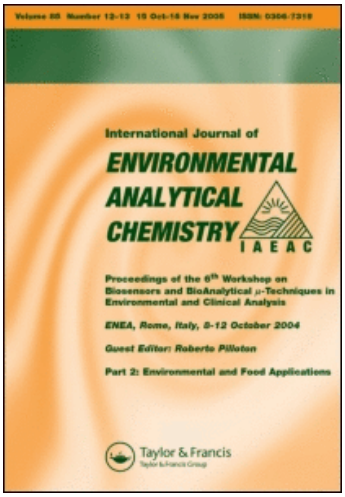

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Trace Enrichment and Chemical Derivatization in Liquid Chromatography; Problems and Potential in Environmental Analysis

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Trace Enrichment and Chemical Derivatization in Liquid Chromatography; Problems and Potential in Environmental Analysis†

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The importance of detection limits in the trace analysis of pollutants is well recognized. Very often selectivity is another criterion and in order to achieve this, a chromatographic separation step may be necessary. The attainable detection level will then also depend strongly on chromatographic parameters and particularly on the injection step.

It has been observed in earlier work that relatively non-polar organics dissolved in a polar solvent such as water are concentrated upon injection onto commercial reversed phase material (C_8–C_{18}) into a small zone on top of the column. This concentrating effect can be adopted for trace enrichment of pollutants in aqueous samples. It permits the use of large injection volumes up to several hundred millilitres without serious band broadening.

The disadvantage of such a procedure is that interferences are also concentrated and one has to trade selectivity for sensitivity. Some of this drawback can be eliminated by using column coupling and step gradient techniques and by combining these methods with chemical derivatization techniques in the post-column mode.

With the proper choice of the post-column reaction, one can further improve the selectivity by choosing a more or less selective reagent. The use of a reagent with reasonable reaction kinetics and a good chromophore or fluorophore will greatly enhance the detection properties.

All these concepts have been demonstrated with a few practical examples. The reproducibility of these techniques (usually below 4% rel. S.D.) permits good quantitation of pollutants.

KEY WORDS: Trace enrichment, step gradient, chemical derivatization, pollutants.

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†Paper presented at the 7th International Symposium on the Analytical Chemistry of Pollutants, Georgia, U.S.A., April 1977.

INTRODUCTION

The possibilities of adapting sample enrichment techniques to trace analytical problems have been discussed earlier[1] and shall be treated in this paper with a view to environmental problem solving. The major disadvantage comes from interferences which are also preconcentrated sometimes to concentration levels, in which an isocratic separation will not enable isolation of the compounds of interest any longer.

In such a case gradient elution and particularly chemical derivatization[1,2] will lead to a solution. Some of these aspects have been investigated and are presented here.

EXPERIMENTAL

Apparatus

The equipment designed for large volume injection and step gradient elution has been described elsewhere.[3] Also discussed in an earlier paper[4] is the design of a typical apparatus for post-column derivatization and fluorescence detection. The same type of equipment was used except that an Altex piston pump with feedback flow control (Altex, Berkeley, Calif., U.S.A.) was used for chromatography rather than a pneumatic amplifier pump.

Reagents and chromatography

The reagents and reversed phase chromatographic techniques used in these experiments have been listed previously.[3,4] Further details are given in the legends of the appropriate figures.

RESULTS AND DISCUSSION

Although peptides with primary amino groups were chosen as model systems for most of the work there is no doubt that the same principles can equally well be applied to other primary amines of interest as industrial pollutants or residue components from agricultural chemicals.

The preconcentration phenomenon

Already in 1974 Kirkland[5] has mentioned the possibility of large volume injection making use of a preconcentration phenomenon on top of the column. Later Little and Fallick[6] reported on the use of very large injection volumes up to 200 ml for relatively non-polar compounds on C_{18} reversed phase columns. Similar observations were recently made with peptides[7,8] and alkaloids[1] injected from aqueous samples.

The essence of all this work is, that when relatively non-polar organic species are injected from an aqueous solution onto a hydrophobic surface they will become immobilized until the elution strength of the solvent mixtures is increased. This means that the compounds are concentrated into a very small zone on top of a reversed phase chromatographic column (trace enrichment effect). These components can then be eluted with a suitable eluent with very little band broadening. Similar phenomena can also be observed on adsorption systems for relatively polar compounds injected from non-polar solvents.

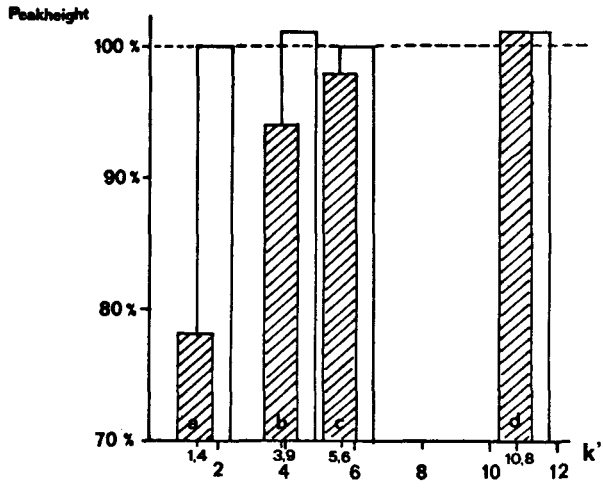

FIGURE 1 Effect of injection volume on peak height. – – – 34 μl injection taken as 100%, □ 340 μl injection from aqueous sample, ▨ 340 μl injection of sample dissolved in mobile phase. a, b, c and d: different peptides ranging in k' from 1.4 to 10.8.

Figure 1 demonstrates clearly what has just been said. In a study with nonapeptides ranging in k' from 1.4 to 10.8 one observed a significant reduction in peak height due to band broadening when 340 μl of the samples are injected in the mobile phase. This phenomenon is particularly dominant for compounds with low retention since they will start moving already during the injection step and consequently band diffusion will be enhanced during the actual separation. For k' above 10 this effect becomes negligible since the mobility of the substance is also slow in the mobile phase.

If the components are injected from an aqueous phase (open blocks) no band broadening is observed for 340 μl injections in comparison to 34 μl injections.

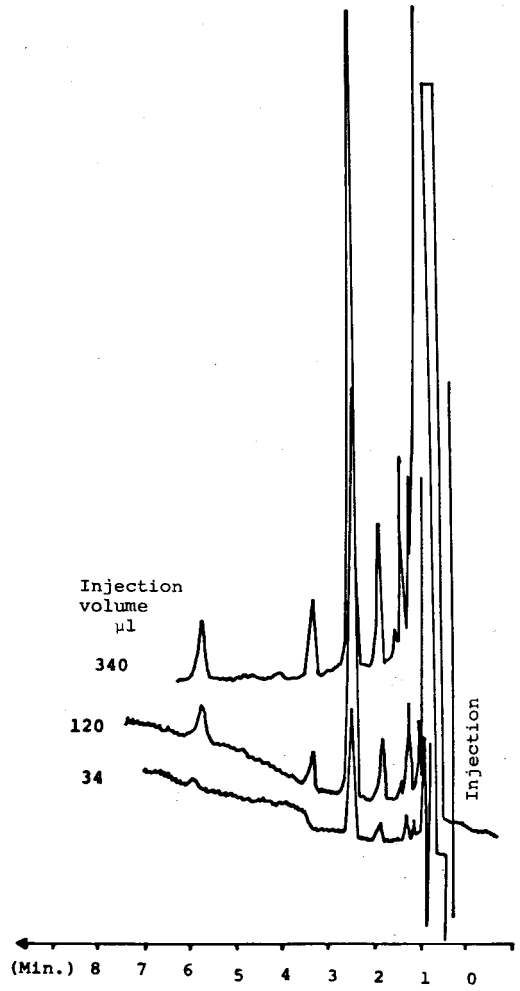

FIGURE 2 Influence of injection volume on peak height and peak broadening for a mixture of nona-peptides. Column: Nucleosil C_8 5 μ, 4 mm I.D., 15 cm length. Mobile phase: 20% v/v acetonitrile in buffered water pH 7. Detection UV at 210 nm; flow ~1 ml/min.

This can be seen also in Figure 2, where 34 μl, 120 μl and 340 μl of a mixture of peptides has been injected. For none of the components can a band broadening be observed. On the other hand, one can appreciate the sensitivity enhancement at 340 μl injection volumes which would permit quantitation of some minor components barely visible at a 34 μl injection.

It is clear that with only 340 μl volumes we are not yet really using this trace

enrichment step to its full potential. In the next figure (Fig. 3) one can see repetitive injections of the same total concentration of a peptide but varying in injection volumes from 100 μl to 300 μl. Except for a shift in base line and a slight change in retention times[1] there is little change of the chromatographic pattern. Band broadening is usually not more than 10–20% in comparison with small (100 μl) volume injections.

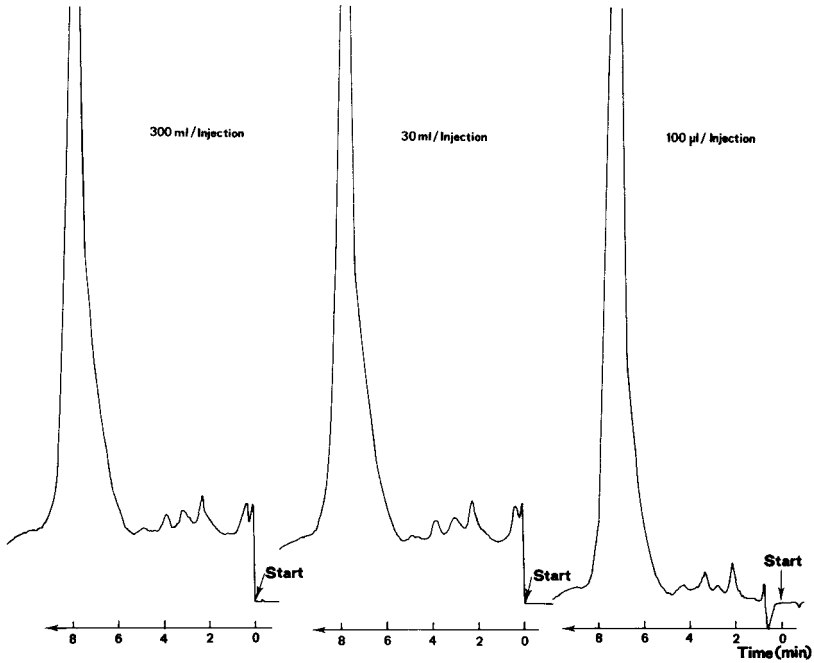

FIGURE 3 Influence of very large injection volumes for a peptide sample on peak broadening, retention and baseline shift. Conditions as in Figure 2.

An application of this concept is also shown in Figure 4 where 165 ml of distilled water have been injected. The two major impurity peaks probably correspond to softeners from a plastic container in which the water has been stored for a short period of time; they were absent when the water was kept in glass containers. The baseline shift is again quite pronounced. When volumes of this magnitude are injected, it is impossible to work with loop injection. Instead the sample is pumped onto the column in a first step followed by isocratic or gradient elution with the same pumping system in the next and subsequent step.

A good reproduction of repetitive large volume injections can be obtained

with the low cost timer controlled gradient apparatus described earlier.[3] The same gradient can then be used for the step gradient separation of the preconcentrated complex mixtures.

A typical example of this nature is shown in Figure 5 with a urine sample of which several millilitres have been injected. The first step after injection consisted of a pure water flushing cycle to elute all the water soluble

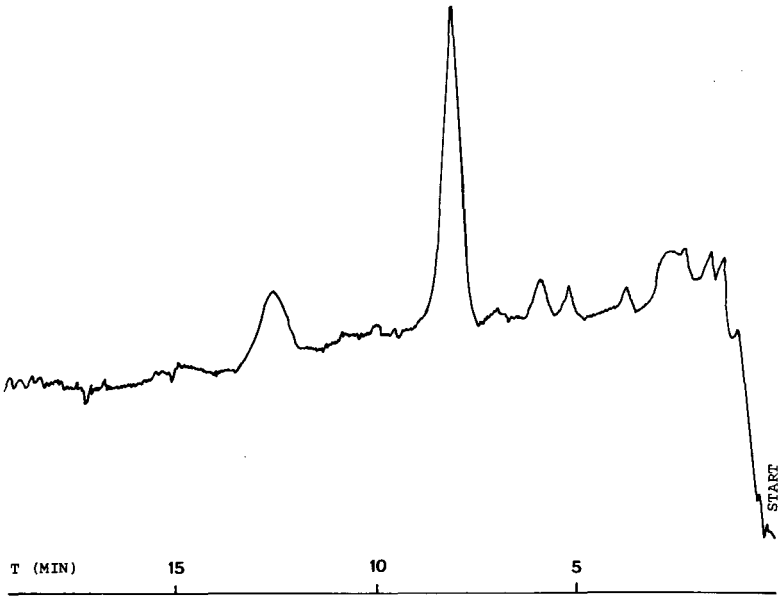

FIGURE 4 Isocratic separation of impurities in distilled water. 165 ml injected; major impurity peaks probably phthalates. Column: Nucleosil C_{18}, 5 μ, 3 mm I.D., 10 cm length. Mobile phase: 40% v/v acetonitrile in 0.1 M ammonium carbonate solution; flow ~1 ml/min. Detection: UV 254 nm.

components and then the elution power is increased by adding increasing amounts of acetonitrile until all the components are removed from the column.

For dirty samples such as this, the problem may arise that after repetitive injection the column may lose its separation performances due to contamination. The loss of costly columns can be avoided by working with a short precolumn which is more easily replaced. Such a precolumn design is shown in Figure 6 and has been used with good success for complex pharmaceutical, biological and environmental samples. The use of such precolumns has also very interesting aspects for field sampling techniques.

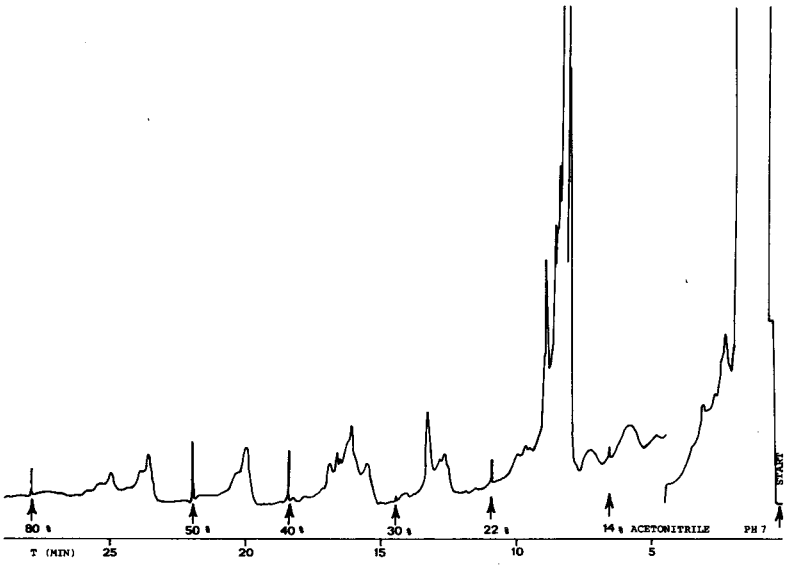

FIGURE 5 Chromatogram of a urine blank (1.777 ml injected). Separation with step gradient[1,3] acetonitrile in 0.1 M ammonium carbonate solution. Column: Nucleosil C_{18} 5 μ, 4 mm I.D., 15 cm length; flow: 3 ml/min; detection UV 237 μm.

Large numbers of samples can be preconcentrated on such columns by simple manual pumping techniques under field conditions and then connected to an analytical HPLC system in the laboratory for final analysis.

Coupling to post-column derivatization

In the foregoing discussion we have seen the potential of preconcentration for trace and ultra trace analytical problems. The disadvantage on the other hand is also quite obvious. Many interferences are also preconcentrated and often to a degree which makes isolation of the compound(s) of interest impossible even with a gradient. In such situations it was suggested earlier[1] to combine the trace enrichment technique with post-column derivatization.

The reasons for using chemical derivatization techniques in liquid chromatography have been discussed before.[2] One of the major arguments was the improvement of selectivity due to the choice of a selective reagent which permits elimination of many interferences. This can be used in the pre-chromatographic mode as a cleanup step or in our case at hand in the post-column mode to eliminate the preconcentrated interferences. The effect of this is shown in Figure 7 for a 1.777 μl loop injection of a peptide solution. The fluorescence trace obtained after derivatization with Fluram shows a normal

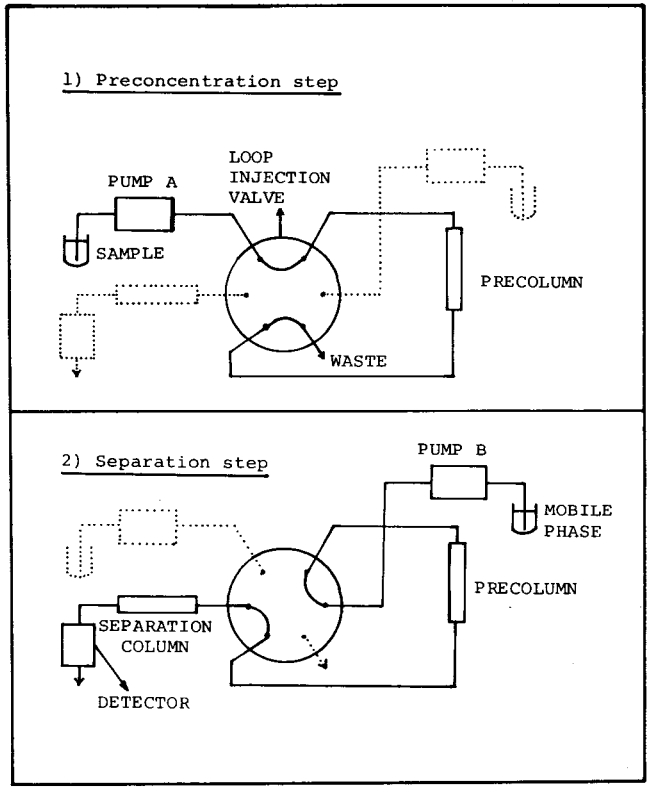

FIGURE 6 Apparative scheme for pre-column trace enrichment technique. Pumps A and B: Altex Model 100 piston pump (Altex, Berkeley, Calif., U.S.A.); Loop injection valve Altex; Pre-column: 3 cm length, 4 mm I.D., slurry packed[10] with reversed phase RP–18 5 μ material (Merck, Darmstadt, GRF).

chromatogram of the main component and some by-products. Essentially no difference was observed between a 30 μl injection and this one. No band broadening or baseline shift was observed. The UV trace by contrast, which has been recorded from the same chromatographic run prior to derivatization, shows considerable interferences from other UV active components. In the next figure (Figure 8) injection of 300 ml of the same peptide solution is shown with p.c. derivatization and fluorescence detection. Compared to a 30 μl injection, this corresponds to a 10,000 fold concentrating factor with only about 20 % band broadening. The main component is here in the 5 ppb concentration range and the by-products are between 5 and 10 ppt if we assume a similar fluorescence yield.

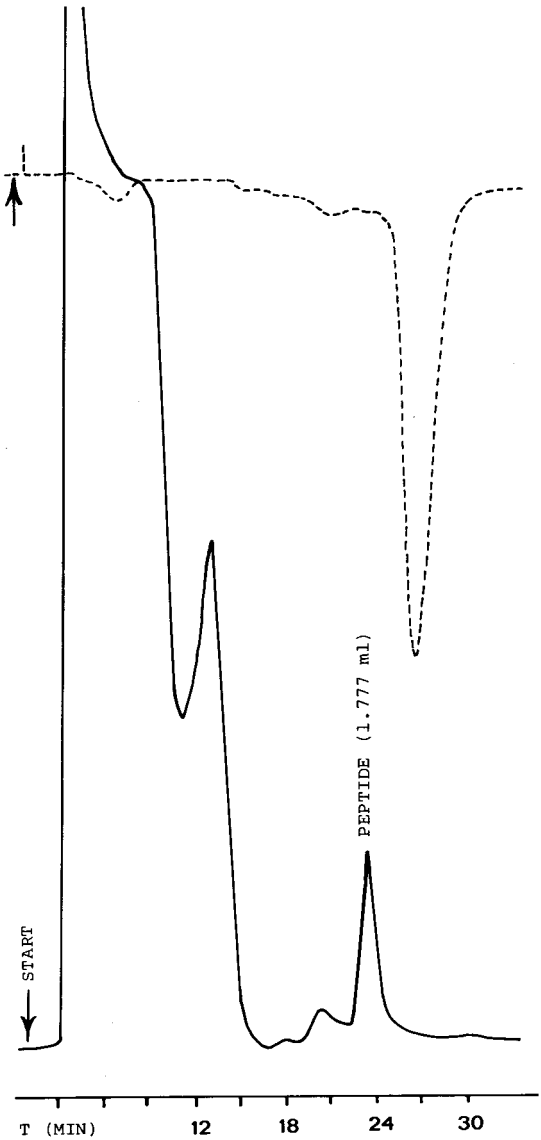

FIGURE 7 Chromatogram for a peptide solution with 1.777 ml sample injection by loop. ——UV detection at 210 nm before derivatization, – – – Fluorescence detection ($\lambda_{ex} \sim 360$ nm; $\lambda_{em} \sim 470$ nm) after Fluram derivatization. Chromatogr. conditions as in Figure 2. Derivatization: 30 mg Fluram in 100 ml acetonitrile added at 0.19 ml/min.

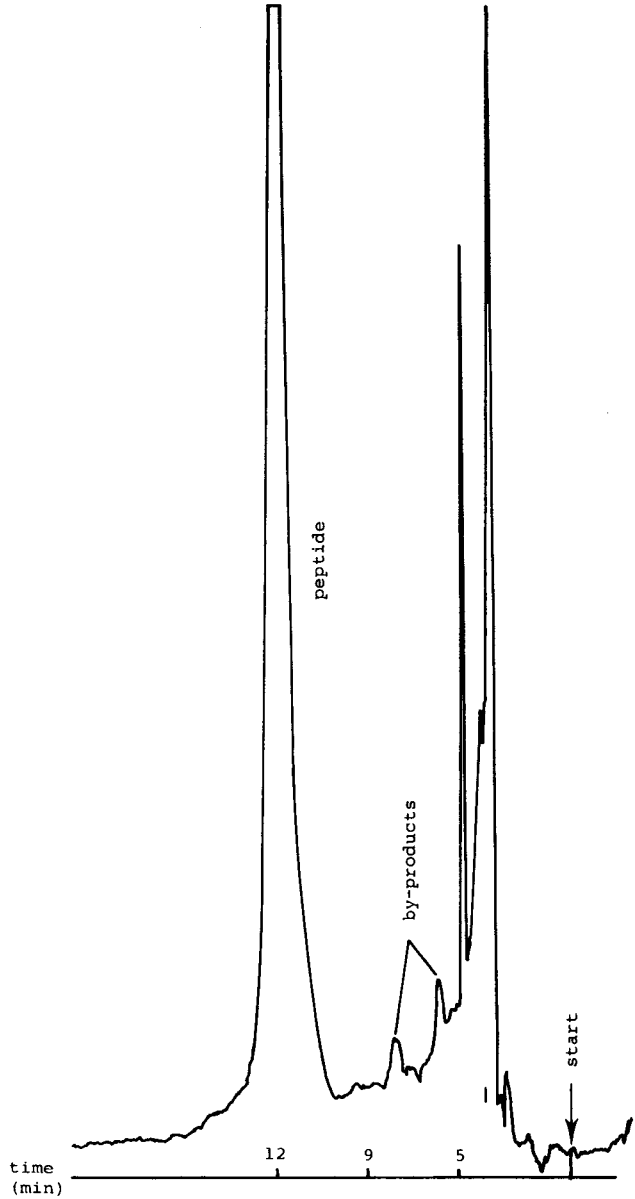

FIGURE 8 Chromatogram for a peptide solution of which 300 ml where injected with reciprocating pump (Waters Model 6000). Chromatogr. and derivatization conditions as in Figure 7.

Combination of a gradient with derivatization

In conjunction with environmental analytical work on complex matrices it may also be desirable to couple gradient separations with post-column derivatization techniques; or even using a combination of trace enrichment (large volume injection) → gradient elution → UV detection → post-column derivatization and fluorescence detection. On first inspection such a combination would be difficult since a constantly changing eluent composition would affect the reaction conditions after the column. It would therefore seem rather difficult to achieve a good reproducibility with a continuous gradient. Some preliminary studies seem to confirm this. With a step gradient, however, such as discussed by Erni *et al.*[3] one obtains a sufficiently good equilibrium condition during each step to render such an

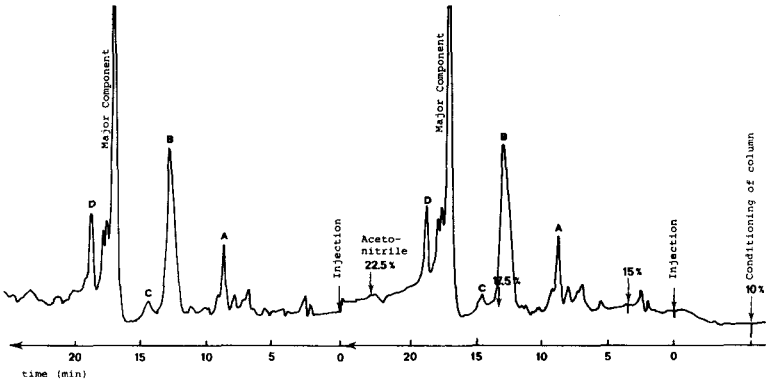

FIGURE 9 Chromatogram in duplicate (repeat injection of 100 μl) of a primary amine mixture. Peaks A, B, C, D are by-products of the major component. Conditions as in Figure 7. Step gradient acetonitrile in water pH 7.

approach quite feasible.[9] A duplicate chromatogram of a complex mixture of primary amines (peptides) as obtained with the step gradient indicated[3] and using the instrument design described earlier[4] for chromatography and Fluram derivatization is shown in Figure 9.

With the gradient steps indicated on the figure the reproducibility of retention values was quite good (< 1.5 % rel. S.D.) for all the peaks marked (see Table I).

The reproducibility of peak heights (Figure 9, Table I) and particularly of the peak areas as determined from Figure 10 and represented in Table I is 2.4 % rel. S.D. for the major component. The corresponding UV trace in Figure 10 taken at 210 nm is not suitable for a study of the minor components in this mixture.

TABLE I
Reproducibility for retention times t_R and peak areas (a) or peak height (h)

	t_R (min)	% rel S.D. for t_R ($n=6$)	h (Fig. 9a)	h (Fig. 9b)	% rel. S.D.* for a ($n=6$)
Peak A	8.10	0.7	5.29	5.13	1.6
Peak B	11.31	1.4	12.80	12.89	
Peak C	16.75	1.5	1.42	1.42	
Peak D	26.14	0.4	8.03	7.80	
Major component	20.35	1.3			2.4

*Data obtained from six repeats of chromatogram Figure 10 and computer integration.

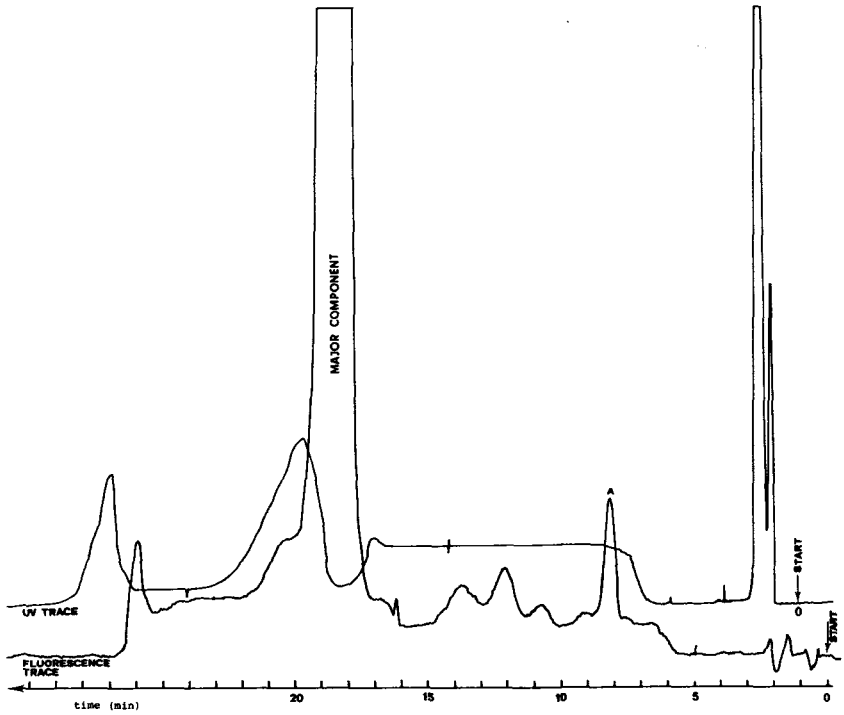

FIGURE 10 Chromatogram of a slightly simpler amine mixture with all conditions the same as in Figure 7. Simultaneous UV-detection at 210 nm.

CONCLUSIONS

It has been shown that preconcentration techniques can be a powerful tool for ultra trace analysis particularly when combined with step gradient elution and (or) post-column derivatization techniques.

The aspect of precolumn use for trace enrichment is also attractive from the field sampling point of view. The fact that all the described techniques can be automated and electronically controlled makes them quite feasible for routine monitoring of pollutants even in relatively complex matrices.

The reproducibility found for all these operations was quite good ($< 4\%$ rel. S.D.). One can claim the techniques to be able to provide truly quantitative information.

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