable and

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reproducible sorbents and the detection process. All three areas have to be considered simultaneously if the overall system performance has to be improved. In addition, the possibility of automating and sometimes miniaturizing such an analytical system is of prime importance when deciding on an optimal combination of sample handling, separation and detection strategies.

On the sample handling side this has resulted in the development of solid–liquid extraction procedures which are often more sophisticated than conventional extraction, evaporation or distillation techniques. A highly promising approach is to enrich trace compounds of interest on suitable sorbents,[1] in order to isolate and preconcentrate them prior to their separation and detection by means of a chromatographic technique. The sorbents, such as silica, alkylsilane-modified silica, alumina, porous polymers with or without ion exchanger groups or carbon materials are usually contained in a short stainless steel or glass column or cartridge; these are called pre-columns when operated on-line with a chromatographic column, and can be operated at elevated pressures. Sample volumes are typically between about 1 ml and 1 L, and the samples themselves are very diverse in nature. They can range from aqueous samples—which include surface waters and biological fluids—to extracts from solid, liquid or even gaseous matrices, which are often encountered in, e.g., residue analysis or even to biological fluids such as urine or plasma injected directly, or after dilution, or deproteination onto a pre-column.

The design of such a pre-column can be quite diverse varying in length from 3 cm to 2 mm and in inner diameter anywhere from 4.6 mm to 1 mm. The trend is to make them in the form of compact cartridges which can be mounted in a suitable holder and inserted into a high pressure system. Several companies are now offering pre-columns. A typical design is described in Figure 1. The cartridge can be loaded off-line (i.e., under field conditions) or on-line and the compounds are transferred on-line to the chromatographic column. The pre-columns can be hand-packed by pushing a slurry of adsorbent into it with a spatula or via a syringe. A syringe could also be used to load samples onto a pre-column. The use of short pre-columns is often recommended to save on expensive adsorbents, to enable an easy manual packing, to reduce backpressure when loading the cartridges and to reduce band broadening in the total system.

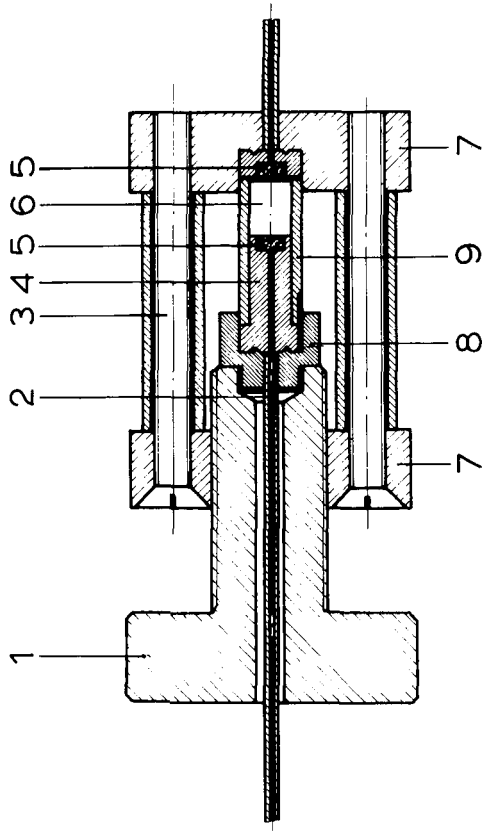

FIGURE 1 New pre-column design aimed at improving performance and flexibility. 1 = Hand-tightened screw; 2 = stainless steel capillary, $\frac{1}{16}''$ O.D., 0.25 mm I.D.; 3 = bolt (of column holder); 4 = PVDF rod, 4.6 mm I.D.; 5 = stainless steel frit (variable diameter, dependent on column diameter); 6 = plug of stationary phase; 7 = column holder; 8 = stainless steel cap; 9 = pre-column (stainless steel tube, length 1 cm, $\frac{1}{4}$ in. O.D., 2.2–4.6 mm I.D.)

The main functions of such a sampling column will be discussed briefly.

Trace enrichment

For compounds with a strong affinity for the sorbent used, adsorption will take place in a small segment of the sorbent bed; large

volumes can be handled at high sampling speed, and breakthrough does not occur even with short columns. A typical example is the trace enrichment of relatively apolar organic compounds from an aqueous solution on hydrophobic sorbents such as porous polymers, alkylsilane-modified silica or activated carbon. With such systems, volumes of up to 1 L can be safely handled on 2–4 mm long precolumns; in other words, compared to the conventional 100-μl injection on, for example, LC columns, concentration factors of up to 10,000 can be obtained. If trace enrichment has to be effected for more polar compounds, sample volumes must be correspondingly lower or pre-columns longer, to prevent breakthrough and thus loss of trace component. Alternatively the choice of a sorbent with higher affinity or derivatization with a suitable reagent to convert the polar component into a less polar derivative can be used.

Clean-up

The more or less selective interaction between the various constituents of a sample and the sorbent will often permit the separation of certain groups from each other, even during the loading step. As an example weakly retained phenolic or acidic compounds will be easily separated from strongly adsorbed phthalate esters on an apolar sorbent. As a result at least a partial removal of interfering compounds will occur. Step-wise desorption of concentrated constituents, by means of step-gradient elution, can further enhance the clean-up function.

The use of more selective sorbents such as ion exchangers, metal loaded phases or supports used in affinity chromatography can further enhance the clean-up potential of our pre-column.

Storage of samples and guard function

Due to the relatively inert character of many of the sorbent materials, particularly the porous polymers, chances that a compound in the adsorbed state will remain unaltered for a prolonged period of time are excellent. Stabilization of otherwise rather non-stable species can be highly useful when storage or transportation of samples is required. This will be important when sampling takes place at remote sites, with the result that long-term storage is

unavoidable. In addition the precolumn also protects our expensive analytical column hence extending the life time of the separation system.

ON-LINE TECHNIQUES

The need for automated sample handling techniques makes it necessary that desorption and transfer of the trace components of interest to the analytical column should be done on-line. A typical scheme for an on-line procedure is shown in Figure 2 with pump A

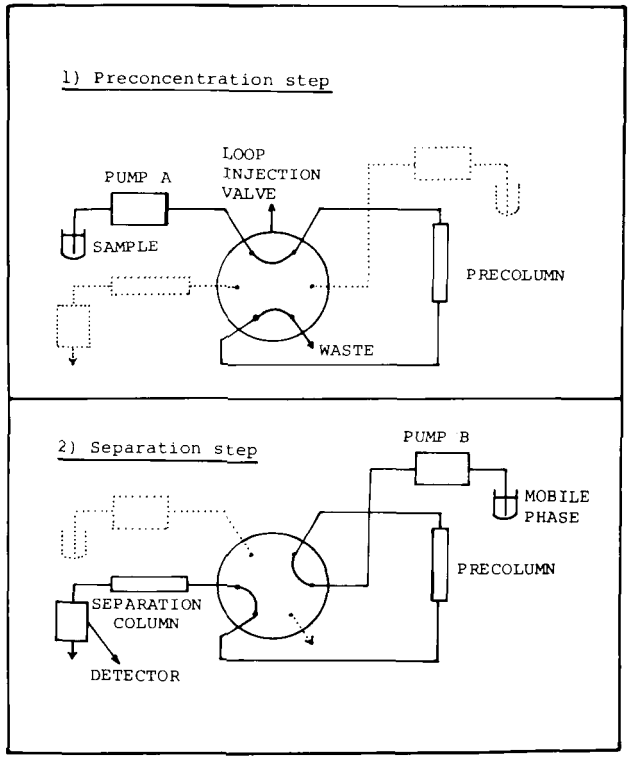

FIGURE 2 Schematic diagram of apparatus for on-line trace enrichment using a pre-column.

being used for the sample-loading or trace enrichment step and pump B for the desorption and on-line transfer of the sample to the separation column.

Handling of low polarity compounds

For the pre-concentration of low polarity compounds on hydrophobic surface, only a very small volume of hydrophobic adsorbent is needed to retain large volumes of aqueous samples. Typical applications of this nature are the trace enrichment of PAH's, phthalates, PCBs, etc., from surface water samples. The loading capacity of a 2×4.6 mm I.D. (internal diameter) typical pre-column is at least about 10 μg. With, for example, PCBs, phthalate esters and (from an acidified aqueous solution) pentachlorophenol, a 2 mm layer of C18 adsorbent is sufficient for handling 0.1–1 l water samples. Even at pumping speed of 10–25 ml min^{-1}, recoveries are over 90%. Here the prime advantage of the use of a short pre-column is the low pressure drop, where rapid large volume sampling is possible.

The above technique has been applied to the analysis of dibutyl and di(2-ethyl-hexyl) phthalate[2] in 1 L volumes of tap and mineral water and soft drinks, and of pentachlorophenol in river and lake water samples. It has also been employed for the trace enrichment of PCB mixtures[2] and for the analysis of pesticides residues.[3] A typical chromatogram is shown in Figure 3. As can be seen, the instalment of the pre-column did not cause additional band broadening.

Handling of medium to high polarity compounds

The situation becomes often problematic when we are dealing with medium to high polarity compounds such as certain substituted phenols or anilines or the more polar metabolites of low polarity parent compounds. Pre-concentration solely based on hydrophobicity is often not sufficient and breakthrough can occur after only a fraction of a milliliter of sample has been deposited. This breakthrough is in the majority of situations a function of the high mobility of our analytes in the pre-column and not of overloading on the adsorbent. In such cases it becomes necessary to study the breakthrough behaviour of the analytes of interest on a particular sorbent.

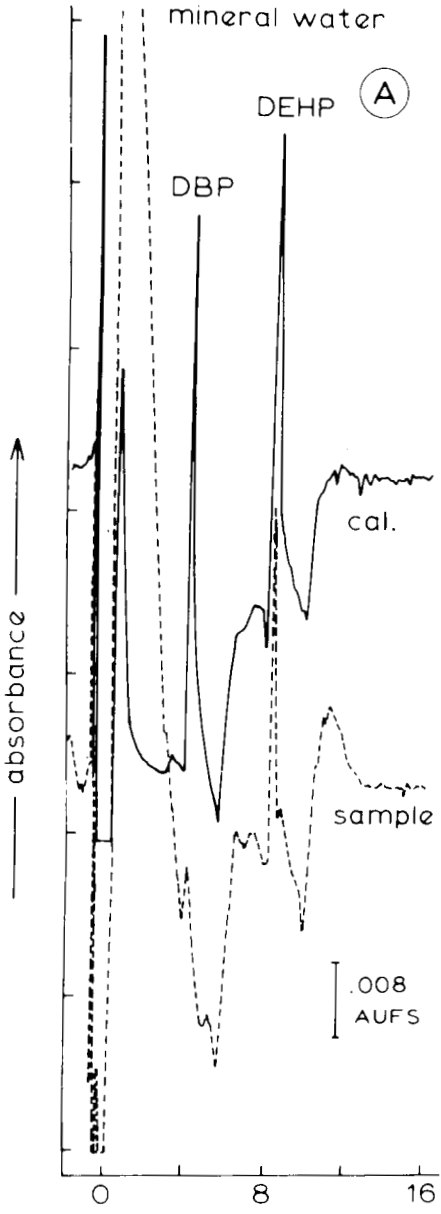

FIGURE 3 HPLC chromatogram recorded for a standard solution (--) of di-n-butyl and di(ethylhexyl)phthalate, and for a sample (—) of mineral water containing 0.08 and 1.23 ng of these phthalates per ml, respectively. Conditions: sample solution, 800 ml; 2 × 4.6 mm 5-μm LiChrosorb RP-18 pre-column; loading at 25 ml.min^{-1} pumping speed; analysis at 2 ml.min^{-1} on a 12.5 cm × 4.6 cm I.D. LiChrosorb RP-18 separation column with 75% (60 s), 85% (300 s) and 95% (270 s) methanol in water as mobile phase; detection at 233 nm (0.08 AUFS) (ref. 2).

Once this is known, the maximum sample volume which can be handled without loss can be determined or the necessary geometry of a pre-column, to pre-concentrate a pre-set volume of sample, can be calculated. Measuring such breakthrough curves can be tedious and time consuming and so Werkhoven *et al.*[4] proposed a simple extrapolation technique which enables the estimation of breakthrough volumes, from rapidly obtained chromatographic data.

Finally, another solution to this problem is, to resort the different types of adsorbents which possess a higher affinity for the compounds to be analyzed.

1. Carbon and polymer based materials Various substituted aromatic compounds were investigated with other adsorbents such as carbon based materials[5] or styrene divinylbenzene copolymer (PRP_1).[6] It was found that these materials have a considerably stronger affinity for, e.g., chlorosubstituted phenols or other chloro- or nitro-substituted aromatics than does C18 chemically bonded silica gel. Table I shows a comparison of breakthrough volumes for selected mono- and dichlorophenols which on C18 were not at all or poorly retained but on pyromodified carbon black (according to Colin and Guiochon[5]) or on PRP_1 material (Hamilton, Reno, NV, U.S.A.) trace enrichment characterization were significantly improved.

2. Ion exchanger materials: For other compounds, with still more polar and ionic characteristics, one can also apply HPLC grade ion exchange materials to carry out a group-wise preconcentration and

TABLE I

Comparison of break-through volumes of lower chlorinated phenols on various sorbents

			Break-through volume (ml)	
Sorbent	d_p	S (m^2/g)	Chlorophenol	Dichlorophenol
C18	5; 10	200	0	10
PMCB	45–50	80	5	30
PRP_1	10	415	30	>200

PMCB = pyrocarbon modified carbon black.
PRP_1 = divinyl benzene styrene copolymer.

isolation of such compounds. Such an example is the preconcentration of polar anilines on strong cation exchangers prior to HPLC analysis. The method was applied to trace determination of these compounds in polluted surface water.[7] The typical chromatogram for a river sample obtained via such an on-line pre-column sampling technique is shown in Figure 4. The high selectivity and sensitivity of the used electrochemical detection principle enabled direct quantitation of sub-ppb concentrations of anilines originally present in the sample. However, a note of caution is necessary when using ion exchangers for treatment of real samples. The ion exchange resins exhibit a hydrophobicity effect and therefore apolar compounds in the sample are also loaded on the surface and can seriously reduce the capacity of the resin. Another cause for problems is the presence of interfering ions which take up the available ion exchange sites and reduce the capacity for analytes. In the present example hard water with its high Ca^{2+} concentration and heavy metals (Fe^{3+} in particular) had a detrimental effect and made regeneration of the pre-column almost impossible.

Hence, the measures of precaution that had to be taken included addition of oxalic acid and EDTA to precipitate and complex the ionic interferences (see also Table III). The apolar constituents can be removed by inserting an on-line PRP_1 or C18 pre-column prior to the ion exchanger cartridge. In this fashion, repetitive analyses of real samples were possible in a fully automated system. An example for the high complexity of samples which can be handled by pre-column technology is illustrated below with the screening for priority pollutants in industrial waste water. Three pre-columns packed with various sorbents are now used in series to permit a group wise extraction of low-polar, medium polarity and ionic pollutants.[8] A schematic of the apparatus used is shown in Figure 5. Table II lists the compounds for which the screening had to be done and indicates their trace enrichment capability on the three adsorbents chosen. It can be seen that the nonpolar compounds (no. 20–29) are trapped on the C18 pre-column but the retention for p-dichlorobenzene and o-dianisidine is rather low. The o-dianisidine plus the chloro- and nitro-substituted aromatic compounds (no. 12–19) are, on the other hand, well retained on the PRP_1 material and can be preconcentrated on the PRP_1 pre-column. Of these, only 3,5-dinitro-o-cresol will also be partly retained on the C18 pre-column and hence be

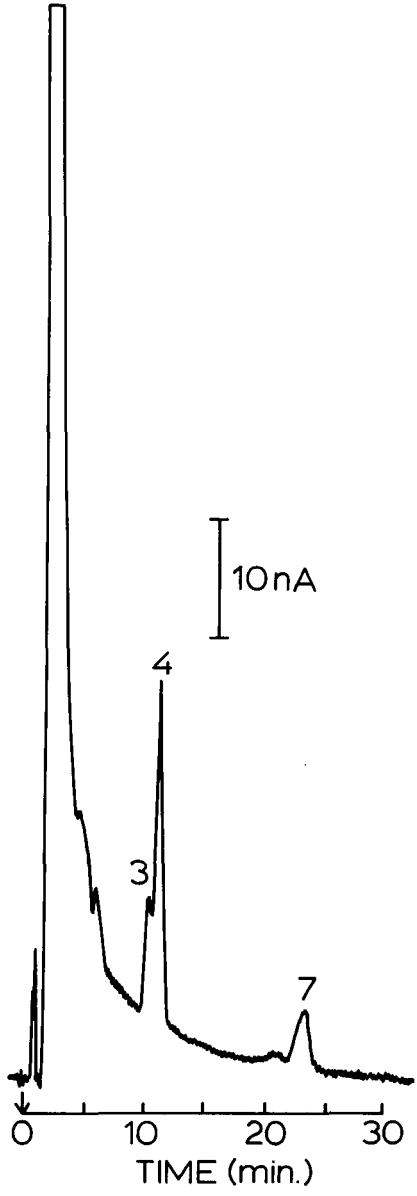

FIGURE 4 Chromatogram of an Amstel river water sample containing (non-spiked) amines; 10 ml of sample concentrated on a 5 × 3.0 mm I.D. Aminex A7 pre-column. Analytical column: 20 cm × 3.0 mm I.D. CP-Spher C18. Eluent: 0.07 M potassium citrate (pH 6)-methanol 7:3) at 0.4 ml min^{-1}. Electrochemical detection at +0.9 V (attenuation, 100 nAFS). (3) p-anisidine (4) aniline (7) o-toluidine. Concentrations approximately 0.5 ppb.

TABLE II

Breakthrough volumes of 29 selected pollutants on short pre-columns packed with various sorbents[a]

No.	Compound	Breakthrough volume (ml) on RP 18, 10 μm 2 × 4.6 mm I.D.	PRP_1, 10 μm 4 × 4.6 mm I.D.	Aminex A5, 13 μm[b] 4 × 4.6 mm I.D.
1	p-aminophenol	0	0	>100
2	p-phenylenediamine	0	0	>100
3	m-phenylenediamine	0	1	>100
4	4-methyl-m-phenylenediamine	0	1	>100
5	o-phenylenediamine	0	1	>100
6	aniline	0	2	>100
7	p-anisidine	0	1	>100
8	p-nitroaniline	1	10	>100
9	3-amino-4-ethoxyacetanilide	1	7	>100
10	o-anisidine	1	6	>100
11	o-toluidine	1	3	>100
12	picramic acid	2	>100	—[c]
13	p-chloroaniline	2	30	—
14	p-nitrophenol	1	25	—
15	3,5-dinitro-o-cresol	10	>100	—
16	m-cresol	1	37	—
17	nitrobenzene	2	>100	—
18	p-chlorophenol	2	72	—
19	p-chloronitrobenzene	3	>100	—
20	pentachlorophenol	>100	—	—
21	o-dianisidine	10	78	—
22	2-aminoanthraquinone	>100	—	—
23	3,3′-dichlorobenzidine	72	—	—
24	3-amino-9-ethylcarbazole	50	—	—
25	p-aminoazobenzene	>100	—	—
26	1-aminoantraquinone	>100	—	—
27	p-dichlorobenzene	17	—	—
28	2-phenylaminonaphthalene	>100	—	—
29	1,2,5-trichlorobenzene	>100	—	—

[a]LC-water samples containing 250 ppb of test solute; pH adjusted to 3.0 with perchloric acid; sampling rate 5 mL min^{-1}.

[b]Maximum values (cf. ref. 7).

[c]Not determined.

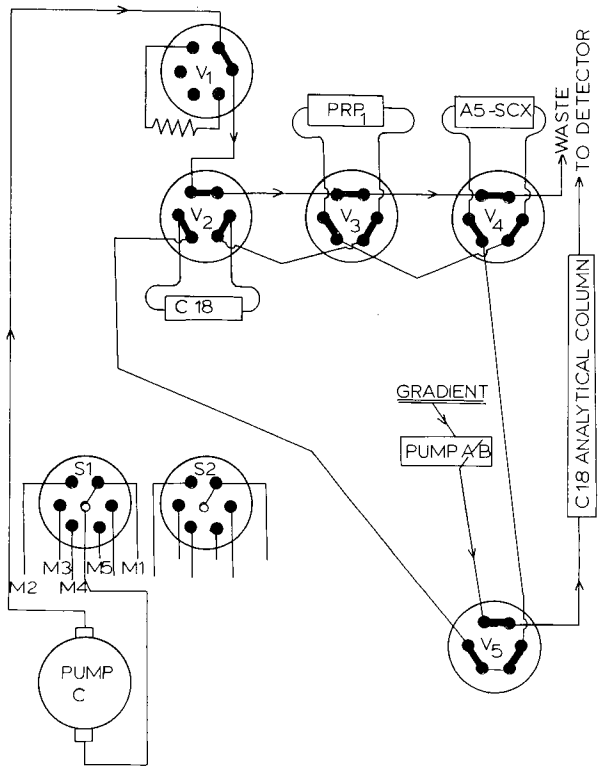

FIGURE 5 Experimental setup for the on-line group separation and trace enrichment of wastewater samples: V, high pressure switching valve; S, low pressure selector valve; M1, sample; M2, 10^{-3} M $HClO_4$; M3, 50% methanol; M4, 0.02 M $HClO_4$; M5, 10^{-3} M $HClO_4$. Pre-columns: 2 × 4.6 mm I.D. (RP 18), 4 × 4.6 mm I.D. (PRP_1), and 4 × 4.6 mm I.D. (Aminex A5). Analytical column: 25 cm × 4.6 mm I.D. CP-Spher C18.

present in the C18 fraction. The remaining 11 compounds (1–11) which are polar anilines, can be successfully preconcentrated at pH 3 on the cation-exchange precolumn from relatively large sample volumes (cf. ref. 7). Of these, only nitroaniline will have been partly absorbed on the PRP_1 precolumn.

A summary of the automated absorption, desorption and regeneration procedure is given in Table III. Diode array detection was used to deal with complexity of the samples. Figure 6 shows a multisignal chromatogram obtained with 5 mL of standard solution (pH 3.0)

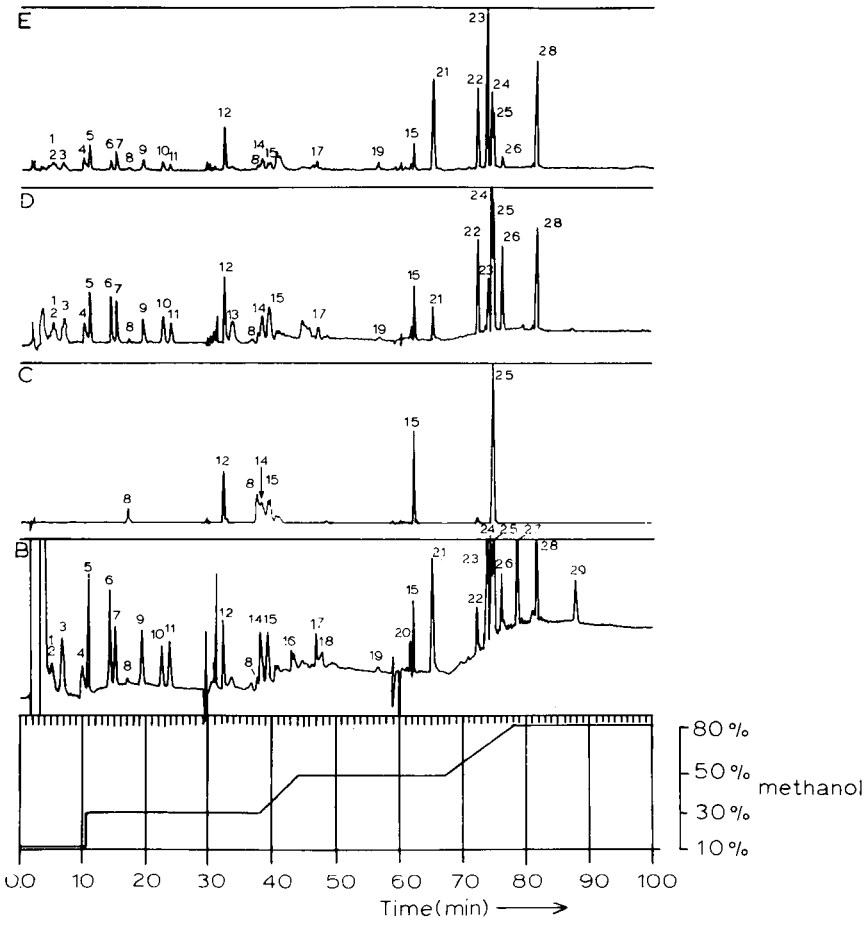

FIGURE 6 Multisignal plot of a 5-ml standard solution containing 200 ppb of the selected pollutants from Table II. Gradient elution with 0.1 M potassium acetate (pH 6.0) and methanol (10–80%) as indicated. Detection at 222 (B), 390 (C), 244 (D), and 295 nm (E). Attenuation was 0.2 AUFS. Peak numbers correspond to the compounds listed in Table II.

TABLE III
General procedure using the setup of Figure 5.[a]

1.	Precipitation and complexation of interferences by oxalate and EDTA, respectively
2.	Filtration and adjustment to pH 3.0 (if necessary)
3.	Group separation and trace enrichment on C18, PRP_1, and Aminex A5 pre-columns (in series)
4.	Flushing the C18, PRP_1, and Aminex A5 pre-columns (in series) with 10^{-3} M perchloric acid
5.	Further clean-up of the cation exchanger by flushing with 50% methanol
6.	Backflush desorption from cation exchange fraction to C18 analytical column
7.	Backflush desorption from PRP_1 fraction to C18 analytical column
8.	Desorption from C18 pre-column to C18 analytical column
9.	Regeneration of the pre-columns in series with 10^{-3} M perchloric acid

[a]Steps 3–9 are fully automated.

containing 200 ppb of each of the 29 pollutants of interest. With the gradient profile, as included in this figure, acceptable resolution was obtained. The first 28 min of the chromatograms correspond to the ion-exchange precolumn (the polar aniline fraction). The period between 28 and 58 min corresponds to the PRP_1 pre-column (other medium polar aromatics) and the final period, from 58 to 90 min, to the C18 pre-column (nonpolar compounds). Trace B, the chromatogram recorded at 222 nm, shows all peaks of interest and can be regarded as a nonselective "total-peak chromatogram". It is clearly shown here that only p-nitroaniline (no. 8) and 3,5-dinitro-o-cresol (no. 15) appear in more than one fraction. Trace C at 390 nm gives a very selective chromatogram, only nitro aromatics and p-aminoazobenzene will appear. Traces D and E (at 244 and 295 nm) give additional information. The polar anilines (no. 1–11) for instance will appear at these wavelengths; their secondary maximum around 290 nm may be an aid for group identification. At 244 nm, p-chloroaniline (no. 13) will be relatively intense as compared to 222 nm which is verified by Trace D. The dichloro- and trichlorobenzene (no. 27 and 29) show only a reasonable absorbance at low wavelengths; they appear only at 222 nm and not at one of the other wavelengths investigated. A typical spectrochromatogram for the PRP_1 fraction is shown in Figure 7. The nitro compounds (no. 8, 14 and 15) are recognized by their maxima at 390 nm. The picramic

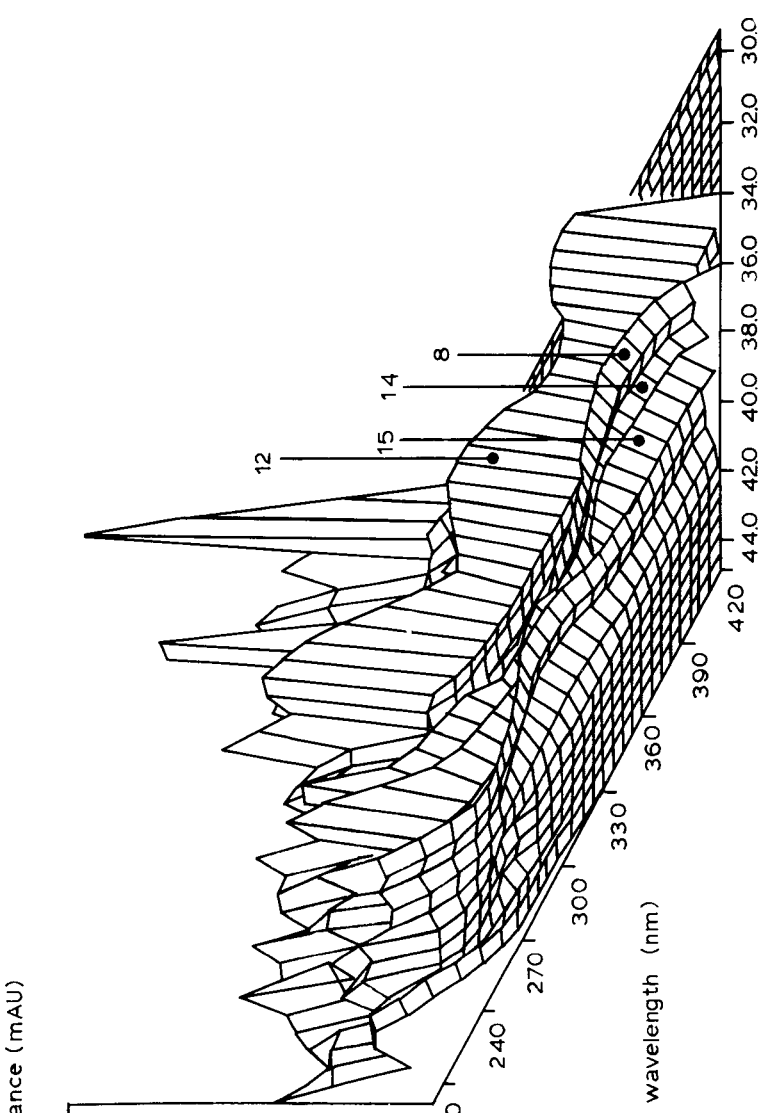

FIGURE 7 Spectrochromatogram of a part of the PRP_1 fraction from the analysis of Figure 6. Spectra were memorized automatically at the peak apex and at the base line after the peak. Conditions are given in Figure 6.

acid (no. 12) spectrum is clearly shown and a good example for the validity and usefulness for such plots for compounds with well-defined UV spectra.

Finally an application to a real sample is demonstrated in Figure 8 which shows a multisignal plot obtained with a 5 mL industrial

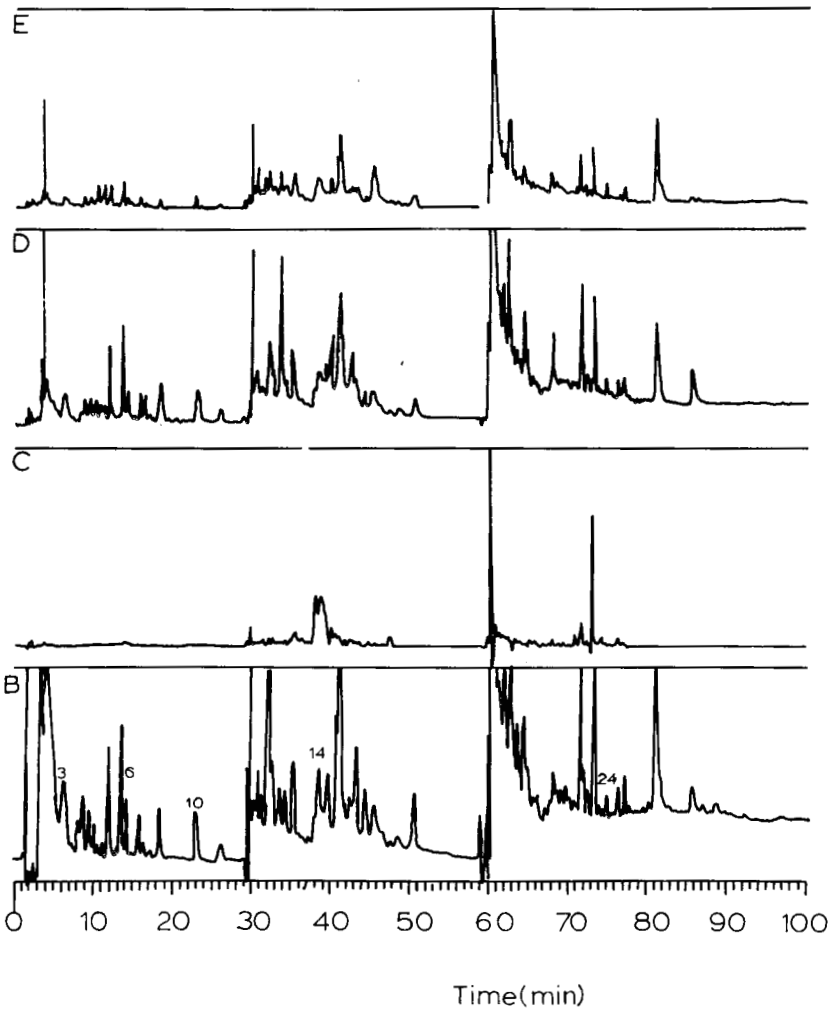

FIGURE 8 Multisignal plot of a 5-ml industrial wastewater sample, taken after a biological treatment plant. Conditions are given in Figure 6.

waste water sample, collected after a biological step in the water treatment plant. The first part (the ion-exchange fraction) shows peaks at 222, 244, 295 nm but not at 390 nm, indicating that there are no nitroanilines present but many other polar anilines. A comparison of retention times of polar anilines with the data obtained with well-defined standard samples will give useful information. m-Phenylenediamine, aniline, and o-anisidine could be identified in this way. The second and third part of the chromatogram (PRP_1 and C18 fraction) contained only two peaks of the pollutants of interest. p-Nitrophenol and 3-amino-9-ethylcarbazole were identified through their UV spectra. Spectra of the other peaks did not resemble the 29 reference spectra of the standard mixture, nor did their retention times.

The compounds tentatively identified in the waste water sample which was collected after biological treatment (see Figure 8) were quantitated by standard addition: 100 ppb of the standard mixture was added to the waste water sample which was reanalyzed. The concentrations were calculated from the relative peak heights with respect to the dilution factor and are m-phenylenediamine (260 ppb); aniline (150 ppb); o-anisidine (290 ppb); p-nitrophenol (260 ppb); 3-amino-9-ethylcarbazol (60 ppb).

3. Metal loaded supports The pre-concentration experiments described in the previous chapters were carried out with medium to non-specific sorbents. With these sorbents solute–sorbent interaction is mainly due to dispersive and electrostatic forces. For certain types of analytes, use can be made of specific sorbents for pre-concentration and/or liquid chromatography. Highly specific and strong solute–sorbent interactions can be obtained by means of ligand-exchange chromatography. Using metal-loaded phases, pre-concentration of organic analytes which possess complexing substituents groups can be performed. In this section an example is given of the potential of metal-loaded phases for on-line pre-concentration of organic analytes, using catecholamines as model compounds.

A Cu(II)-loaded iminodiacetate-modified silica (IDA) has been described (see Figure 9). This phase was used for the separation of some amino acids and dopamine. A strong influence of pH and ionic strength of the buffer solutions used as mobile phase on solute retention was observed. In the present study we investigated the

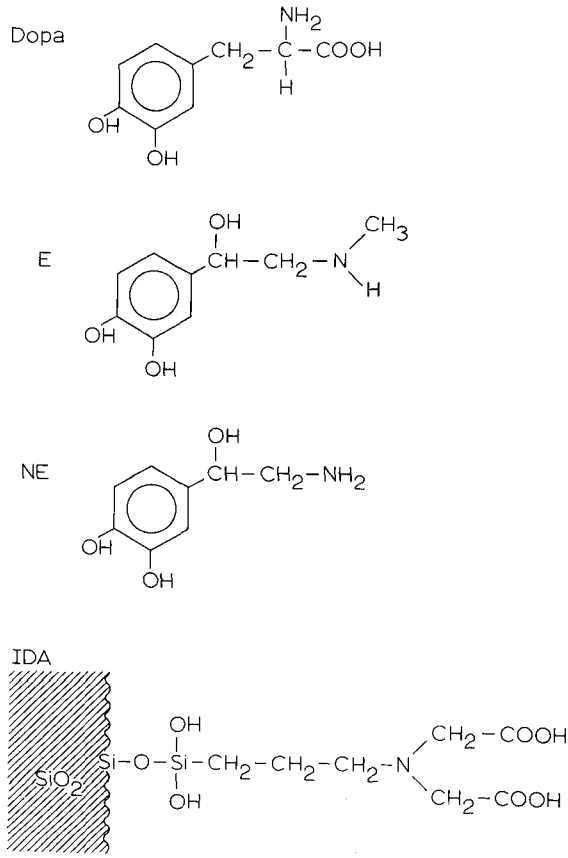

FIGURE 9 Structures of the catecholamines dopamine (Dopa), epinephrine (E) and norepinephrine (NE) and of the iminodiacetate phase (IDA).

applicability of the IDA-Cu phase for pre-concentration of the catecholamines dopamine, epinephrine (adrenaline) and norepinephrine (noradrenaline).

Retention behaviour of solutes on the IDA-Cu phase is primarily based on complexation with the fixed Cu(II) ion and is influenced by the properties of the mobile phase. Retention of amino acids was found to be low at low pH values, which was explained by the decreased complex formation tendency of the ammonium and carboxyl groups. Retention of negatively charged compounds such as

carboxylate anions decreases again at high pH values, due to the influence of negatively charged iminodiacetate and dissociated silanol groups of the IDA phase. The potential for using similar phases for, i.e., organic acids or complexing pollutants in water samples is currently being explored with promising results.[11]

Another application of metal loaded surfaces has recently been worked out by combining a cartridge with platinum loaded material with a C18 pre-column for the group separation of phenylurea pesticides from their aniline metabolites and final separation and quantitation of the intact herbicide.[12]

In the determination of the phenylureas, a special problem is the discrimination between these analytes and the substituted anilines which are their main (bio)degradation products. In addition, the latter are generally present in abundance as they are bulk chemicals as well as products of several industrial activities. Discrimination between a single herbicide and its corresponding aniline can easily be achieved by means of LC. However, severe problems are encountered, when a relatively large number of phenylureas and anilines have to be screened. Recently, several combined LC-GC analysis schemes have been elaborated for the determination of 15 phenylureas and the corresponding anilines.[13] These are, however, rather complicated and there is a need for simpler procedures which, in the case of water samples, should involve the use of pre-column technology. To solve this problem chemically modified (ACDA) silica gel surfaces according to the following structure (see Figure 10) were studied.

When Pd or Pt have been complexed to the ligands the substrate is able to act as an efficient filter for all the anilines investigated. The herbicides which did not interact with the metal were then preconcentrated on a C18 loaded pre-column and transferred on-line to a C18 reversed phase column for final separation. The schematic

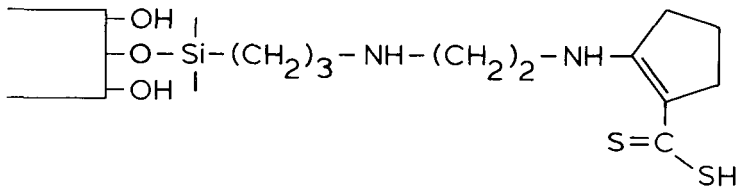

FIGURE 10 Structure of the ACDA-Pt phase.

drawing for the valve switching unit which had been used for a fully automated processing of water samples is shown in Figure 11. The analysis of a river water sample for these herbicides with and without the use of such an ACDA-Pt filter is demonstrated in Figure 12. It can be seen that the metal loaded cartridge also removes other complexing interferences from this polluted water sample since impurities as seen in the unspiked sample (chromatogram C) are significantly reduced in chromatogram B. One problem which frequently occurs when using relatively strong and specific forces to retain compounds, is the desorption step. Irreversible adsorption can occur and the (often expensive) substrate has to be discarded. Desorption of the anilines from an ACDA-Pt surface was also problematic and it took a flushing step with 1 ml of pure acetonitrile (see positions c

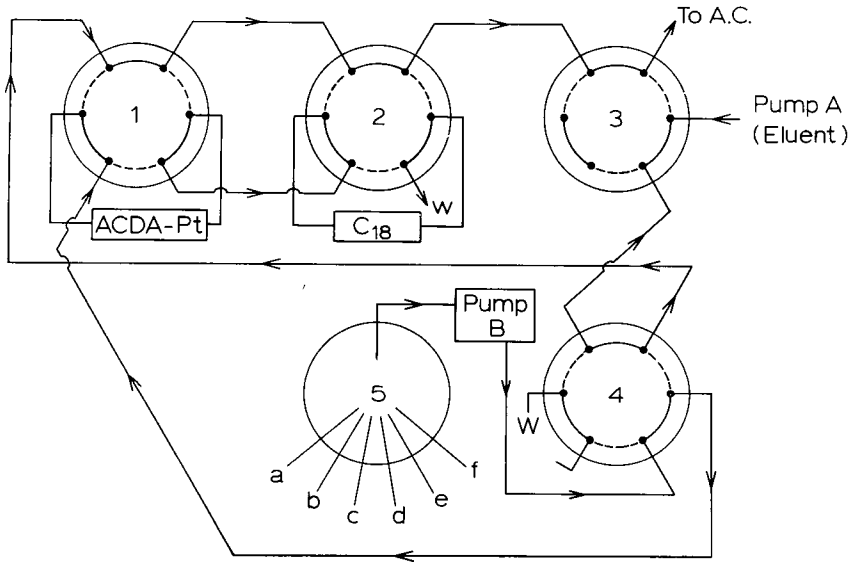

FIGURE 11 Switching-valve configuration of the on-line removal of anilines and pre-concentration of phenylurea herbicides prior to LC analysis. 1, 2, 3, 4 = High-pressure switching valves; 5 = low-pressure six-port selector valve (a = water; b = sample 1; c = acetonitrile; d = water; e = sample 2; f = acetonitrile); ACDA-Pt and C18, pre-columns (11 × 2 mm I.D. packed with the indicated material); A.C. = analytical column; A and B = HPLC pumps, W = waste.

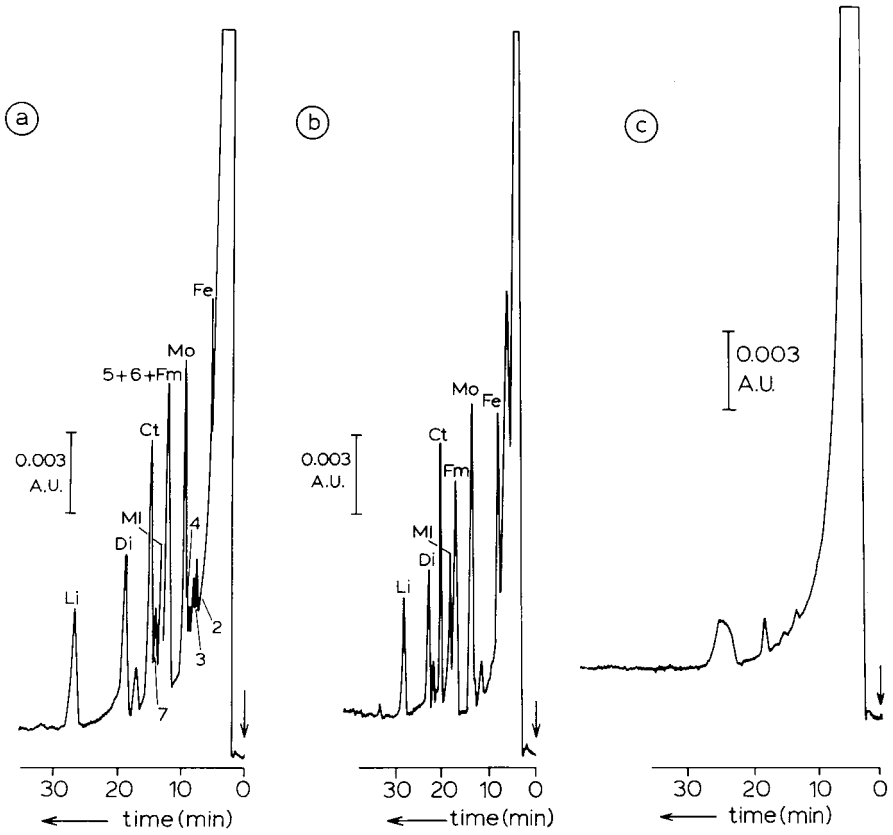

FIGURE 12 Pre-concentration of 10 ml of a river water sample, spiked with seven herbicides and seven anilines. (a) Pre-concentration on a C18 pre-column; (b) as (a), but with insertion of an ACDA-Pt pre-column upstream from the C18 pre-column; (c) river water blank pre-concentrated on a C18 pre-column. UV detection at 245 nm. Fe: fenuron; Mo: monuron; Fm: fluometuron; Ct: chlortoluron; M1: monolinuron; Di: diuron; Li: linuron. Peaks 2–7: different anilines. Anal. column CP-sphere, C18, 8 μ 250 × 4.6 mm I.D. MeOH/H_2O (60:40), 0.85 ml min^{-1}.

and f in Figure 11) to quantitatively remove all anilines. The acetonitrile seemed to act as displacer, whose removal required reconditioning with several milliliters of water prior to the next use. However, since all these steps can be done in a fully automated form, the time loss in this analytical scheme is not very critical.

COUPLING OF PRE-COLUMN TECHNOLOGY WITH SELECTIVE DETECTION

One way of improving the overall selectivity of the total system, besides using more specific adsorbents, is the choice of a selective detection mode. Simple replacement of a UV detector by an electrochemical detector can already significantly improve for example the detection of phenolic or anilinic compounds in complex water or biological samples.[6,7] The result is often a simplified sample handling and hence automation becomes feasible. A particularly interesting line of detectors are the so-called reaction detectors[14] which involve an on-line post-column reaction to produce better fluorescence, UV or other detection properties. In such reactors it is the chemical reaction which produces a selectivity effect. Reagents are pumped to the effluent stream of the column and the reaction takes place in a suitable reactor. Detailed descriptions of reaction detectors and their use have been given elsewhere.[14,15] For kinetically slow reactions of several minutes often segmented-flow reactors are used to suppress band broadening during the reaction step. The segmentation principle can also be used to advantage when the excess reagent used interferes with the signal of the product. This is the case for a large number of reactions potentially useful from a kinetic point of view. It holds also for ion-pairing and complexation reactions. Fortunately the reaction products are usually much lower in polarity than the reagent which permits an extractive separation of the two. This can be done in a continuous flow mode by segmenting with nonmiscible organic solvent plugs (solvent segmentation) which act as extractants and suppress the band broadening.

An ion-pairing system was studied to test the possibility for the development of a reaction detector based on such a dynamic micro extraction principle. It consisted of tertiary amines of pharmaceutical and agrochemical importance which were ion-paired with a fluorescent counterion dimethoxy anthracene sulphonates (DAS).

The schematic drawing of such an extraction detector is shown as a subunit (pump C, detector IV) in Figure 13. The ion-pairing reagent can also be added to the mobile phase which permits considerable simplification of the design. In addition, the same microextraction system can be used for pre- or post-column clean-up[16] without requiring an ion-pairing or chemical reaction.

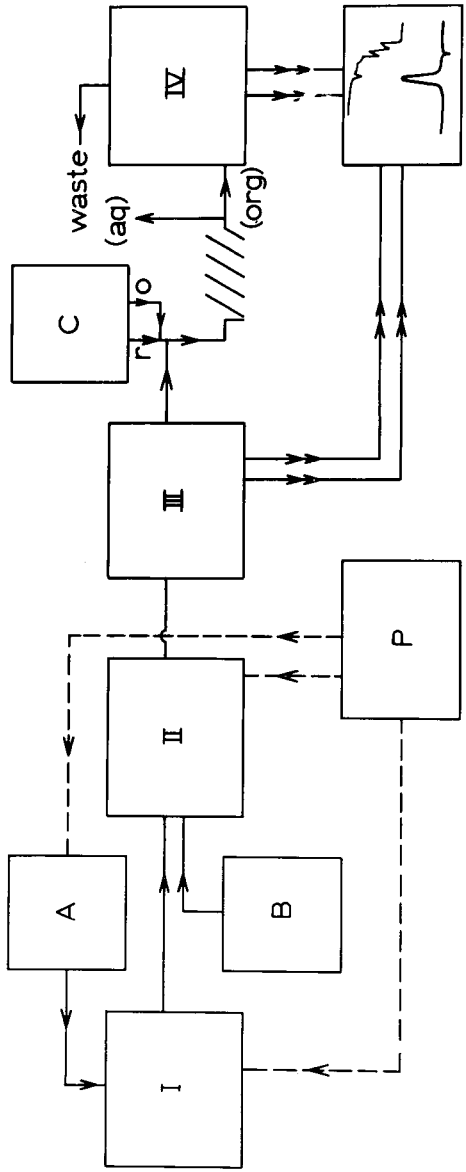

FIGURE 13 Apparatus for automated pre-concentration and HPLC analysis. A, B = pumps; P = programmer; C = peristaltic pump; I = autosampler; II = column-switching apparatus; III = UV-detector; IV = fluorescence detector; r = reagent solution; o = organic phase.

ON-LINE TECHNIQUES AND AUTOMATION

The feasibility and use of fully automated analytical systems for trace pollutants analysis in water samples has already been shown with several examples using hydrophobic (C18, PRP_1) metal loaded and ion exchange materials.

In this section an example for the completely automated analysis of biological fluids based on the principles discussed above is demonstrated. It involves the analysis of a tertiary amine drug, secoverine (see structure in Figure 14), in plasma and serum samples.[17] Secoverin is pre-concentrated from a 1 ml plasma or serum sample on a pre-column filled with CN bonded phase material. The analyte is then flushed on-line to a reversed phase column filled with the same material and separated. Since in this example UV detection does not yield the necessary selectivity and sensitivity, a post-column ion pairing with DAS and dynamic extraction is performed and the fluorescent ion pair secoverine-DAS detected in the organic phase. The schematic for the automatic performance of this analytical sequence is shown in Figure 13. Two pre-columns are operated in tandem. While one pre-column is loaded with a sample, the other pre-column is flushed on-line to the separation column and regenerated while the separation takes place. To avoid clogging of the pre-column after repeated injection of 1 ml serum sample often a pretreatment with an enzyme (Subtilisin-A) is carried out which breaks down the proteins to shorter chain peptides and amino acids. The repetitive injection of 1 ml of serum samples spiked with secoverin at the low nanogram level and treated with Subtilisin-A is shown in Figure 15.

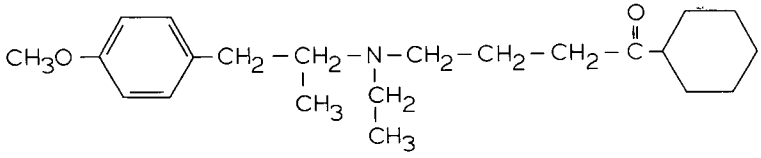

FIGURE 14 Chemical structure of secoverine.

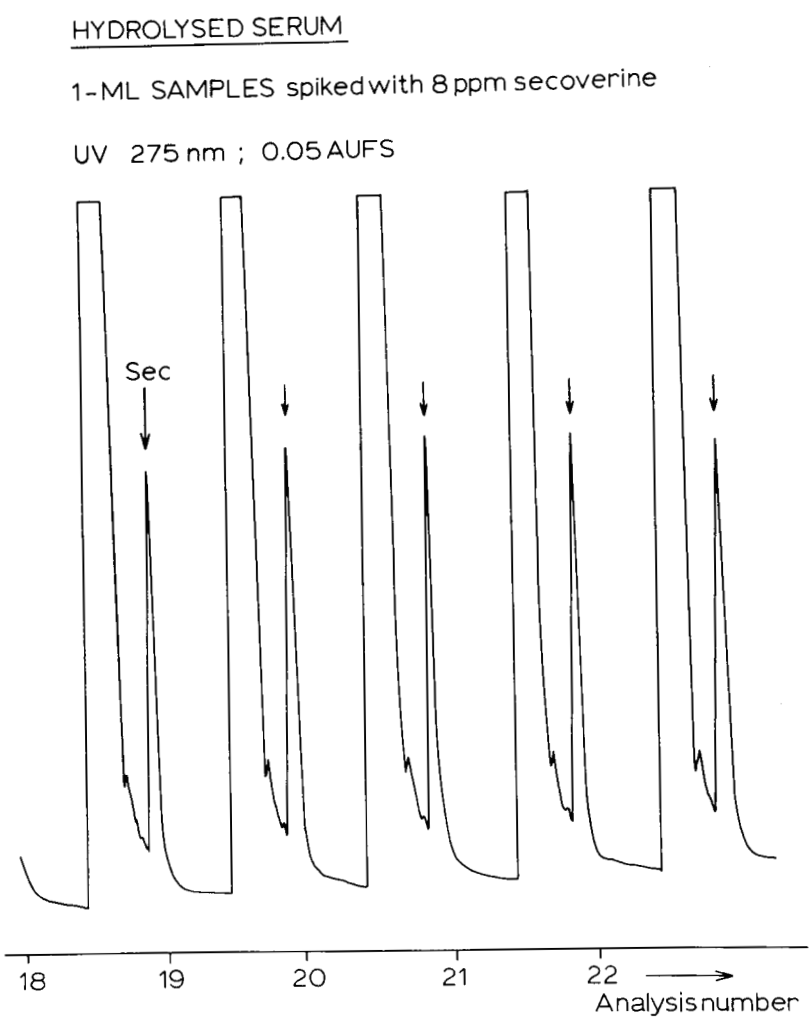

FIGURE 15 Repetitive injection of 500-μl pre-concentrated secoverine-spiked and Subtilisin-A treated serum. Cycle-time: 12 min. Detection: post-column ion-pairing + fluorescence detection.

MINIATURIZATION AND AUTOMATION

Narrow-bore liquid chromatography (LC)—featuring separation columns of about 1 mm I.D.—offers advantages over conventional-size LC such as relatively low solvent and stationary phase consumption and high mass sensitivity. However, due to the limited injection volume of about 1 μl and the reduced pathlength in, e.g., UV adsorption detector flow-cells, concentration sensitivity is often rather unsatisfactory. To overcome some of these drawbacks we have recently developed a simple micro-pre-column which can be packed manually and used in on-line systems.[18] Critical parameters such as pre-column length, inlet capillary I.D. and the use of screens instead of frits were studied and the applicability to the direct analysis of plasma and serum samples—i.e., without any off-line clean-up—was demonstrated. Unfortunately, extra-column band broadening caused by this pre-column was rather serious and resulted in a 3.5-fold increase in detection limit and a 12-fold loss in plate number. Therefore the actual enrichment factor was 60 instead of the expected 200 for a 100 μl trace enrichment experiment versus a 0.5 μl loop injection. Automated switching was also rather difficult with this design.

An improved design was described[19] where a micro-pre-column was inserted within the axis of a common six-port switching valve. In this way, extra-column band broadening could be reduced without losing the benefits of the previous design. The system has been applied to the determination of clobazam and its active metabolite desmethylclobazam in plasma samples without any off-line sample pretreatment.

Design of the switching valve with internal pre-column The switching valve with its internal precolumn (Figure 16) consists of three main parts:

—The valve body (1) is constructed from a standard Valco six-port switching valve. All ports were drilled to 1 mm. The analytical column fits directly into one of these ports without any connective tubing in between;

—The seal (2) is home-made and fits in the valve body. Note that this seal is fixed whereas the seal in the standard switching valve is not;

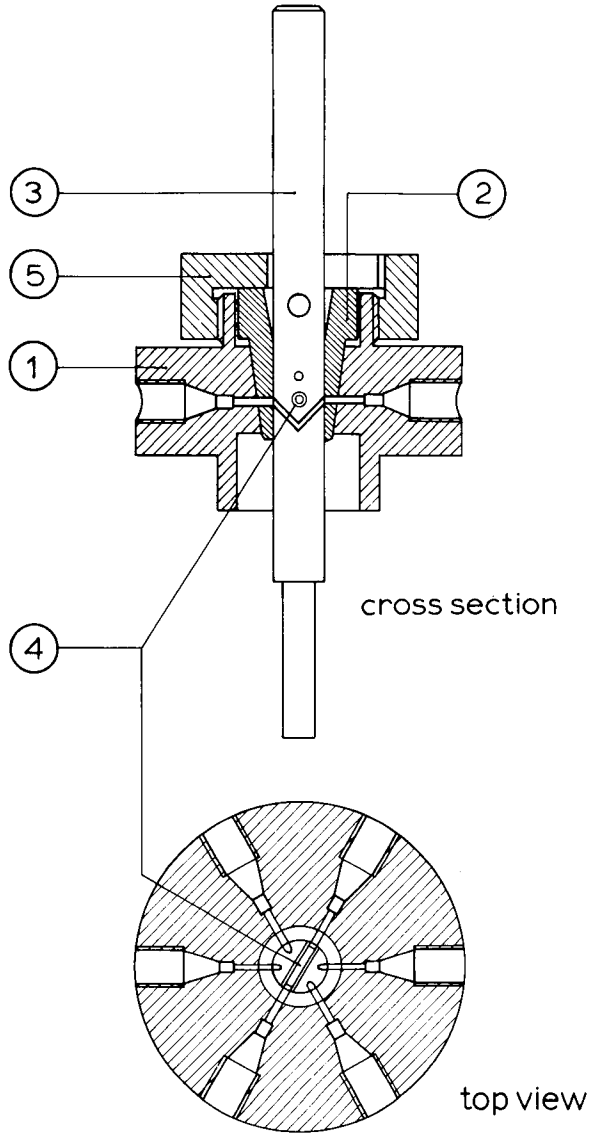

FIGURE 16 Switching valve with internal micro-pre-column. For explanation, see text.

—The axis of the valve (3) was drilled through, to get a 4.5 mm × 1 mm I.D. hole which can be filled with packing material to obtain the actual micro-precolumn (4). The axis contains one permanent screen; the other screen is held by a PTFE ring. The axis also contains two dummy channels for the other flow-lines of the six-port valve. These channels are drilled in such a way as not to touch each other, nor the micro-pre-column. The valve is leak-tight up to 250 bar when the wheel (5) has been tightened. The valve rotates extremely easy, thanks to the very small contact area between the rotating and the fixed part, and is easily pneumatically activated.

The geometrical volume between pre-column and separation column is about 2.5 μl, which is a relatively high value for narrow-bore LC. However, because of the absence of inner diameter changes—all parts are 1 mm I.D.—the final band broadening caused by this dead volume is extremely small. The pre-column can again be filled manually with a syringe with a thin slurry of the reversed phase material in methanol.

Automated sample handling of plasma samples As an example the micro-pre-column was used for the automated sample handling of untreated plasma samples spiked with the tranquilizer clobazam and its active metabolite desmethylclobazam; the apparatus used is shown in Figure 17. After filling the loop, the switching program was started. A 100 ng/ml standard solution of clobazam and desmethylclobazam in water was diluted with an equal volume fresh human plasma and concentrated on the micro-pre-column and analyzed (Figure 18). During each analytical separation the pre-column was flushed on-line with, arbitrarily, about 2 ml of water to remove the remaining water-soluble protein fragments and then re-used for the next sample. From the chromatogram it can be seen that the sorption/desorption procedure on the pre-column provides excellent clean-up: even with the non-selective UV detection at 254 nm, no interferences show up. Recoveries were 91% (1.7% rel S.D., $n=6$) for desmethylclobazam and 71% (1.1% rel. S.D., $n=6$) for the less polar and more strongly protein-bound clobazam. Detection limits are 2.5 and 5 ng/ml in real samples for desmethylclobazam and clobazam, respectively.

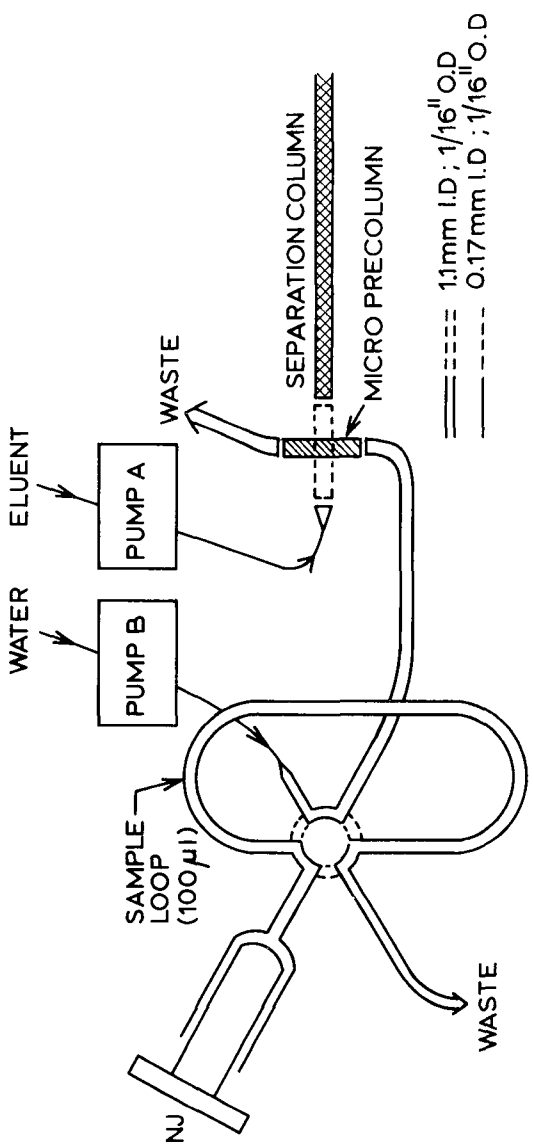

FIGURE 17 Setup for the automated sample handling and trace enrichment of plasma samples. Conditions: 20 cm × 1 mm I.D. 3-μm Spherisorb ODS-2 column, acetonitrile-water (50:50) at 50 μl/min; detection at 254 nm, 0.02 AUFS. Micro-pre-column, 4.5 × 1.0 mm I.D. 40-μm Octyl (C_8). Sampling at 200 μl/min.

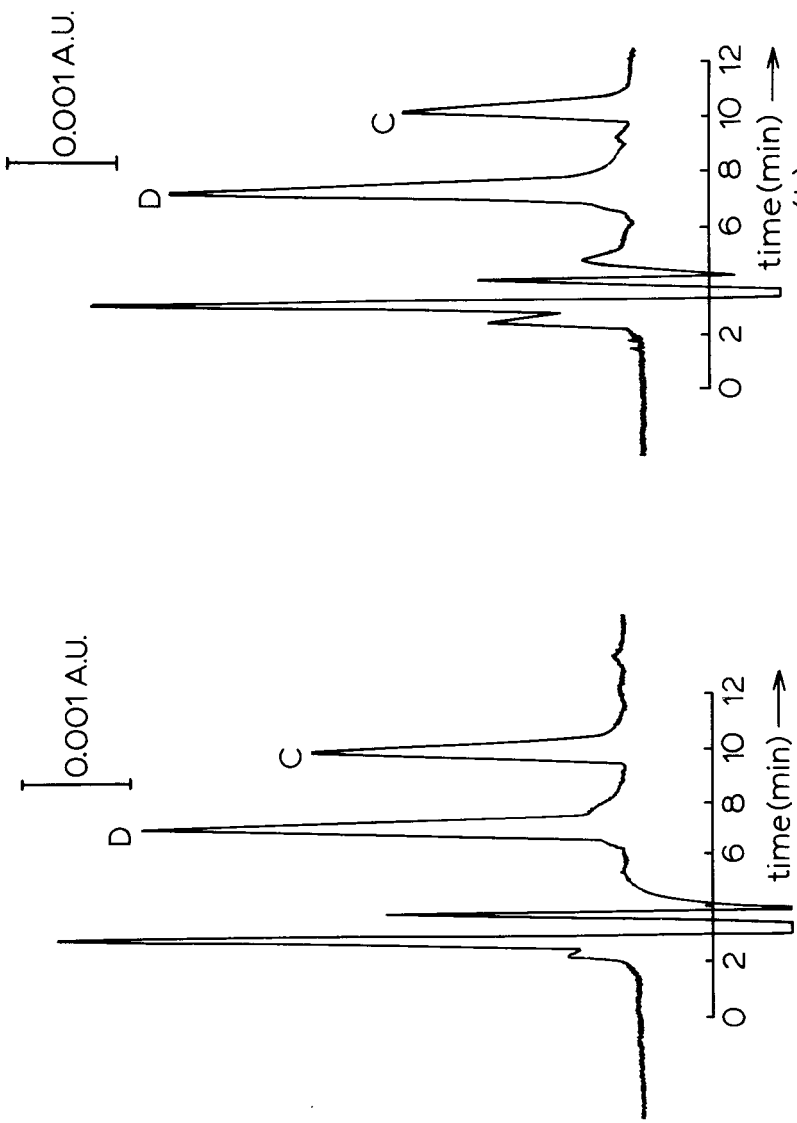

FIGURE 18 Chromatograms of a 100 ng/ml standard solution of desmethylclobazam (D) and clobazam (C) diluted 1:1 with blank plasma. Conditions as in Figure 17.

CONCLUSION AND FURTHER DEVELOPMENTS

We can state that coupling of pre-column sample handling techniques with selective detection can provide us with powerful analytical systems. They can be fully automated to handle samples as complex as biological body fluids or polluted water sources and they can be conceived as tailor made systems to solve well defined problem areas.

One of the serious disadvantages of trace enrichment on sorbents displaying rather low selectivity is that many sample constituents will be concentrated on the pre-column. As a consequence, clean-up may well be insufficient and LC separation of the components of interest from the many interfering substances—which obviously display rather similar chromatographic characteristics—will be difficult. There are at least three possible routes to eliminate this problem, namely by introducing column-switching techniques, by developing more selective adsorbents and using different pre-columns in series, or, as already mentioned, by combining pre-column trace enrichment with selective detection modes such as post-column derivatization.

Derivatization can, of course, also be effected in the pre- or on-column instead of in the post-column mode. In on-column derivatization, the sorbent will usually act simply as substrate on which reaction of the pre-concentrated solute takes place. The sorbent can, however, also participate in the reaction itself, as occurs, for example, in the catalytic hydrolysis of phenylurea herbicides of the corresponding anilines.[20] At about 160°C, hydrolysis is unexpectedly rapid due to the presence of the silanol groups on the surface of the silica sorbent. In this case, subsequent derivatization is either done off-line, after elution of the anilines from the catalytic sampling column, or on the column itself with dansylchloride as reagent to form fluorescent dansyl amides.[20]

The above examples serve to show that techniques such as pre-column trace enrichment combined with a derivatization step are powerful and versatile means of simplifying laborious sample-handling procedures by means of increased selectivity and sensitivity. Moreover, many of the published off-line procedures, such as those involving the efficient XAD-type porous polymers, can in all probability, be adapted to on-line operations. It is obvious that the

potential of on-line trace enrichment can be further enhanced by developing more selective sorbent materials, such as metal-loaded phases, selective ion exchangers and phases containing immobilized enzymes. The automation potential of the pre-column and column switching techniques is evident, and several automated or semi-automated modules for sample handling have been commercialized, i.e., automated sample handling/LC apparatus for the analysis of body fluids. It has also been demonstrated that miniaturization of pre-column designs and total systems is possible and can even be automated. The advantages of miniaturized systems becomes particularly obvious when dealing with expensive and/or highly toxic chemicals.

Finally, it should be emphasized that the sample-preparation step cannot be regarded as a separate entity. Sample handling should always be viewed in terms of the total analytical procedure and be brought in tune with the separation system and the detection mode used, e.g., as regards the mobile-phase composition. Such a total-system approach offers the best possibility to achieve simplified sample handling and/or reduced sample size and, eventually, faster analyses at reduced cost.

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