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DETECTION INVOLVING POST-CHROMATOGRAPHIC ADDITION OF REAGENTS

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SUMMARY

Unlike the approach to reagent addition as used in AutoAnalyzer methodology, we have developed a packed bed mixer which can be used without recourse to a gas-segmented stream.

The very low contribution of the mixer to the overall dispersion of the chromatographic peak is compatible with high-performance liquid chromatography. The mixer is particularly suitable for use with fast post-column reactions such as the fluorimetric determination of amines with fluorescamine or *o*-phthalaldehyde as reagents. The mixer is ideally suited for use in conjunction with electrochemical detection where addition of reagents to control pH and electrolyte composition is very important.

INTRODUCTION

Derivatization in modern high-performance liquid chromatography has evolved due to the lack of very sensitive, universal detectors. Consequently, it has become necessary to utilize existing detection systems more fully by forming derivatives of hitherto undetected or poorly detected species. Additionally, chromatographers are becoming more realistic and recognize that the rapid separation of complex biological samples is often extremely difficult, if not impossible. Specific detection, resulting from derivative formation, provides an alternative approach to this problem.

Derivatization can be achieved either pre- or post-chromatographically[1]. To date, adequate mixing of the eluent and reagent streams has been performed with gas-segmented reactors[2,3] and columns slurry packed with small glass beads[4]. The gas-segmented reactor is based on AutoAnalyzer methodology and is best suited to long reaction times. However, there are many instances when the reaction is fast and the smaller dispersion arising from the packed column is to be preferred. This paper involves developments of the latter technique.

Pre-chromatographic derivatization has many favourable aspects such as wide solvent compatibility, improvement in chromatography and usefulness when

long reaction times are involved. However, there are many instances when it is desirable to use post-chromatographic derivatization, such as:

(i) when preparative recovery of the starting material is required;

(ii) when derivatization adversely affects the chromatography; this is very important in the separation of amino acids by well characterized ion-exchange techniques;

(iii) when the derivative is unstable; and

(iv) when the derivative is very bulky and not easily chromatographed.

The dearth of applications in this area is, in part, a reflection of the lack of suitable post column mixing devices. The packed bed mixer (PBM) described in this paper goes a long way towards meeting these requirements.

EXPERIMENTAL

Reagents

o-Phthaldialdehyde was obtained from Sigma (St. Louis, Mo., U.S.A.) and fluorescamine (from Hoffmann-La Roche, Basle, Switzerland).

Equipment

Waters (6000A), Constametric II and Milton-Roy (Duplex) pumps were used. Injection was accomplished with a Rheodyne 7120 syringe loading valve. The following detectors were used:

(i) Cecil CE 212 UV (variable-wavelength) detector with a 10-μl flow-cell;

(ii) DuPont Model 836 fluorescence detector; and

(iii) LCA-10 electrochemical detector (EDT Research, London, Great Britain).

Post-column mixers and telescopic jackets were made in collaboration with Magnus Scientific Instruments (Sandbach, Great Britain).

Construction of post-column mixer

The mixer is essentially a packed, porous bed (Fig. 1) supported on a porous stainless-steel frit (15 μm). Low-dead-volume end fittings are used. The eluent is fed centrally into the packed bed and reagent is added via the diffuser (Fig. 2), which ensures that a split stream flow surrounds the injection point, thus preventing unwanted upwards diffusion of the eluent. This design has been found to minimize dispersion when injection is made into a non-adsorbing packed bed such as that used in the mixer.

On-column valve injection

It has long been recognized that the sample injection process and column end fittings can, if poorly designed, contribute markedly to solute dispersion[5]. This factor becomes more critical as the efficiency of the column increases. For some years, syringe injection at the centre of the packed column, just penetrating the packed bed, has been found to give the least dispersion at the sample injection stage. However, repetitive injection leads very quickly to damaged column packings, resulting in the need to top up the column periodically or even to re-pack it.

With the introduction of low-dead-volume, high-pressure sampling valves, highly reproducible sample injection can now be achieved. There has been a tendency

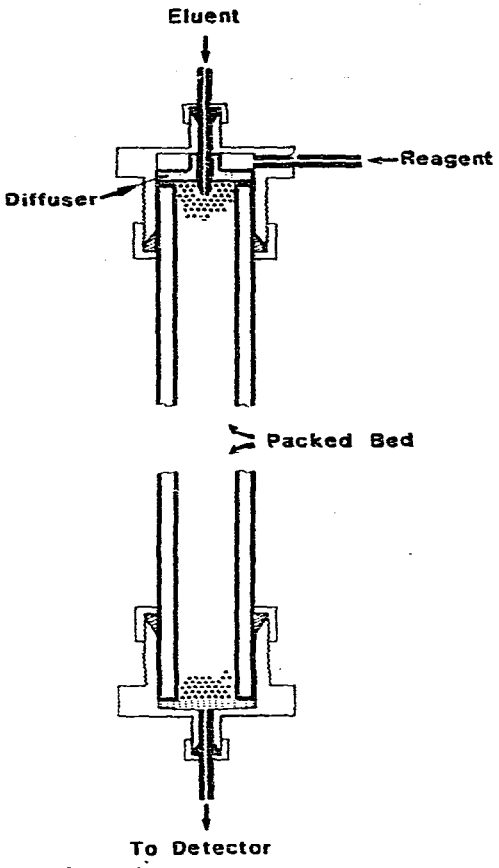

Fig. 1. Post-column mixer.

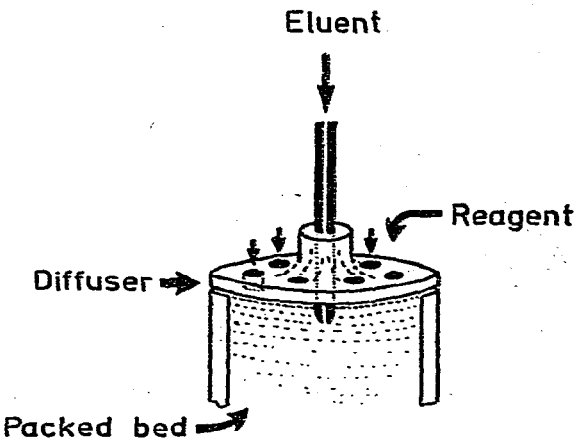

Fig. 2. Split stream addition using reagent diffuser.

to use these injectors in conjunction with columns sealed with a frit at the top end or with wire mesh and a porous PTFE plug. Under these conditions, the split stream flow previously described is advantageous. However, more efficient point injection is achieved if the capillary tubing leading from the valve is conically ground and positioned such that the conical end just penetrates the packed bed (Fig. 3). Under these conditions, the process approaches "on-column" syringe injection but without damaging the column packing. The split stream is unnecessary as the solute is immediately adsorbed with no tendency for back-diffusion to occur. Consequently, we have used on-column injection (Fig. 3) at the chromatographic column stage and on-column injection with split stream flow (Fig. 2) at the mixer stage.

Construction of telescopic jacket

The design of the water jacket is shown in Fig. 4. It is particularly useful (in conjunction with a water circulating bath) for maintaining the required column and packed bed temperatures. The design allows for complete immersion of the column and end fittings in the circulating water without the need to remove ferrules in order to remove the column.

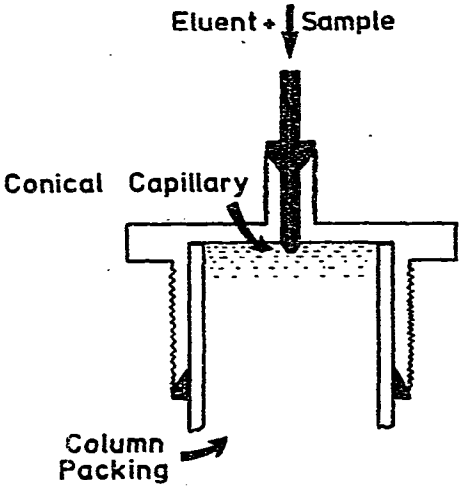

Fig. 3. On-column injection with valve.

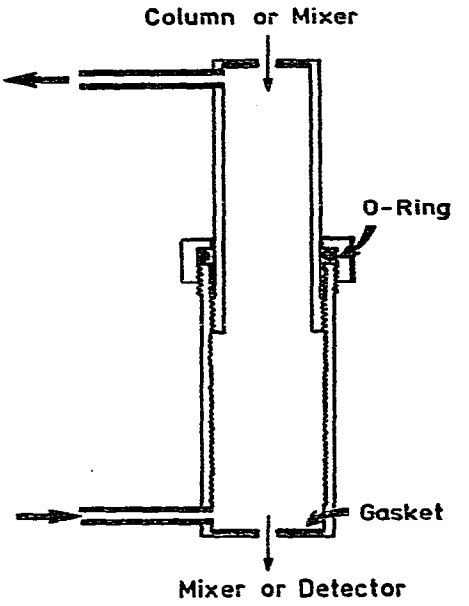

Fig. 4. Heating jacket for column and mixer.

RESULTS AND DISCUSSION

Prior to the evaluation of the component parts of the PBM, it was necessary to determine the variance (σ^2_{xc}) of the extra-column effects. In order to determine σ^2_{xc}, a Rheodyne syringe loading valve (20-μl loop) was connected directly to a UV flow-cell (Cecil CE 212, 10 μl). Connections were made with low-dead-volume fittings and capillary tubing (0.010 in. I.D.).

Methanol–water (1:1) was used as eluent and 10 μl of a 0.1% solution of acetone in methanol was injected into the system. σ^2_{xc} was obtained from measurements of the width of the peak at 0.606 of the peak height, which is related to the dispersion, σ.

These extra-column components were kept constant and the variance arising from them was subtracted from all subsequent measurements.

Composition of the packed bed

A packed bed of length 3 cm and I.D. 0.42 cm was constructed. Methanol as eluent and water as reagent were pumped at 1 ml/min through the system comprising the extra-column components (already described) and the PBM. Acetone (0.1% in methanol) was injected as the sample and its variance (σ^2_{Tot}) measured as previously described. The variance due to the PBM (σ^2_m) was calculated as $\sigma^2_{Tot} - \sigma^2_{xc}$ and is shown in Table I for a range of packed bed materials.

From these data, it is obvious that small, spherical, impermeable glass beads give rise to the best PBM. Further experiments with smaller glass beads are in progress but in all subsequent work reported in this paper 120-μm glass beads were utilized. The back-pressure due to such a PBM is very low.

TABLE I

COMPOSITION OF THE PACKED BED

Bed material	*Variance due to mixer, σ_m^2 (sec²)*
Glass spheres (120 μm)	1.16
Glass spheres (720 μm)	6.69
PTFE spheres (40 mesh)	7.4
Crushed glass (<150 mesh)	7.6
CPG-10 (200–400 mesh)	12.7

Effect of packed bed diameter, d

Packed beds of 0.2, 0.3 and 0.4 cm diameter and of length varying between 5 and 50 cm were constructed and evaluated as previously described.

The variance at high bed diameters was unacceptable (Fig. 5), whereas at a diameter of 0.2 cm the variance of a 50-cm PBM is still significantly less than the extra-column effects.

Effect of packed bed length, L

Data obtained from the previous experiment was studied with respect to L (Fig. 6). Again, it is obvious that wide-bore packed beds contribute significantly to dispersion as the length of the bed increases, whereas at a diameter of 0.2 cm the effect of increasing the bed length has a minimal effect on dispersion.

These data, together with the preceding data, suggest that narrow packed beds, preferably less than 0.3 cm in diameter, should be used whenever possible. All subsequent work was carried out using packed beds of 0.2 cm diameter, although development is under way to investigate the effect of decreasing the diameter still further.

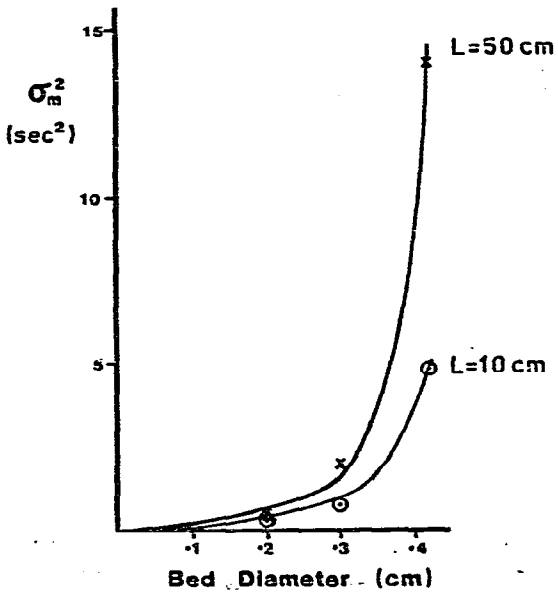

Fig. 5. Effect of packed bed diameter on variance.

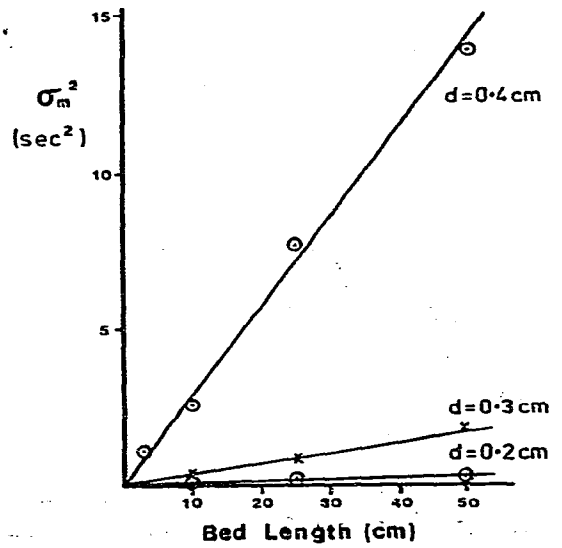

Fig. 6. Effect of packed bed length on variance.

Effect of flow-rate

In order to extend the residence time in the packed bed such that derivatization may proceed to completion, either the bed length can be increased or the flow-rate through the PBM decreased. In practice, both parameters may be adjusted. However, it is to be expected that as the flow-rate is lowered so the dispersion due to diffusion will increase. It was found that a combined eluent plus reagent flow of 0.2 ml/min was limiting for the 0.2-cm diameter PBM. At lower flow-rates, dispersion increased rapidly and it was more advantageous to increase the length of the PBM. Reaction times of 2–3 min can be obtained before dispersion starts to become a problem.

Effect of temperature

An alternative to increasing the residence time in the packed bed is to increase the reaction temperature. Using the column jacket previously described and a circulating water system, the temperature of the PBM was increased and the corresponding dispersion measured (Fig. 7). Surprisingly, the dispersion dropped significantly. This indicated the possibility that the glass beads, supposedly non-adsorbing, were contributing to retention in a small but measurable manner. Consequently, elevated temperature might be considered an advantage, even when fast post-column reactions are being used. It is proposed to silanize the beads in order to see if a reduction in adsorption occurs.

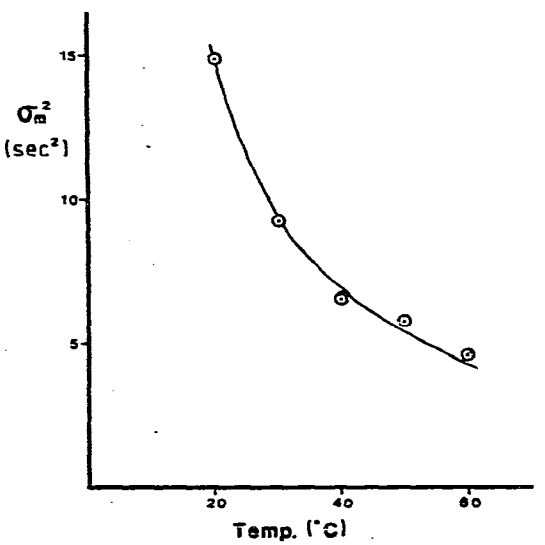

Fig. 7. Effect of temperature on variance.

APPLICATIONS

Amino acids

The technique of reagent addition described in this paper has been applied successfully to the fluorimetric determination of amino acids using either *o*-phthaldialhyde[6,7] or fluorescamine[8] as reagent (Fig. 8).

Both reagents necessitate buffering the eluent with strong alkali. *o*-Phthal-

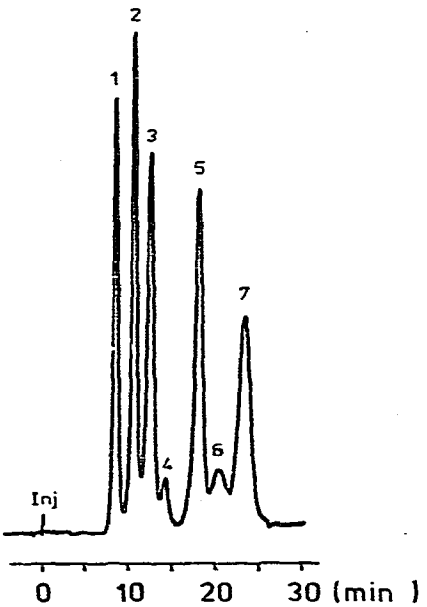

Fig. 8. Fluorimetric detection of amino acids. Column: 15 × 0.4 cm LCR-2 resin (Jeol) (12 ± 0.5 μm); 50°. Eluent: sodium citrate (0.2 *M*), pH = 3.25; 90% aqueous, 10% methanol; flow-rate = 0.46 ml/min. Reagent: *o*-pthaldialdehyde (1 g) dissolved in methanol (25 ml) added to solution of boric acid (12.37 g) + sodium hydroxide (10 g) + 2-mercaptoethanol (2 ml), and made up to 1000 ml with water; pH ≈ 10; flow-rate = 0.67 ml/min. Peaks: 1 = asp; 2 = ser; 3 = glu; 4 = cys; 5 = ala; 6 = $(cys)_2$; 7 = val.

dialdehyde is stable in aqueous solution and can be added at the same stage as the buffer. On the other hand, fluorescamine is unstable in aqueous solution and must be added in acetone solution after the eluent has been made alkaline. In this case, two PBMs are used in series. A comparison of these reagents[9] indicates advantages for each, but a significant advantage of the fluorescamine reaction is observed in the simultaneous monitoring of the fluorescence of primary amines and the UV absorption of secondary amines[10].

It is obvious that these reagents have enormous scope for amino acid analyses in the picomole range. They have also been applied to the trace analysis of other amino compounds such as anilines[9], diamines and polyamines[10], and basic antibiotics such as ampicillin and streptomycin[11]. As such, we have devoted most of our post-column application work to this area of chromatography.

Electrochemical detection

It is in conjunction with electrochemical detection[12–15], that we feel the major developments of post-column reagent addition lie. Electrochemical detection is still in its infancy in high-performance liquid chromatography, but is assuming a rapidly increasing importance in the trace analysis of drugs and metabolites in samples of biological origin[16,17]. Its fundamental advantages are very high sensitivity coupled with a certain degree of specificity as required. Additionally, a vast range of compounds are electroactive or can be made electroactive by derivative formation. In

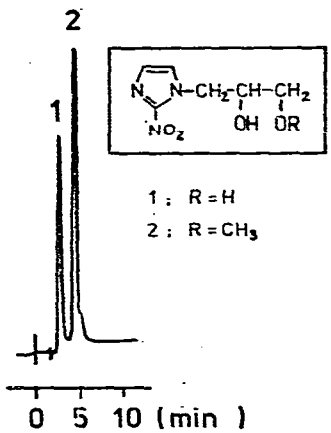

Fig. 9. Electrochemical detection of misonidazole (2) and its metabolite (1). Column: 10 × 0.4 cm Partisil-10 bonded with C_{18}. Eluent: Methanol–water (15:85); flow-rate = 1.0 ml/min. Reagent: 0.1 *M* sodium phosphate (pH 7.4); flow-rate = 1.2 ml/min. Detector: LCA-10 electrochemical detector at −0.8 V *vs.* Ag/Ag$^+$.

principle, the range of applications should exceed that of UV detection, but this has yet to be realised in practice.

There are two major reasons for using post-column reagent addition for electrochemical detection, outlined below.

Adjustment of pH and supporting electrolyte. It is not generally recognized by chromatographers that the choice of pH and electrolyte is critical in electrochemistry.

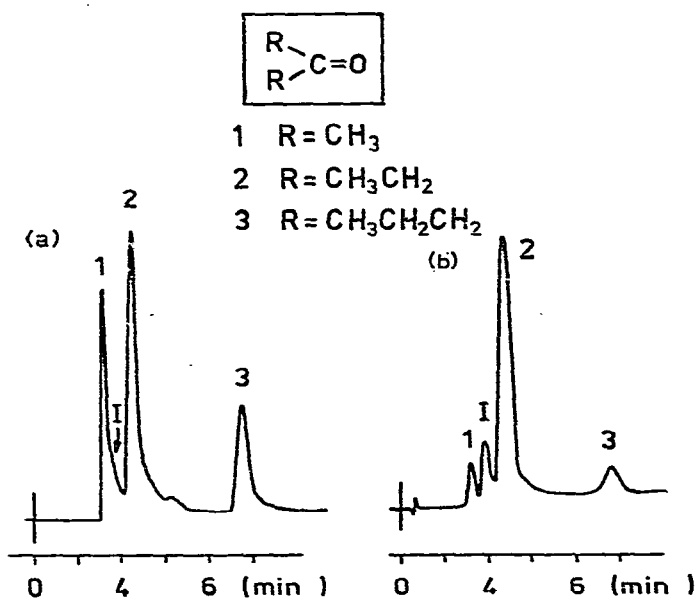

Fig. 10 10. Comparison of (a) UV and (b) electrochemical detection of ketones. Column: 10 × 0.4 cm Partisil-10 bonded with C_{18}. Eluent: methanol–water (40:60); flow-rate = 1.0 ml/min. Reagent: semicarbazide hydrochloride (10^{-3} *M*) in eluent; flow-rate = 1.0 ml/min. Detector: LCA-10 electrochemical detector at −1.1 V *vs.* Ag/Ag$^+$.

An eluent which is optimal for electrochemistry may be far from optimal for the chromatography. To illustrate this, we separated the radiosensitiser "misonidazole" from its metabolite using a well established eluent system. Post-chromatographically we added 0.1 *M* sodium phosphate buffer (pH 7.4), which sets up ideal conditions for the electrochemical detection of these compounds (Fig. 9).

Addition of derivatizing reagent. It has been shown[15] that the electroreduction of carbonyl-containing species such as aldehydes and ketones is totally repressed at a solid electrode such as vitreous carbon. If the carbonyl group is converted into its semicarbazone derivative, electroreduction readily takes place. We have achieved this derivatization by the post-chromatographic addition of a semicarbazide hydrochloride solution which enabled us to determine a mixture of ketones electrochemically (Fig. 10). Comparison of the UV trace with the electrochemical trace shows different response characteristics which are attributable to different reaction rates of the ketones with semicarbazide hydrochloride. The inhibition of the acetone response allows determination of the impurity I_1, but the impurity I_2 is not recorded electrochemically.

In both of the above examples, the electrochemical detection limit is at least one order of magnitude lower than the UV detection limit. Both examples were

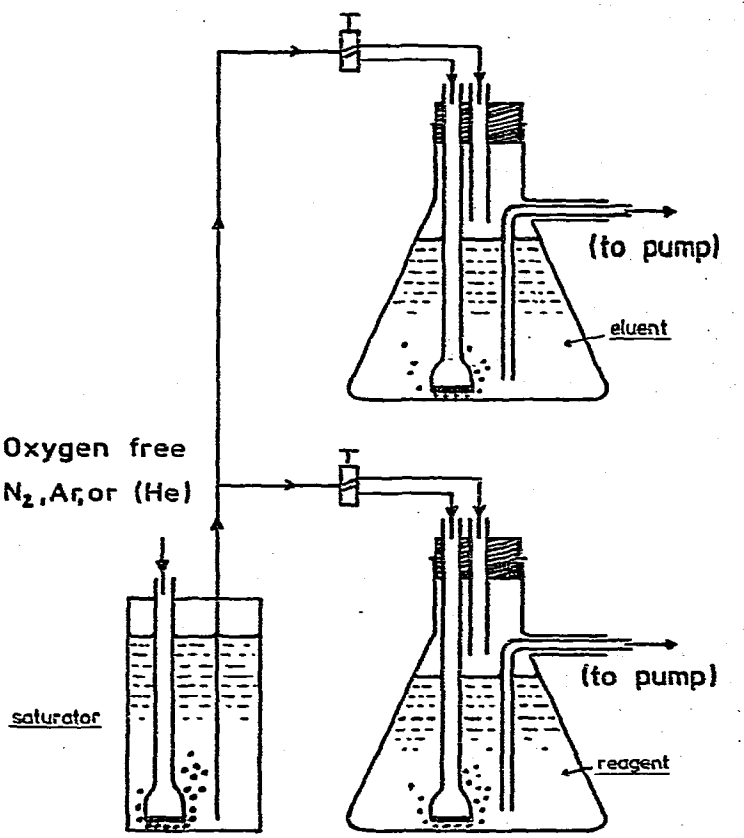

Fig. 11. Apparatus for deoxygenating the eluent and reagent. The saturator contains the aqueous and non-aqueous components of the eluent and reagent.

monitored by electroreduction techniques. This means that all dissolved oxygen must be removed from the eluent. The experimental set-up for achieving oxygen-free eluent is shown in Fig. 11.

Deoxygenation is achieved by "scrubbing" the eluent and reagent with oxygen-free nitrogen (or better, helium) for about 10 min. Thereafter, the nitrogen purge is directed above the eluent and reagent solutions to prevent ingress of oxygen. The "scrubbing" gas is previously saturated with water and methanol (or other solvent) to prevent concentration of the solutions by evaporation.

CONCLUSIONS

The packed bed mixer has been shown to be suitable for the post-chromatographic addition of reagents. Its application is limited to fast chemical reactions or the straightforward addition of a non-reacting species. It could be argued that for reactions involving long reaction times and elevated temperatures that gas-segmented reactors should be used. However, we feel that a better approach is to look for new rapid reactions that open up the possibility of detection by visible and fluorimetric spectroscopic methods or by electrochemical methods. There is still a vast uptapped literature of colorimetric spot tests[19]. This process is already under way if one considers that the fluorimetric detection of amino acids previously described is slowly but inexorably replacing ninhydrin as the detection method of choice.

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