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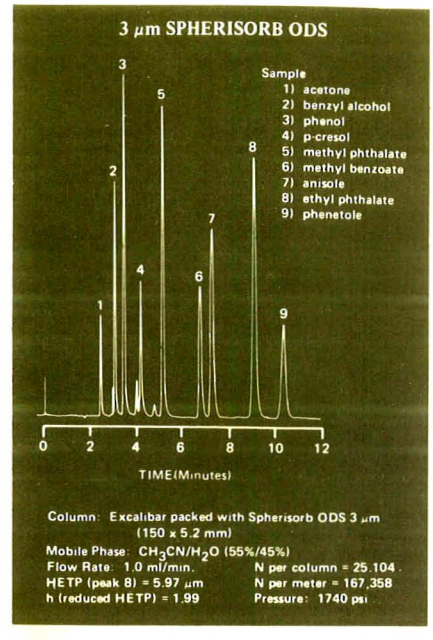

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JOURNAL OF LIQUID CHROMATOGRAPHY

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JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

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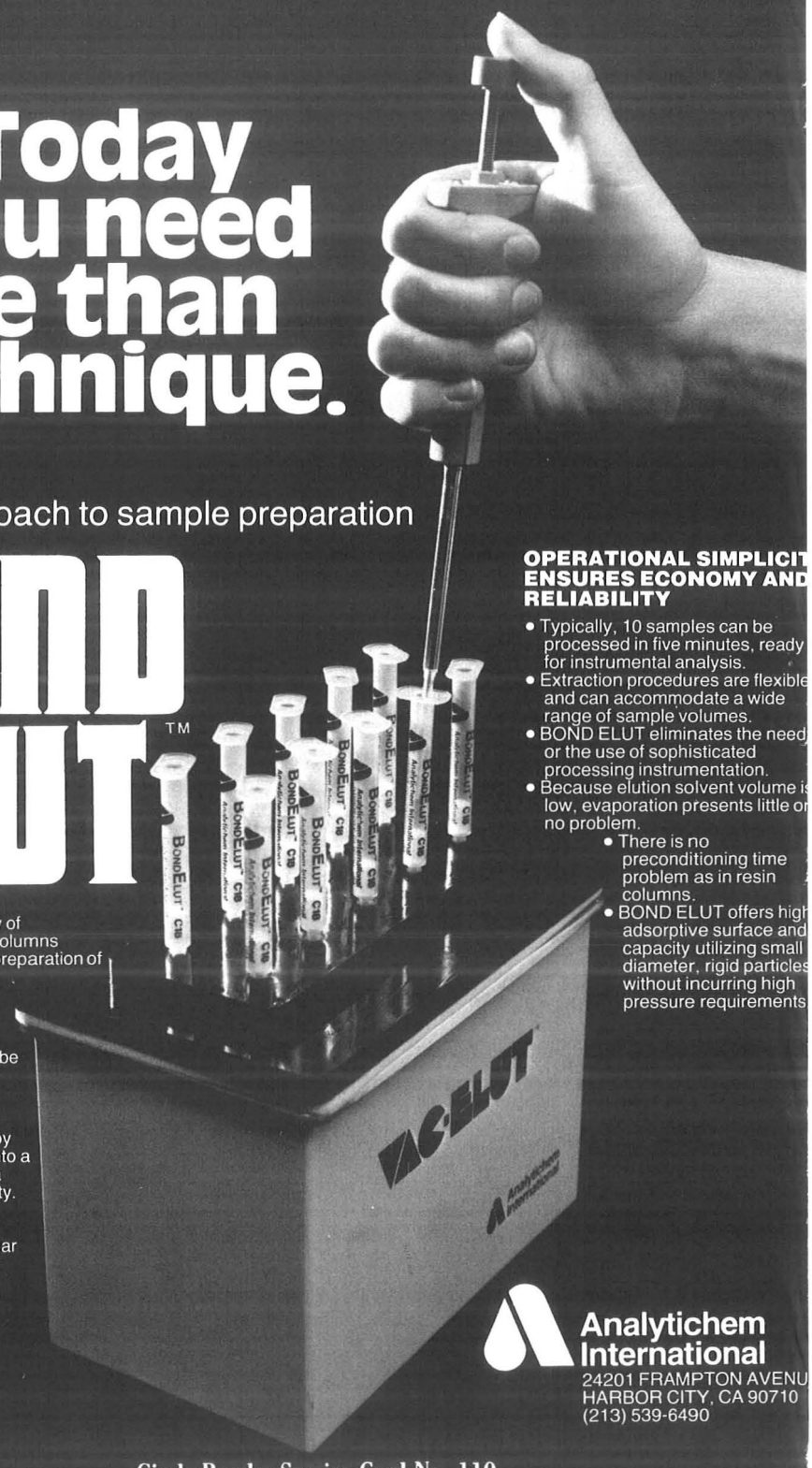

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(8), 1297-1309 (1981)

GEL PERMEATION CHROMATOGRAPHY OF POLYELECTROLYTES

M. Rinaudo, J. Desbrières, C. Rochas
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ABSTRACT

The mechanisms are discussed which control the GPC elution of ionic solutes, both the polyelectrolytes and the low molecular salts. The processes involved are quite general and valid in organic and in aqueous solvents. The conclusion is that gel permeation chromatography is a powerfull method to characterize polyelectrolytes ; and it is shown that the correct data on molecular weight distribution can be obtained when the ionic content in the eluent is larger than 5.10^{-2} M and when the concentration injected is lower than the critical overlapping concentration. The interpretation of chromatograms can be performed using the universal calibration and a viscosimetric detector.

INTRODUCTION

This paper deals with the mechanism of gel chromatography on ionic polymers in organic and aqueous solvents. We have previously shown that the processes involved were controlled by electrostatic mechanisms whatever is the solvent (1 - 4). One will propose a general treatment for characterization of polyelectrolytes by gel permeation chromatography (GPC). Much papers concern GPC on

synthetic neutral polymers ; it is well established that the steric exclusion is controlled by the hydrodynamic volume $[\eta]$ M of the polymers (5). When the chromatography of ionic solutes is performed, even with low molecular weight solutes electrostatic exclusions are observed (2, 3, 6, 9). As consequence the volume of elution depends on the ionic concentration of the solute injected but also on that of the eluent. On polyelectrolytes, universal calibration has been validated for polystyrenesulfonate and polyacrylate sodium salts (9).

One wants : 1/ to dissociate electrostatic and steric processes as soon as the secondary adsorptions on the gel are avoided ;

2/ to propose best conditions to obtain steric exclusion mechanism on ionic polymers such as to get their molecular weight distribution.

MATERIAL AND METHODS

The porous silica gels are Spherosil from Rhône-Poulenc ; a set of columns (height = 147 cm ; inner diameter 0.8 cm) are filled up with gels of following average pores diameters 70, 150, 300, 500, 1250, 3000 and 5000 Å.

The solvents are dimethylformamide (DMF) or water freshly distilled and degazed. With H_2O, the tank of solvent is maintained at 60°C during elution. Constant flow-rate is maintained with a Milton Roy pump (11 ml/h in DMF - from 12 to 60 ml/h in water). The detectors are adapted on line : a differential refractometer R 401 Waters, a conductivity cell built up in our laboratory and connected to a Wayne Kerr B 642 Conductimeter and an automatic viscosimeter from Fica filled with constant effluent volume delivered by a special buret built up in the laboratory (4). The signals of the refractometer (Δn) and of the conductimeter ($\Delta \chi$) are associated to get informations 1/ on the purity, osmotic coefficient and charge distribution of polyelectrolytes, (10)

2/ on electrostatic exclusion of low charge solutes or polyelectrolytes.

The differential refractometer signal (Δn) is coupled with the automatic viscosimeter data (η) when steric exclusion is investigated. All the concentrations are expressed in equivalent ionic charge per unit volume. The standards used to calibrate the set of columns are : polystyrenes in DMF, dextrans and sodium polystyrenesulfonates in water ; the total volume V_T of the system is determined with D_2O, whatever is the eluent.

RESULTS AND DISCUSSION

A/ Electrostatic exclusion on simple salt

First, we shall discuss the results obtained with a low molecular weight solute generally NaCl in water (11) and $NaNO_3$ in DMF (10).

Dependence with the ionic concentrations

When a constant volume (v) of a given electrolyte at a constant concentration C_s is injected, the elution volume V_e decreases when the ionic concentration of the eluent (C_e) decreases.

At a given ionic concentration of the eluent, the elution volume V_e decreases to a limit called V_{ex} when the ionic concentration (C_s) injected decreases. The figure 1 gives a schematic representation of this behaviour.

In addition, the form of the peaks eluted is modified when the ionic content varies ; they are symetrical as soon as their elution volume reaches V_{ex} for very dilute solution and V_T for high concentration (figure 2).

The total volume (V_T) of the set of columns is given wathever is the solvent by D_2O used as reference ; the volume of elution of D_2O is independent on the ionic concentration of the eluent. On the contrary, when samples of H_2O are injected, one obtains an elution peak at a limiting volume of exclusion V_{ex} depending only on C_e.

The set of results recalled in this paper is directly related to electrostatic exclusion ; the conclusion is that over 5.10^{-2}M

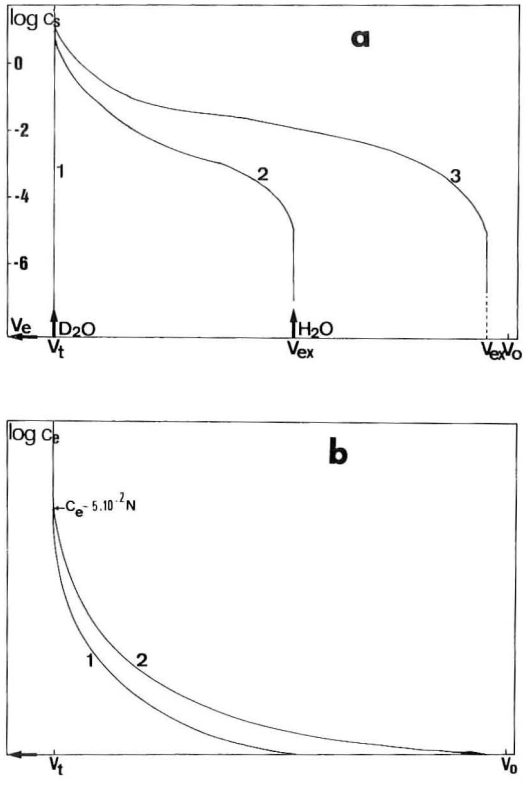

Figure 1 : Schematic dependence of the ionic concentration on the elution volume (V_e) for a constant volume (v) of electrolyte injected.

(a) influence of C_s at constant C_e
1. $C_e = 10^{-1}$ N ; 2. $C_e = 10^{-4}$ N ; 3. water

(b) influence of C_e at constant C_s
1. $C_s = 10^{-2}$N ; 2. $C_s = 10^{-4}$N.

or better 10^{-1}M for the ionic concentration of the eluent, the repulsions are screened (Figure 1b).

Dependence with the ionic content injected.

As previously mentionned, the elution volume is dependent on the concentration of the solute injected. In fact, ones demonstra-

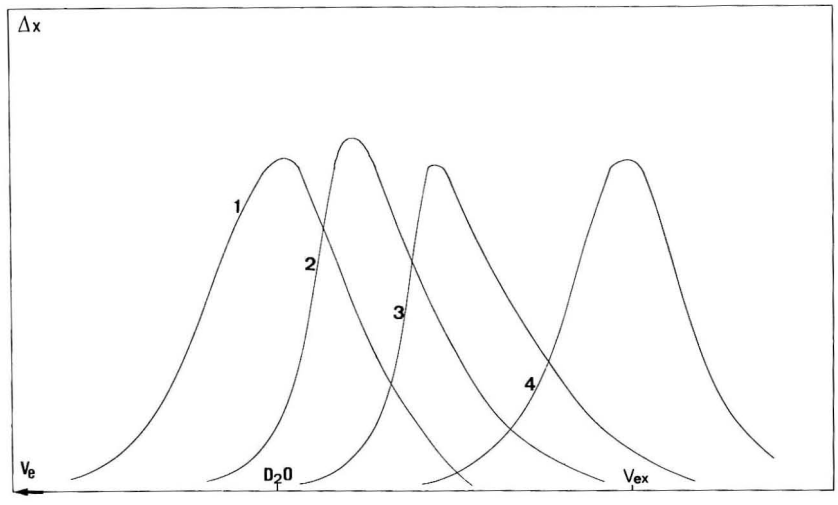

Figure 2 : Conductimetric traces for various concentrations C_s injected and eluted by water (v = 50µl).
1. C_s = 1 M - sensitivity x 1 ; 2. $C_s = 4 \times 10^{-2}$M - sensitivity x 20
3. $C_s = 8 \times 10^{-3}$M - sensitivity x 100 ; 4. $C_s = 2 \times 10^{-6}$M - sensitivity x 5000.

te that V_e is imposed by the product (volume x concentration) (11); as the volume of solution (V_S) in which a given salt is eluted is approximatively constant, one proposes to plot the dependence of average salt concentration $C_S = \frac{VC_S}{V_S}$ as a function of the partition coefficient $K = \frac{V_e - V_o}{V_T - V_o}$ (V_o is the void volume determined from the elution of a very high molecular weight polymer). When the eluent is an electrolyte, the area of the peak as well as its position is imposed by the excess of electrolyte $\Delta\overline{C_S} = (C_S - C_e) \times \frac{V}{V_S}$ (C_e is the electrolyte concentration of the solvent). For each eluent, a only curve correlates the average excess salt concentration $\Delta\overline{C_S}$ to the elution volume expressed by K.

In addition, it is shown that 1) the curve is dependent on the average pore diameter if the elution volume of NaCl is tested on

a column filled up with one type of gel (one compares Φ = 300 Å and 5000 Å (Figure 3); 2) the curve obtained for elution of Na_2SO_4 in water is nearly identical to that obtained with NaCl. Each curve is characterized by 3 parameters :

- [A] is the higher average salt concentration necessary to screen the electrostatic repulsion ($\Delta \overline{C_S} > [A] \rightarrow K = 1$). [A] depends on the porosity of the gel.

- [B] is the lower average concentration under which the elution volume remains constant ($\Delta \overline{C_S} < [B]$, $K = \text{const} = K_{ex}$) ; [B] depends on the ionic content of the eluent.

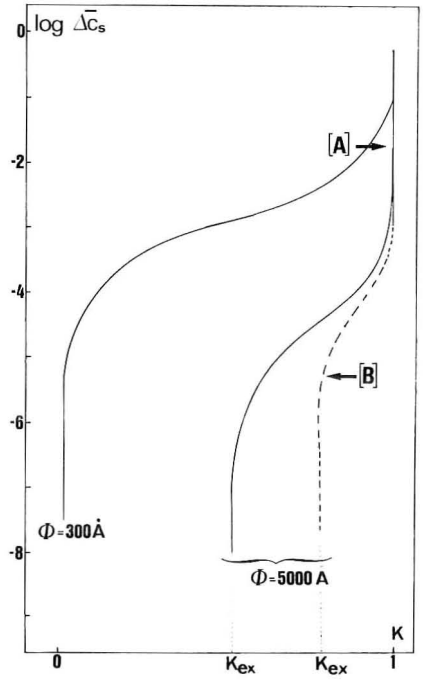

Figure 3 : Influence of the pore size (Φ = average pore diameter) on the volume of elution of NaCl expressed by the partition coefficient K ——— eluent H_2O ; — — — — eluent NaCl 10^{-4}M.

- K_{ex} is the limiting partition coefficient corresponding to the electrostatic exclusion with $0 < K_{ex} < 1$. K_{ex} depends on the porosity of the gel, on the ionic content of the eluent but not on the valence of the ions. One proposes to interpret qualitatively this set of results by introduction of the screening length of Debye-Huckel $\delta \sim \mu^{-1/2}$ (μ is the ionic strength) compared with the pore size Φ (Φ is the average pore diameter) :
For the ionic concentration B, when $\delta_{[B]} > \Phi$, one gets $K_{ex} = 0$; when $\delta_{[B]}$ is of the same order than Φ, K_{ex} corresponds to the volume fraction of the pores whose diameters are larger than δ ; it implies a sort of steric exclusion without dependence with the net charge of the ions. In the range of porosity investigated, the limit ionic concentration [B] is imposed by the ionic content of the eluent ; it is around 10^{-4} in NaCl 10^{-4} M and 10^{-6} in water.
At last, the ionic concentration [A] is attained when $\delta_{[A]}$ is lower than the lower pore diameter in the gel (3).

B/ Electrostatic exclusion on polyelectrolyte.

When a polyelectrolyte is injected and eluted by pure solvent (water or DMF), a only peak is obtained if the polymer is free of low molecular weight salt. It is a good way to test the purity. The ratio of conductimetric and refractometric peak gives an information on the charge distribution on the polymer ; this point has been previously discussed (1-2).

When two samples of sodium polystyrenesulfonate (M_w = 21.000 and 145.000 respectively) are injected at various concentration C_p and eluted by water , results like that given in figure 3 with NaCl are obtained ; the limit K_{ex} depends on the molecular weight of the polymer (3). This result is interesting and means that for very low concentration ($C_p < C_e$) the chromatograms should be interpreted in terms of molecular weight distribution even if electrostatic exclusion is not at all screened ; the curve $\Delta\chi/\Delta n$ (V_e) gives the charge density as a function of the molecular weight distribution.

When an electrolyte is used as eluent, two peaks are observed in the chromatogram due to Donnan equilibrium previously discussed (1-12). One peak corresponds to elution of the polymers and the second to that of low molecular weight solute contained in the solvent and excluded by the polyelectrolyte. Both peaks separate as a function of the concentration of polymer injected (Fig. 4a).

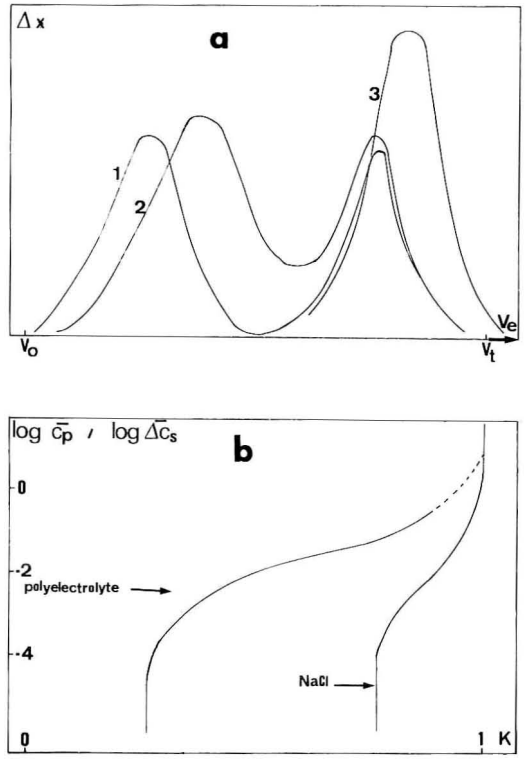

Figure 4 : Influence of the polyelectrolyte concentration injected on the chromatogram (eluent NaCl 10^{-4}M)

(a) Chromatograms obtained for variable polyelectrolyte concentration (C_p) dissolved in the same solvent (NaCl 10^{-4}M) (v = 50 µl) 1. $C_p = 10^{-1}$ eq.l^{-1} ; 2. $C_p = 5.10^{-3}$ eq.l^{-1} ; 3. $C_p = 10^{-3}$ eq. l^{-1}

(b) Schematic dependence of the volume elution for NaCl and the polyelectrolyte injected separately.

In the figure 4b, one gives the volume of elution of NaCl and polyelectrolyte separatly as a function of the average ionic concentration ; it demonstrates how to interpret the evolution of chromatogram (Figure 4a). The area of the peak eluted to higher elution volume allows to calculate the osmotic coefficient Φ of the polyelectrolyte in presence of external salt.

The number of equivalent of salt Q_s under this peak is correlated to that of the polyelectrolyte injected (Q_p) by the relation :

$$Q_s \simeq \frac{\Phi}{4} Q_p = \frac{\Phi C_p v}{4}$$

By GPC, the coefficient Φ can be obtained as a function of the ionic strength (C_e) and of the polymer concentration (C_p) ; when eluent is water, the conductimetric signal allows to deduce Φ directly (1-2) but also the mobility of the polyelectrolyte. The dependence of Φ as a function of the polymer concentration obtained by GPC has been previously compared with direct measurements (2).

C/ Steric exclusion.

One demonstrates that the same calibration of a set of silica gels is valid in water and organic solvent (4-11) ; the calibration in DMF was performed with standards of polystyrene and in water with dextrans (using 1 % ethyleneglycol in the eluent to avoid adsorption (13))and sodium polystyrenesulfonates. Coupling refractometric and viscosimetric detectors, one interprets the chromatograms obtained for a polyelectrolyte for different eluents. It is very important to control the nature of the counterions and to adopt an univalent electrolyte with same ion than the counterions of the polyelectrolyte. Using the universal calibration ($[\eta] M (V_e)$) one deduced an apparent molecular weight which decreases to a constant value as the ionic content of the eluent increases (Table I). This effect corresponds to the screening of the electrostatic exclusion of the polyelectrolyte.

TABLE I

Influence of the ionic concentration in eluent ($NaNO_3$ in DMF) on the apparent molecular weight obtained from GPC experiments.

$[NaNO_3]$	M_W [I]	M_W [II]
5×10^{-3}M	450,000	350,000
1.5×10^{-2}M	350,000	290,000
5×10^{-2}M	250,000	200,000
10^{-1}M	230,000	170,000
2×10^{-1}M *	250,000	170,000

* Light scattering measurements.

[I] and [II] are two samples of poly(acrylonitrile-co-metalylsulfonate) sodium salt. Ionic content : 0.6×10^{-3} and $1,2 \times 10^{-3}$ equivalent $-SO_3^-$ per gram of dried polymer respectively.

When $C_e > 5.10^{-2}$M, the molecular weight M_W obtained by GPC is equal to that obtained by light scattering (1-10).

In addition as the ionic content of the eluent increases over 5.10^{-2}M, the peak is still displaced to higher elution volume due to decrease of the classical hydrodynamic volume of the polyelectrolyte.

In the Table II, an exemple for the characterization of polystyrenesulfonates in aqueous solvent is given ; the agreement between molecular weight obtained by GPC and direct measurements is good ; the intrinsic viscosities given by the viscometric detec-

TABLE II

Characterization of polystyrenesulfonates (sodium salt form) by GPC ; comparison with direct measurements.

Samples	M_w Light scattering	GPC M_w	GPC M_n	GPC $[\eta]$ ($ml.g^{-1}$)	$[\eta]$ Static ($ml.g^{-1}$)
I	23,400	25,000	18,000	15.2	15.2
II	188,000	194,000	109,000	102	102
III	646,000	651,000	303,000	301	303

Solvent : H_2O - 0.1M NaCl.

tor is also very comparable to that determined in a separate experiment with an Ubbelohde viscometer.

At end, it is important to control the polymer concentration C_p injected ; in fact, one observes a dependence of the elution volume with C_p : up to a critical concentration C^*, V_e is constant; over C^*, it increases (4). This concentration corresponds to the transition between dilute to semi-dilute regime (14). It is a quite general behaviour obtained with neutral and ionic polymer ; so, it is essential to determine the elution volume extrapolated to infinite dilution to plot the calibration and it is necessary to inject a polymer at a initial concentration $C_p \leqslant C^*$. This should be a limitation for the GPC characterization of very high molecular weight polymers.

CONCLUSION

This paper is a synthesis of our contribution to the gel permeation chromatography on polyelectrolytes ; results are quite

identic in organic and aqueous solutions. The dependence of ionic concentration of the solute injected, of the eluent, of the porosity of the gel on the elution volume has been discussed.

The electrostatic exclusion from the gel is suppressed with eluent with salt content higher than 5.10^{-2}M at least for gels whose pores diameter is larger than 50 Å ; then the mechanism of steric exclusion is the only one which controls the elution and the universal calibration is convenient to analyze chromatograms. Nevertheless, it is shown that the concentration of the polymer injected must remain lower than the overlapping concentration C^*.

All the results are established on anionic polymers ; it is clear that up to day with partially negative charged gels, it is impossible to elute polycations. The GPC on polyelectrolytes seems to be a very important technic of characterization 1/ to determine the purity of a sample, the ionic charge distribution on the polymer, the osmotic coefficients when the eluent is free of external salt or at a low salt content, 2/ to establish the molecular weight distribution based on steric exclusion using the universal calibration.

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A NEW PACKING FOR AQUEOUS SIZE EXCLUSION CHROMATOGRAPHY POLYVINYLPYRROLIDONE-COATED SILICA.

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ABSTRACT

Adsorption of polyvinylpyrrolidone on silica in aqueous medium is studied. Adsorption isotherms, thermogravimetry and elementary analysis show that a complete surface coverage is reached with large pore microbeads. The coating stability and its ability to prevent non-steric effects when used as packing in exclusion chromatography are demonstrated. Four different pore size packings were prepared for chromatographic evaluations. It is shown that water soluble polymers like polyethylene oxide, polyvinylalcohol, polyacrylamide and hydroxyethylcellulose can be chromatographied in pure water according to the steric exclusion mechanism.

INTRODUCTION

For rapid size exclusion chromatography of water soluble polymers, inorganic gels (microparticles of porous silica or controlled pore glass) seem to be the best way for high mechanical strength packings. In order to prevent the strong adsorption of samples on the surface of these materials, silica particles bonded with various molecules, have been recently prepared (1-3). Two micropackings are now commercially available (4-5) but the

nature and the covalent structure of these bonded phases were not published. Another technique consists in pretreating silica beads by polyethylene oxide (6), but the coverage is not very stable.

By contrast, it is well known that polyvinylpyrrolidone (PVP) is strongly adsorbed onto silica (7-8). The presence of this polymer on a silica surface must reduce the adsorption of macromolecular solutes in GPC experiments ; this led us to check the chromatographic properties of PVP-coated silica in exclusion chromatrography of water soluble polymers.

EXPERIMENTAL

Apparatus

A Waters Associates model ALC/GPC 201, equipped with a M 6000A solvent delivery system, a U6K injector and a R 401 differential refractometer, was used throughout this work. Stainless steel columns (30cm length, 48mm I.D.), capped with Parker end fittings (2 micron frits) were packed by the slurry technique.

Materials

The silica used in our experiments was Lichrospher , purchased from Merck (Darmstadt, RFA). This spherical porous material has an average particle diameter of 10 microns and a controlled, narrow pore size distribution. To cover the whole range of molecular weight solutes, four different porosities were studied : 100 Å, 500 Å, 1,000 Å and 4,000 Å. The coating material was polyvinylpyrrolidone (PVP K 15), supplied by Fluka (Buchs, Switzerland) with a molecular weight near 10,000. The chromatographic properties of our PVP-coated silicas were mainly investigated with two sets of standards : dextrans from Sigma (St Louis, USA) and polyethylene oxides from Toyo Soda (Tokyo, Japan). In all experiments, desionized water was produced by a Milli-Q system supplied by Millipore (Bedford, Mass, USA).

PVP Adsorption

Adsorption of PVP onto silica was studied by stirring a silica microbead suspension in aqueous solutions of PVP. Some kinetic experiments have shown that adsorption equilibrium was reached within 6 hours. After this time, the suspension was centrifuged at 6,000 rpm so as to separate the supernatant, and the polymer concentration was measured by refractometry via a calibration curve. Adsorption isotherms (9) were determined on 500mg of silica in 5ml solutions of PVP from 0.1% to 10%. For each kind of silica, the weight of adsorbed polymer per weight of silica was calculated ; This was then plotted as a function of polymer concentration in solution at equilibrium (Figure 1). These curves have a different adsorption limit, depending upon the bead pore size. The higher the porosity, the smaller the amount of coated polymer. As the specific surface area of these materials is given by the supplier, we have calculated the amount of the coated polymer per surface unit. The results are given in Table 1.

The same value ($1mg/m^2$) is obtained for 500 Å, 1,000 Å and 4,000 Å materials. Conversely, the value found for the 100 Å silica is smaller ($0.8mg/m^2$). These results are in good agreement with the chromatographic properties described below, and lead us to believe that a complete coverage of the silica surface is reached except in the case of the 100 Å material. Other measurements were achieved by thermogravimetry and elementary analysis of carbon and nitrogen and gave the same values with regards to experimental errors. (Table 2).

The coating stability was checked by measuring the amount of coated polymer via elementary analysis and thermogravimetry after several washings with pure water. No desorption could be detected. The same result was obtained by checking the coating before chromatography and after eluting more than 20 liters of water.

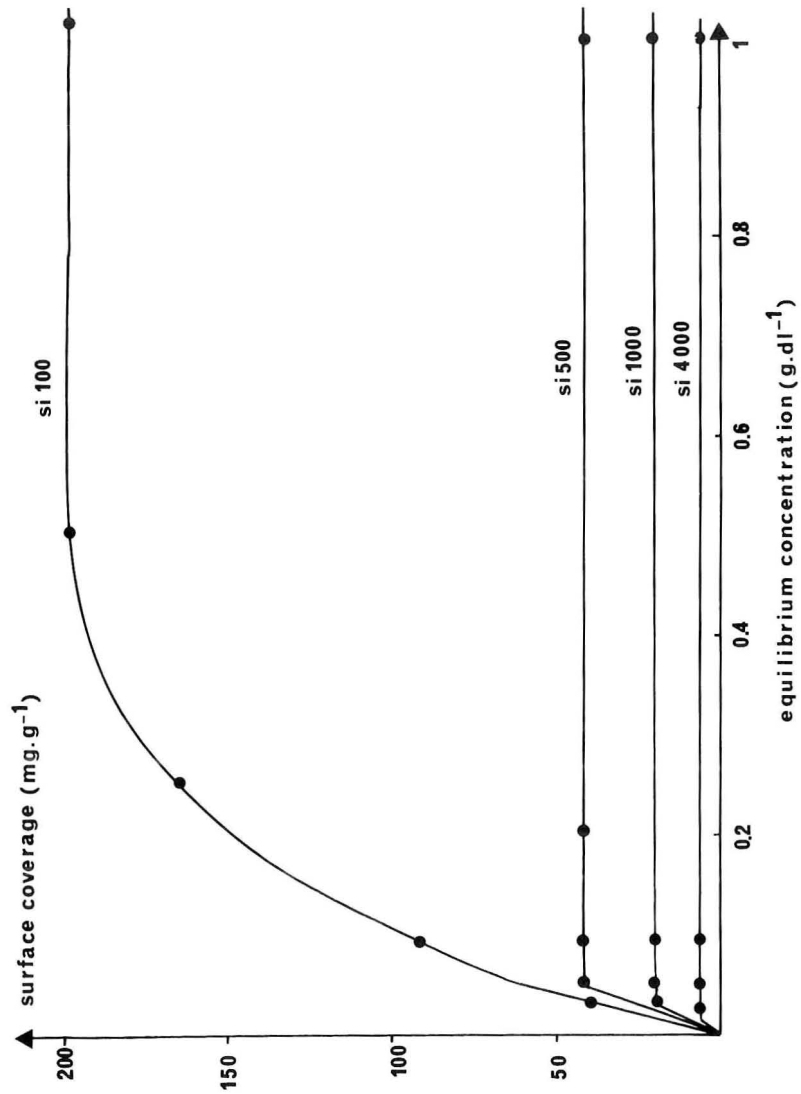

FIGURE 1

Adsorption isotherms for PVP K 15 on silica porous Lichrospher from H_2O.

TABLE 1

Porosity (in Å)	Surface area (in m^2/g)	amount of coated polymer (in mg/g)	in mg/m^2)
100	256	200	≃ 0,8
500	40	41	≃ 1
1,000	19	20	≃ 1
4,000	5	5	1

TABLE 2

Porosity (Å)	Amount of coated polymer (in mg/g)		elementary analysis
	isotherms	thermogravimetry	
100	200	170	164
500	41	40	43
1,000	20	23	24
4,000	5	--	9

Chromatographic experiments

We first tried to fill chromatographic columns with coated silicas by the slurry technique using pure water as solvent. The resultant efficiency was generally poor and did not exceed 3,000 plates per foot. After several trials in other solvents, the best efficiency (≃ 4,000 plates per foot) was obtained with a chloroform-methanol (75:25 v/v) mixture. Unfortunately, the coating is not very stable in this solvent and is partially removed from silica. The columns packed under these conditions still present some adsorption effects.

We obtained efficient PVP-coated silica columns, by the following method. Unmodified silica was packed in chloroform-methanol mixture under a pressure of 300 bars, then reconditioned in pure water via THF, methanol and methanol-water (50:50). The plate count decreased from 4,000 to 3,000 plates per foot. The silica coating was obtained by eluting a PVP solution, through the column at a level of 10% in water during 24 hours, with a flow rate of 0.5 ml/mn. No decrease was observed in the efficiency. Some measurements were performed by elementary analysis and thermogravimetry and it was demonstrated that the amount of adsorbed polymer per surface unit is the same either by this method or by the adsorption isotherm method.

The chromatographic experiments were performed at 30°C. At a flow rate of 1ml/mn, the pressure did not exceed 500 PSI per column for pure water. The polymer samples were run with injection volumes of 15µl at concentrations about 0.5%. The columns were characterized by plotting the logarithm of molecular weight of various polymer samples versus the partition coefficient, Kd defined by :

$$Kd = (V_e - V_o)/(V_t - V_o)$$

where V_e is the elution volume, V_o the void volume and V_t, the total volume of solvent.

DISCUSSION

We studied the chromatographic properties of our columns by injecting two sets of standards : dextrans and polyethylene oxides (PEO). As these two sets of compounds have different polydispersities (about 1 for PEO and about 2 for dextrans), we characterized their elution volumes by their "peak molecular weight" $M = \sqrt{(\bar{M}n.\bar{M}w)}$, which is the most representative parameter of the peak apex (10). In order to use Benoit's universal calibration, intrinsic viscosities [η] were measured by Ubbelohde viscometry.

The ability of the PVP coating to prevent adsorption of samples on silica surface is shown in Figure 2 : a classical universal

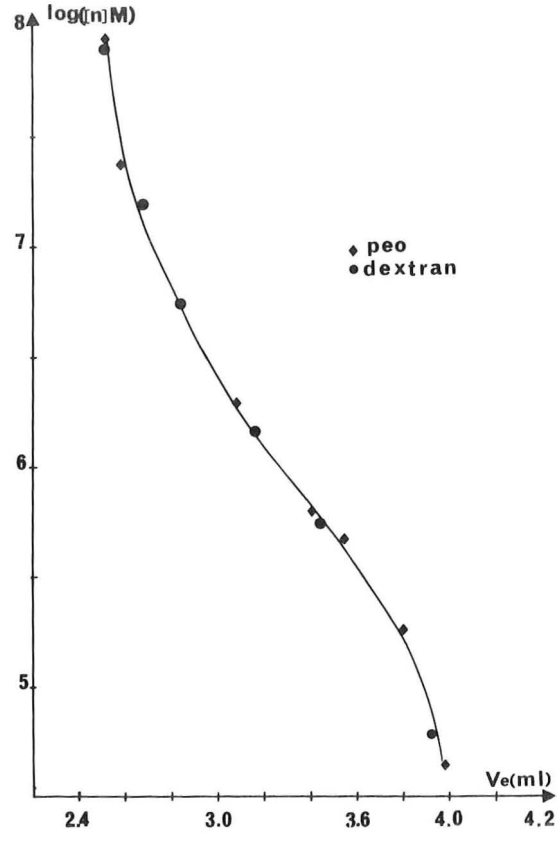

FIGURE 2

Universal calibration curve on 500 Å column : flow rate 1ml/mn.

calibration curve for PEO can be obtained on the 500 Å column, whereas these compounds are strongly retained on unmodified silica. Besides, the two universal calibration curves for dextrans and PEO are identical (Figure 2) and prove the absence of non-steric effects in the elutions. The same result was observed with the 1 000 Å and 4 000 Å columns.

On another hand, the 100 Å material presents a particular behaviour. A universal calibration can be obtained for dextrans but adsorption occurs with PEO. Excluded PEO samples, which are

strongly adsorbed on unmodified silicas, are eluted here at the void volume on coated materials, while lower molecular weight samples, which penetrate into the pores, are retarded. This phenomenon, in connection with adsorption isotherm results, can be explained by incomplete surface coverage. The coating material has, in solution, too large a hydrodynamic volume ($\bar{M}w \simeq 10,000$) to enter completely into the pore structure during the coating procedure and

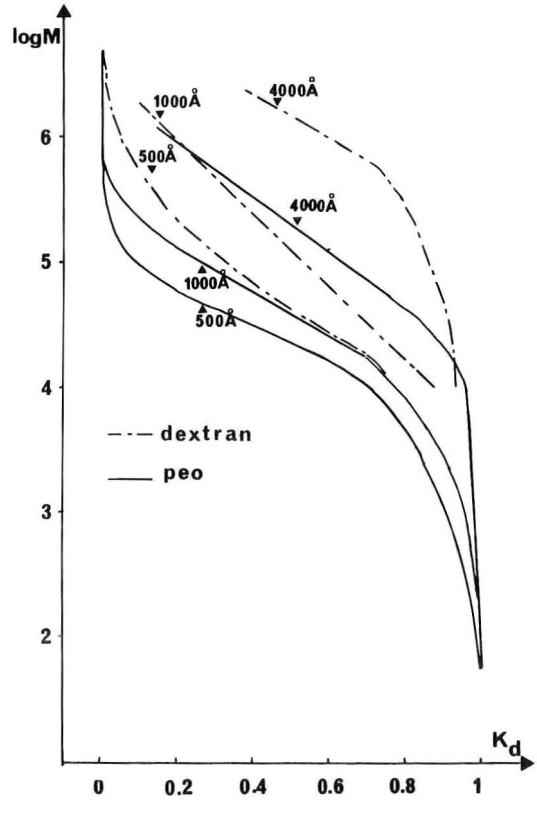

FIGURE 3

Calibration curves for PEO and dextran on 500, 1,000 and 4,000 Å columns. Solvent : H_2O. Flow rate : 1ml/mn.

TABLE 3

Porosity (Å)	Exclusion limit	
	Dextrans	PEO
500	6.10^5	2.10^5
1,000	3.10^6	5.10^5
4,000	10^7	2.10^6

the surface coverage is probably complete outside the beads but partial within the pores. The use of a lower molecular weight PVP could probably overcome this drawback and we are studying this molecular weight parameter.

In Figure 3, we have plotted the calibration curves $\log\bar{M}w=f(Kd)$ for dextrans and PEO on 500 Å, 1,000 Å and 4,000 Å coated packings in pure water. These curves enhance the difference of behaviour for the two sets of standards since the hydrodynamic volume of PEO is larger at a given molecular weight than the dextran one. In addition these curves show that the coating procedure does not significantly affect the packing porosity and that PVP coated silicas have a very wide range of molecular weight selectivity. The different exclusion limits with dextrans and PEO are given in Table 3.

As an example, Figure 4 represents the separation of a PEO sample mixture on a 1000 Å column.

Many water soluble polymers such as polyvinylalcohol, polyacrylamide and hydroxyethylcellulose, have been successfully eluted in pure water without non-steric effects. However, PVP samples seem to be retarded, because of dipolar interactions between coated PVP and PVP chains in solution, leading to an apparent increase of their elution volumes. This non-steric effect does not permit the accurate analysis of PVP samples.

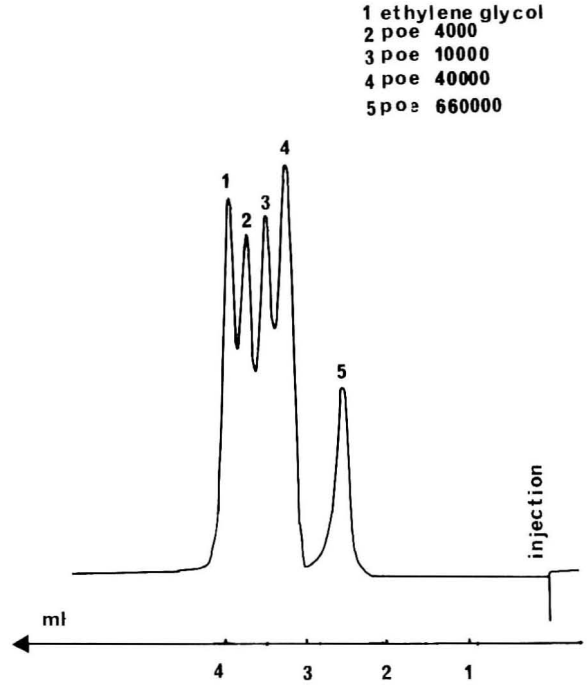

FIGURE 4

Separation of a mixture of PEO on SI 1,000 (30cm). Mobile phase : H_2O. Flow rate : 1ml/mn.

Finally, molecular size separation of charged polymers such as proteins or polymethacrylic acid were achieved with phosphate buffer as eluent. Figure 5 shows the separation of some proteins on the 500 Å column.

CONCLUSION

We have shown that the porous silica surface properties could be dramatically modified by coating it with a polymer such as poly-

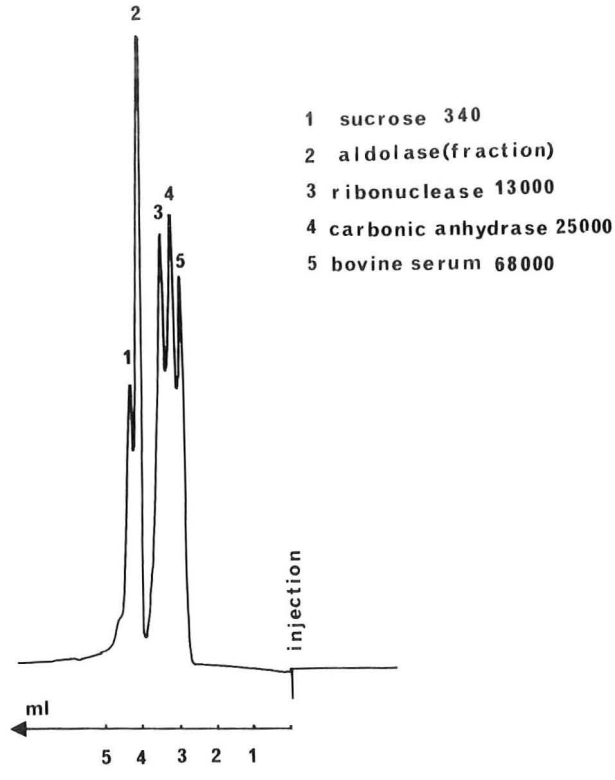

FIGURE 5

Separation of proteins on SI 500 (30cm). Mobile phase : phosphate buffer 0,1M. Flow rate : 0,5ml/mn.

vinylpyrrolidone using a very simple procedure. These PVP-coated silica packings are stable in water and may replace commercial bonded phases, that are chemically more difficult to obtain.

The direct application of these coated materials in aqueous steric exclusion chromatography is highly interesting and promising since the absence of non-steric effects enables the exact molecular weight characterization of many water soluble polymers.

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REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING CARBOXYLIC ACIDS IN THE MOBILE PHASE

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ABSTRACT

This report describes the use of different carboxylic acids as mobile phase modifiers. The effect on retention of acid chain length, pH, and eluent composition for a series of phenylalkanols, phenol, and the amines aniline, N-methylaniline, and benzylamine is discussed. The retention of both neutral and positively charged compounds is influenced by the dissociation equilibrium of the carboxylic acid in the mobile phase. By using 1-pentanol to coat excess exposed silanol groups on the reversed phase column used, the inflection in the retention of both neutral and charged solutes as pH is changed occurs at the pK_a of the acid in the mobile phase. In addition, by using an acid and amine with the same or similar pK_a values, selective ion-pairing of this pair over others with dissimilar pK_a values can be promoted. Application of this technique to the selective retention of amino acids and peptides was unsuccessful.

INTRODUCTION

Ion-pairing partition high-performance liquid chromatography, as introduced by Eksborg and Schill (2,3) has been applied to

numerous separations in recent years (4-6). The theory of the retention mechanism of this process involves various equilibria including ionization of both sample and counter-ion species, partitioning of both species and their ion-pair between stationary and mobile phases, in-situ ion exchange equilibria, adsorption processes, micelle formation, and complexation between the species involved (7). Control of the separation process can be accomplished through careful selection of eluent pH, counter ion concentration, and solvent composition to establish an optimum equilibrium position for separation.

The counter ions used for retention of basic samples have typically been the alkyl sulfonic acids (8-12) or sulfates (13, 14), di- or tricarboxylic acids (15,16), picric acid (17), or perchloric acid (18,19). The pH of the mobile phase has been chosen for protonation of the analyte base and full ionization of the counter ion. By choosing counter ions with low pK_a values, such as the sulfonic acids or alkyl sulfonates, complete ionization could be assured over a wide pH range. Therefore, ion-pairing of a number of basic samples with different pK_a values could be attained in complex mixtures (20-22).

In the present study, we have investigated the use of carboxylic acids as ion-pair reagents under conditions of partial ionization. While the ionized species was expected to act as an ion pairing reagent, it was thought that the neutral species would act as an organic modifier of the mobile phase, similar to methanol or

acetonitrile when used as organic modifiers in reversed phase high-performance liquid chromatography.

EXPERIMENTAL

Materials: Solvents and reagents were gold label spectrophotometric grade and reagent grade respectively, from Aldrich Chemical Company (Milwaukee, WI), and were used without further purification. Columns were 30 x 0.42 mm (i.d.) packed with 10$_\mu$ Sherisorb ODS (Phase Separations, Queensferry, Clwyd., UK) as described below. The chromatographic system consisted of a Waters ALC 202 liquid chromatograph (Waters Associates, Milford, MA, USA), equipped with a model 660 solvent programmer, two model 6000 pumps, a 254 nm differential UV detector, R401 differential refractive index detector, and a U6K 2 mL sample loop injector. Retention volumes were measured by collecting and measuring the eluent at the exit port from the time of injection to the top of each peak using 10 mL graduated cylinders. The void volume was measured using a saturated NaCl/H_2O solution. In general, the void volume was independent of the mobile phase composition, and the standard value of 2.92 mL was used in all calculations. Mobile phases were prepared for a pressurized solvent reservoir (24) in 500 mL aliquots. For all aqueous mobile phases, the organic salt or acid was weighed and diluted to about 450 mL, the pH adjusted to the desired value using 1.0 M HCl or concentrated NH_4OH, and then the solution was diluted to exactly 500 mL. The

pH did not change after this second dilution. When using 1-pentanol stationary phases, the mobile phase was saturated with stationary phase before this second dilution. The 70%/30% (v/v%) H_2O/MeOH solutions were prepared by diluting the organic salt or acid with 150 mL methanol and 350 mL H_2O, and then adjusting the pH. This "apparent pH" was measured using a Beckman Zeromatic pH meter (Fullerton, CA). Mobile phases were degassed for 10 minutes using an ultrasonic bath (L & R Manufacturing, Kearney, NJ) before pumping.

Stationary Phase Coating

Coating with the stationary phase 1-pentanol or 1-octanol was performed using a modified in-situ method of Kirkland and Dilks (25). The column was equilibrated with absolute ethanol, followed by 100 mL of a 10% (vol %) solution of the stationary phase in acetone at a flow rate of 1.0 mL/min. Finally, the mobile phase (aqueous solution) saturated with the stationary phase was pumped through the column at a flow rate of 2.0 mL/min, until a stable baseline was obtained. The passage of about 100 mL of mobile phase was usually required. Control of the temperature was essential, and each mobile phase was kept at the same temperature as the column.

The amount of stationary phase on the column was determined by gas chromatography after elution of the stationary phase with absolute ethanol. A Hewlett Packard HP3380A integrating gas chromatograph (Hewlett-Packard, Palo Alto, CA) was used for analysis.

The loading was 0.095 gram/gram of support for 1-pentanol and 0.077 gram/gram of support for 1-octanol.

Column Packing

30 x 0.42 mm (i.d.) columns were slurry packed as follows. 4.0 grams of dried 10μ Sherisorb ODS was weighed into a 75 mL graduated test tube. 15 mL of dry spectrophotometric grade methanol was added to the support and the mixture shaken for 30 min. on a rotary shaker. This mixture was allowed to settle on an angle and the top layer of methanol was removed by pipette as much as possible. 25 mL of redistilled cyclohexanol was then added to the wetted support, and this mixture placed on a rotary shaker for 30 min. The column interior was cleaned with conc. HNO_3, distilled H_2O, and acetone, and dried in a stream of $N_{2(g)}$. The cyclohexanol-support mixture was degassed in an ultrasonic bath for 8 min. The mixture was then added to a solvent reservoir connected between a model 6000 pump and the column with "no dead volume" fittings between the column and reservoir. The reservoir was completely filled with fresh cyclohexanol, leaving no air in the reservoir. The pump was primed with n-heptane, and this solution pumped through the column and reservoir as quickly as possible maintaining maximum pressure at or near 6000 psi. The column was heated from the bottom as the support was packing with a heat gun (Master Appliance Corporation, Racine, WI) being careful only to heat already packed portions of the column. The packing was completed when pump pressure dropped to normal values

(1000 psi). This packing procedure showed excellent results, with column plate counts in excess of 18200/m, obtained using naphthalene as solute and 80%/20% (v/v%) MeOH/H_2O as eluent.

RESULTS AND DISCUSSION

The chromatographic behavior of phenol and phenylalkanols (i-iii) was investigated as a function of pH in a reversed phase system with an aqueous 0.4 M ammonium acetate solution as mobile phase. The pronounced change in retention as pH is changed is shown in Figure 1.

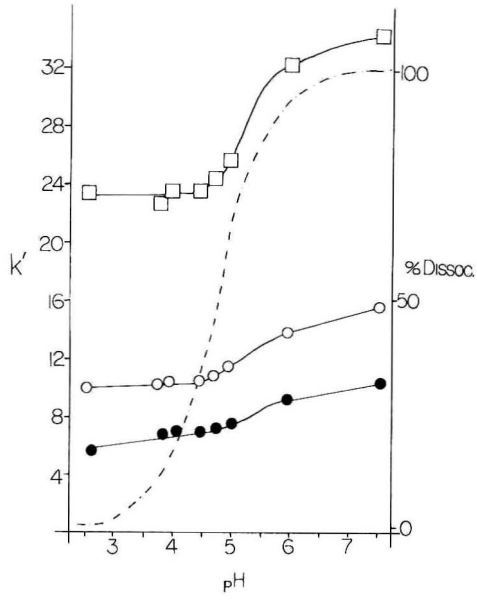


FIGURE 1

Effect of pH on the capacity factor for phenol (●), benzyl alcohol (O), and phenethyl alcohol (□) using a 0.40 M ammonium acetate mobile phase. A calculated dissociation curve for acetic acid is also shown (—·—).

The capacity factor shows a sigmoidal increase with pH and an inflection point at about pH = 5.2. A calculated dissociation curve for acetic acid is also shown, with its inflection point at 4.75. An explanation for this change in retention at or near the pK_a of the organic acid modifier of the mobile phase is found in the degree of dissociation of the acid at various pH's. At low pH, a neutral species exists in solution acting as an organic modifier of the mobile phase and lowering its polarity. At high pH, the carboxylate salt is the predominant species acting to increase the ionic strength of the mobile phase and increase retention of these neutral solutes through a "salting out" mechanism or by increasing the surface tension of the mobile phase (23).

To mimic the behavior of the equilibrium system in Figure 1, mobile phases were prepared containing calculated concentrations of ethanol and ammonium chloride equal to the equilibrium concentrations of acetic acid and acetate ion, respectively, at various pH's. Results for the retention of phenol (i) and phenylalkanols (ii, iii) using these solutions are shown in Figure 2. The shape of the curves and the magnitude of the effect exhibited by ethanol and ammonium chloride are similar to that of the ammonium acetate system.

The same comparison was made for the retention of (i-iii) using a 1-butanol and ammonium chloride system to mimic the retention of valeric acid at various pH values. The curves were also similar in shape and the magnitude of their effect.

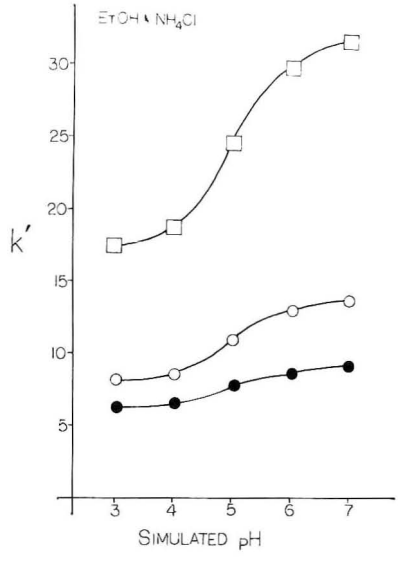


FIGURE 2

Capacity factor for phenol (●), benzyl alcohol (○), and phenethyl alcohol (□) using solutions containing ethanol and ammonium chloride in the same concentrations as acetic acid and acetate ion of Figure 1.

As additional evidence that retention is controlled by dissociation of the acid, the retention of the alcohols (i) and (ii) was examined as a function of pH using a mobile phase containing malonic acid. These results are shown in Figure 3. The curves show two inflection points at pH's of 3.3 and 5.8. These inflection points correspond to the two pK_a's for malonic acid of 2.83 and 5.69 respectively. As before, calculated dissociation curves for the two ionized species are also shown for comparison.

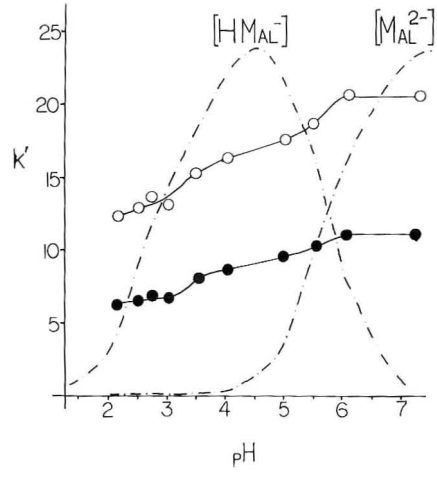
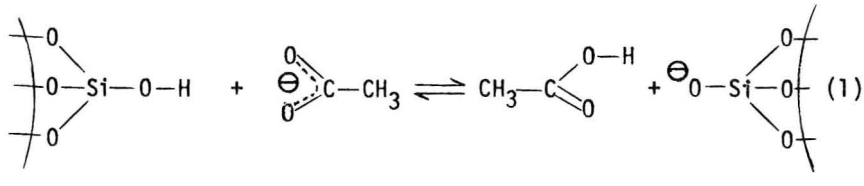


FIGURE 3

Effect of pH on the capacity factor for phenol (●) and benzyl alcohol (o) using an aqueous 0.4 M malonic acid mobile phase. The dissociation curves for the mono and dianion species of malonic acid are also shown (—·—).

The noticable difference between the point of inflection in these graphs, using both malonic and acetic acid, and the pK_a values for these acids can be explained by the acidity of residual exposed silanol functions on the column which supress the ionization of the acid through their own ionization, as is shown in Equation 1 below.



Coating the column with a 1-pentanol or 1-octanol stationary phase, in addition to the C-18 bonded phase, succeeded in blocking the effect of these acidic silanol moieties and the inflection in the curves for retention of both neutral and positively charged solutes in the presence of carboxylic acids, more closely paralleled the dissociation of the acid used. An example is shown in Figure 4 for the effect of pH on the retention of amines. By using 1-pentanol in the stationary phase, the inflection for the retention of these amines occurs at the pK_a of valeric acid (pK_a = 4.82), which was used in the mobile phase.

The use of 1-pentanol in the mobile phase also serves to solubilize valeric or octanoic acids used as well as maintain a saturated coating on the stationary phase. The effect of a sat-

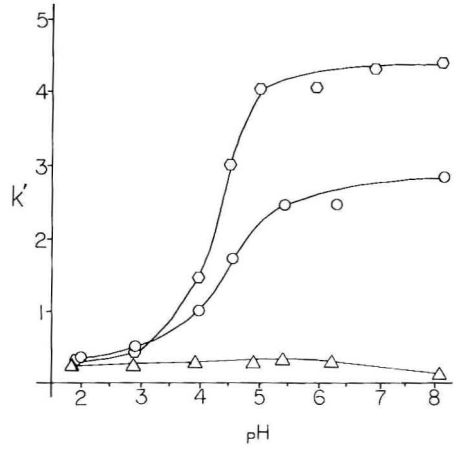


FIGURE 4

Effect of pH on the capacity factor for phenylalanine (△), benzyl amine (o), and aniline (⬡) using an aqueous 0.4 M sodium valerate mobile phase and a 1-pentanol coated stationary phase.

urated pentanol mobile phase alone causes a marked reduction in the retention of all solutes by approximately half their value in a pure water mobile phase, and its effect on the ion-pairing and in-situ ion exchange processes is unknown. However, it does not seem to affect the ionization equilibria of the species in solution as is evidenced by the foregoing data for the pK_a's of the various acids. An attempted use of a nonaqueous solvent system resulted in a suppression of ionization of the acid. When a 70%/30% (v/v%) H_2O/MeOH solvent system was used in conjunction with ammonium valerate as the mobile phase for the separation of phenol and phenylalkanols (i-iii), the inflection in the curve of pH versus k' occurred at about 6.5, and an even greater shift in the dissociation equilibrium was found for acetic acid. Use of a non-aqueous solvent in conjunction with carboxylic acids as mobile phase modifiers is therefore not useful for ion-pairing because too high a pH is needed to produce replete ionization of the acid. Present reversed phase columns preclude the use of pH's above 8.

Finally, it was of interest to explore the case where pK_a's of solute bases were similar to the pK_a of the acid in the mobile phase. From the preceding results for alcohols and amines, one would expect that a maximum in retention can occur at a pH equal to the pK_a of both species. At this pH, a maximum amount of both ionized species of the ion-pair exist in solution. Such a maximum did occur for the retention of amines (vi) and (vii), as is shown in Figure 5. Both aniline (vi) and N-methylaniline (vii)

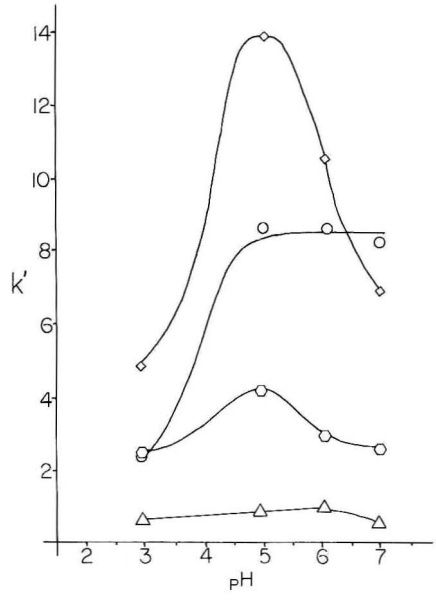


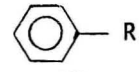
FIGURE 5

Selective ion-pairing using a 10 mM sodium octanoate mobile phase and a 1-pentanol coated stationary phase: (△) phenylalanine; (⬡) aniline; (○) benzylamine; (◇) N-methylaniline.

have similar pK_a's to the pK_a of octanoic acid used in the mobile phase. The pK_a of benzylamine (v), on the other hand, is much higher (see Table 1). Over the entire pH range shown, benzylamine exists as its protonated ammonium species, and therefore it exhibits similar behavior to that discussed earlier, being controlled by the dissociation of the acid. For aniline and N-methylaniline new factors enter into the equilibria. The curves in Figure 5 for these compounds can be thought of as containing three different sections; below, at, and above pH 4.8, which is

TABLE 1

Analyte Species And Their Ionization Constants

cmpd.	name	R	K_a	pK_a
				
i	phenol	OH	1.28×10^{-10}	9.89
ii	benzyl alcohol	CH_2OH		
iii	phenethyl alcohol	CH_2CH_2OH		
iv	phenylalanine	$CH_2CH(NH_2)COOH$	2.63×10^{-3}	2.58
			5.75×10^{-10}	9.24
v	benzylamine	CH_2NH_2	4.67×10^{-10}	9.33
vi	aniline	NH_2	2.34×10^{-5}	4.63
vii	N-methylaniline	$NHCH_3$	1.41×10^{-5}	4.85

the common pK_a of both the bases and the octanoic acid in the mobile phase. At pH's below 4.8, the amines are protonated, and neutral octanoic acid exists in the mobile phase. At pH's above 4.8, the octanoic acid exists as its octanoate ion and the amines are neutral species. At a pH equal to about 4.8, the maximum amount of both ionized species exists in the mobile phase. Maximum ion-pairing of these species at this pH, or maximum in-situ ion-exchange is responsible for the maximum in retention observed. By choosing appropriate acid-base pairs of this type, with common pK_a values, one should be able to selectively promote

ion-pairing of this pair over others in a mixture by adjusting the pH of the eluent.

Attempted use of decanoic acid as a paired-ion reagent failed due to the insolubility of this acid in saturated 1-pentanol or 1-octanol solutions.

Using these same carboxylic acids as ion-pair reagents for the retention of amino acids or peptides was unsuccessful. Examples of these experiments are shown in Figures 4 and 5 for the retention of phenylalanine (iv). In general, these amphoteric compounds were unaffected by the type of acid used or pH, and their retention volumes were short in all cases. This behavior is consistent with the foregoing explanations given for the retention behavior of neutral and charged solutes. These amphoteric species exist as charged solutes at all pH's, and cannot exhibit the "salting out" effect of neutral solutes. Ion-paired species, if formed, contain a second charge and are therefore repelled by the hydrophobic column. Low retention is therefore shown at all pH values.

Current research is underway to develop ion-pairing reagents for amino acids and peptides. It is expected that this technique will prove useful in the separation of compounds on the basis of their pK_a values.

Reversed phase columns with stability at high pH values will be needed for development of the use of alkylamines as organic modifiers of the mobile phase in a partial ionization mode for the separation of ionized acids and other anionic species.

ACKNOWLEDGMENT

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SELECTIVE QUENCHOFLUOROMETRIC DETECTION OF FLUORANTHENIC POLYCYCLIC AROMATIC HYDROCARBONS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The phenomenon of fluorescence quenching was used for selective HPLC detection of fluoranthenic polycyclic aromatic hydrocarbons (PAH). Termed a "Quenchofluorometric" detection system, it employs a filter fluorimeter or spectrofluorimeter and nitromethane in the mobile phase as the fluorescence quenching reagent. Chromatograms obtained with and without the quenching reagent are compared for PAH standards, a coal tar extract, and a shale oil sample. The quenchofluorometric detection system provides an inexpensive method to achieve selective detection for fluoranthenic PAH as a group.

INTRODUCTION

Since the advent of modern high-performance liquid chromatography (HPLC), a variety of detection systems have been used to enhance both sensitivity and selectivity. Recently, efforts have focused on the development and use of selective detectors, which

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are specific for various groups of compounds or individual compounds, rather than a "universal" detector. The need for selective detectors in HPLC is often the result of the inability of the chromatographic column to separate the compound(s) of interest from the other constituents in the complex mixture. This is particularly true for the analysis of mixtures of polycyclic aromatic hydrocarbons (PAH) from air particulates, coal tar, petroleum, coal liquids, tobacco smoke condensate, etc.

Due to the complexity of such mixtures, i.e., a large number of both unsubstituted and alkyl-substituted PAH, co-elution of compounds is a major problem. Wise *et al.* (1,2) have advocated the use of multi-dimensional chromatographic techniques, i.e., separation on normal-phase followed by reverse-phase columns, to separate such complex mixtures. The use of UV absorption and fluorescence detection for the HPLC determination of PAH also improves the selectivity.

In 1964 Sawicki *et al.* (3) described the use of selective fluorescence quenching of certain PAH in the presence of nitromethane and termed this phenomenon "quenchofluorometric" analysis. They found that in the presence of nitromethane, the fluorescence spectra of non-fluoranthenic PAH (i.e., those not containing the fluoranthene structure) were quenched and the spectra of fluoranthenic PAH were not quenched. Sawicki *et al.* (3) employed this quenchofluorometric technique, following column chromatography and directly on thin-layer chromatographic (TLC) plates, in characterizing fluoranthene and benzo[*k*]fluoranthene in air particulate extracts. Later, Dreeskamp *et al.* (4) studied the nitromethane fluorescence quenching of 22 PAH and observed some exceptions to Sawicki's rule (3), i.e., some fluoranthenic PAH (particularly fluoranthene, benzo[*b*]fluoranthene, and benzo[*k*]fluoranthene exhibited some quenching. In a brief note, Blumer and Zander (5) reported the application of nitromethane fluorescence quenching as a selective detection system for PAH in HPLC. The selectivity achieved by the addition of 5 percent nitromethane to the mobile

phase was illustrated for the analysis of a technical pyrene fraction from coal tar.

In this paper further application of the use of nitromethane as a selective quenchofluorometric HPLC detection system for PAH is described, particularly for the determination of the benzofluoranthenes in the presence of perylene and the benzopyrene isomers in coal tar and shale oil. The selectivity achieved in the HPLC determination of PAH with the quenchofluorometric detection method is compared to normal UV and fluorescence detection.

EXPERIMENTAL

Experiments were conducted on an HPLC system with gradient elution capability, a loop injector, and a fixed-wavelength UV detector (254 nm). Reverse-phase C_{18} columns (Vydac 201TP and Zorbax ODS) were used for the chromatographic separations.

The quenching reagent (a solution of 2.5 percent or 5 percent nitromethane in acetonitrile) was pumped into the system after UV detection via a "T", followed by a 3 m x 0.51 mm i.d. mixing cell. The mobile phase flow was 2.0 mL/min and the reagent flow was 0.5 mL/min. After mixing, the eluent and reagent entered a filter fluorimeter with an excitation filter passing $\sim$ 250-380 nm (color specification number 7-54) and an emission filter passing > 380 nm (color specification number 0-52). For comparison, a spectrofluorimeter was used in place of the filter instrument to evaluate the selectivity achieved by varying the excitation and emission wavelengths. For the analysis of the shale oil sample, a fluorimeter was employed with an excitation monochromator set at 300 nm and an emission filter passing > 400 nm. For this application the nitromethane was added directly to the mobile phase instead of after the UV detection. This small amount of nitromethane in the mobile phase did not affect the chromatographic separation and produced only a small shift in baseline due to its UV absorbance.

The percent of the PAH fluorescence quenched was determined by injecting the PAH standard in the presence and absence of the

nitromethane reagent. The peak heights were measured and used in the following equation:

$$\frac{\text{Percent Quenched}}{100} = 1 - \frac{\text{peak height in presence of } CH_3NO_2}{\text{peak height in absence of } CH_3NO_2}$$

RESULTS AND DISCUSSION

The structures of the PAH used in this study are shown in Figure 1. These compounds are found in most naturally occurring PAH mixtures. The six PAH isomers of molecular weight 252, i.e., the three benzofluoranthenes, benzo[*e*]pyrene, benzo[*a*]pyrene, and perylene, are generally isolated as a group using normal-phase liquid chromatography or TLC on silica or polar bonded phases (1,2,6). In addition, baseline resolution of all six of these isomers is difficult to achieve on reverse-phase C_{18} columns (2).

The use of the quenchofluorometric technique for the selective HPLC determination of fluoranthenic PAH was investigated. The effects of nitromethane on the fluorescence of the PAH shown in Figure 1 are summarized in Table 1.

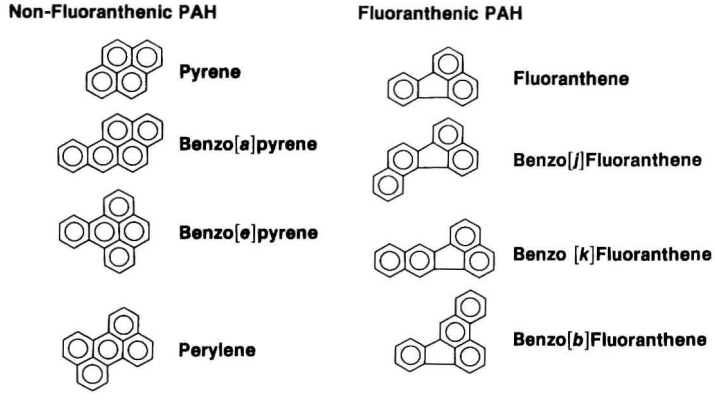

FIGURE 1 Structures of polycyclic aromatic hydrocarbons used in this study.

TABLE 1

Fluorescence Quenching of PAH by Nitromethane

CH_3NO_2 concentration in the mobile phase (volume/volume):	0.5 percent	1 percent
	Percent Quenched	
Fluoranthenic PAH:		
Fluoranthene	14	27
Benzo[*b*]fluoranthene	28	37
Benzo[*j*]fluoranthene	18	27
Benzo[*k*]fluoranthene	42	56
Non-fluoranthenic PAH:		
Pyrene	98	100
Benzo[*a*]pyrene	92	96
Benzo[*e*]pyrene	88	96
Perylene	71	86

Mobile phase: 90% CH_3CN/10% H_2O
Detector: Filter fluorimeter

With 1 percent nitromethane present in the mobile phase, the non-fluoranthenic PAH are almost completely quenched, whereas less quenching is observed for the fluoranthenic PAH. These results agree with Dreeskamp *et al.* (4) indicating that some quenching does occur even with the fluoranthenic PAH. At 0.5 percent nitromethane in the mobile phase, greater selectivity was achieved between the non-fluoranthenic and fluoranthenic PAH than at 1 percent. Thus, the samples were analyzed using the lower percentage of nitromethane in the mobile phase.

The selectivity of quenchofluorometric detection is illustrated in Figure 2 using a sample of benzo[*b*]fluoranthene (B[*b*]F), benzo-[*j*]fluoranthene (B[*j*]F), benzo[*k*]fluoranthene (B[*k*]F), and perylene in acetonitrile. Chromatogram (A) is without nitromethane; chromatogram (B), at the same attenuation, is with 0.5 percent nitromethane present. In chromatogram B the nitromethane quenches the B[*a*]P, almost all of the perylene, and presumably the B[*e*]P (since it coelutes with B[*j*]F, it is not observable).

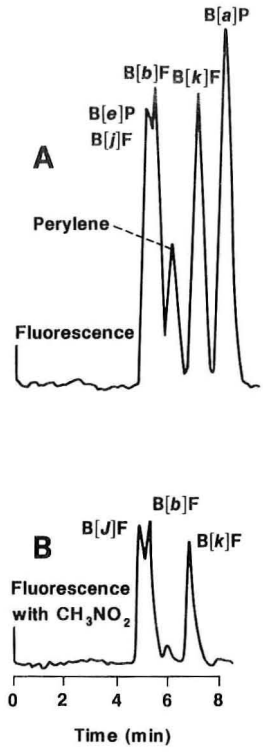

FIGURE 2 Reverse-phase C_{18} HPLC separation of six isomeric PAH standards with (A) fluorescence detection and (B) quenchofluorometric detection with 0.5 percent nitromethane in the mobile phase. Column: Vydac 201TP, mobile phase: 90 percent acetonitrile in water at 2 mL/min, detector: filter fluorimeter.

In Figure 3, chromatograms of a naturally occurring sample, a coal tar extract, are shown using three different detection methods: (A) UV at 254 nm, (B) filter fluorimeter, and (C) filter fluorimeter with nitromethane as the quenching reagent at the same attenuation as in (B). Both the UV and fluorescence chromatograms are quite complex; however, excellent selectivity is obtained for the fluoranthenic PAH using the nitromethane quenchofluorometric detection scheme as shown in chromatogram (C). Due to the large

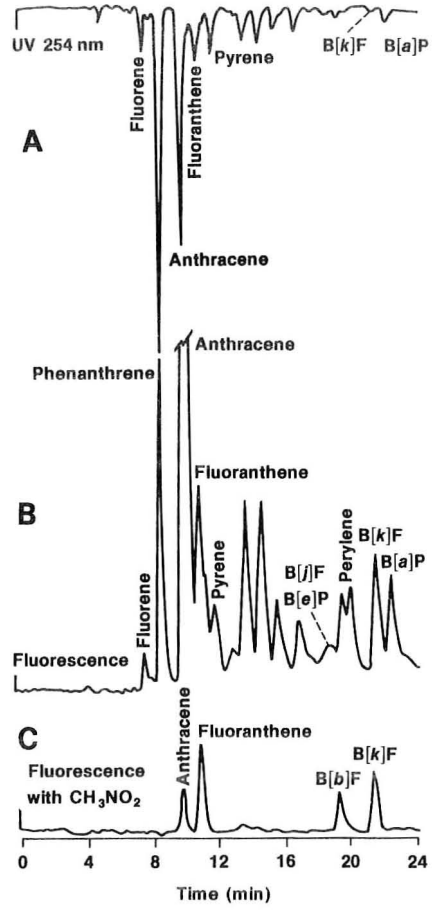

FIGURE 3 Reverse-phase C_{18} HPLC separation of a coal tar extract with (A) UV detection at 254 nm, (B) fluorescence detection, and (C) quenchofluorometric detection with 0.5 percent nitromethane in the mobile phase. Column: Vydac 201TP, mobile phase: linear gradient from 50 to 100 percent acetonitrile in water in 25 min at 2 mL/min, detector: filter fluorimeter.

fluorescence response of anthracene in this sample, a small peak for anthracene is observed in chromatogram (C) even though the great majority of its fluorescence is quenched.

The chromatograms in Figure 4 are of the same coal tar extract as in Figure 3. A spectrofluorimeter was used with the excitation and emission monochrometers set to optimize the response for fluoranthene in chromatogram (A) and benzo[*k*]fluoranthene in chromatogram (B). The selectivity for individual compounds using a spectrofluorimeter (e.g., fluoranthene in Figure 4A) is far greater than using a filter fluorimeter. Recently, May *et al.* (7) described the use of a spectrofluorimeter as an HPLC detector to achieve optimum selectivity for the determination of individual PAH in shale oil. The nitromethane quenchofluorometric method, however, provides a

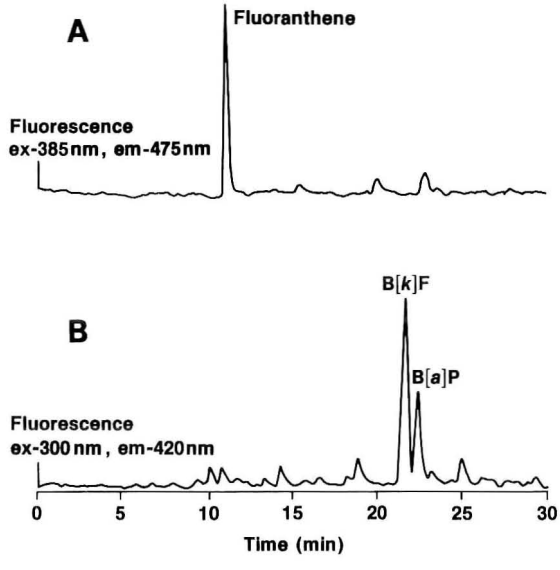

FIGURE 4 Reverse-phase C_{18} HPLC separation of a coal tar extract with selective fluorescence detection (A) λ_{ex} = 385 nm, λ_{em} = 475 nm and (B) λ_{ex} = 300 nm, λ_{em} = 420 nm. Column and mobile phase same as Figure 3, detector: spectrofluorimeter.

"fluoranthenic group specific" detector using a relatively inexpensive filter fluorimeter.

The nitromethane quenchofluorometric HPLC detection system was employed to quantitate three fluoranthenic PAH (i.e., fluoranthene, benzo[*b*]fluoranthene, and benzo[*k*]fluoranthene) in a shale oil sample [National Bureau of Standards (NBS) Standard Reference Material (SRM) 1580, "Organics in Shale Oil"]. The chromatograms in Figure 5 compare the reverse-phase C_{18} separations

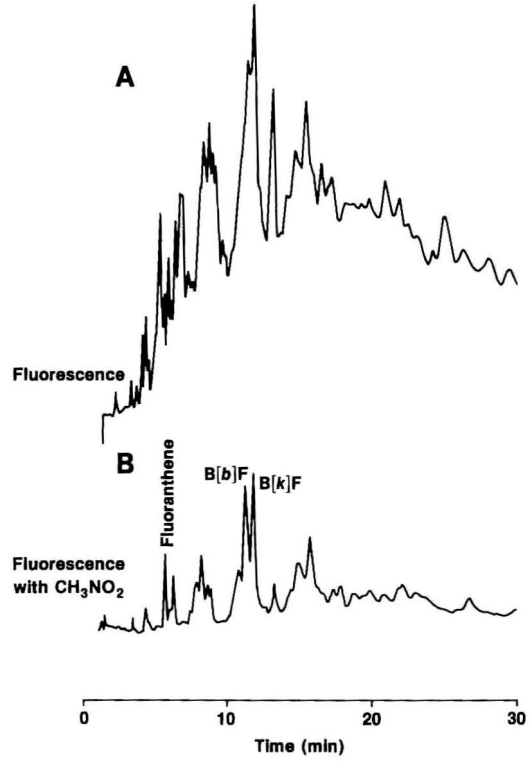

FIGURE 5 Reverse-phase C_{18} HPLC separation of PAH in shale oil (A) with fluorescence detection and (B) quenchofluorometric detection with 0.5 percent nitromethane in the mobile phase. Column: Zorbax ODS, mobile phase: 80 percent acetonitrile in water at 3 mL/min, detection: filter fluorimeter, λ_{ex} = 300 nm, λ_{em} > 400 nm.

of a total PAH fraction, which was collected from a normal-phase HPLC separation on a bonded amine column (8), (A) without the addition of a quenching reagent and (B) with the addition of nitromethane. The fluoranthene, benzo[*b*]fluoranthene, and benzo[*k*]-fluoranthene are easily quantitated from the chromatogram using the quenchofluorometric detection. Concentrations (μg/g) of these fluoranthenic PAH in the shale oil sample were found to be fluoranthene (48 ± 4), benzo[*b*]fluoranthene (12 ± 2), and benzo[*k*]-fluoranthene (5 ± 1) (uncertainty is one standard deviation of the mean). The concentrations of the fluoranthene in NBS SRM 1580, as determined by direct injection GC/MS with single ion monitoring and sequential normal- and reverse-phase HPLC with fluorescence detection, are 55 ± 5 and 53 ± 2 μg/g, respectively. The certified concentration is 54 ± 10 μg/g (9).

In summary, the quenchofluorometric HPLC detection system provides an inexpensive method to achieve selectivity for fluoranthenic PAH as a group. A spectrofluorimeter has the capability of providing a high degree of specificity for a single compound (e.g., fluoranthene in Figure 4A), however, excitation and emission wavelengths cannot be selected to provide a "group specific" chromatogram for the fluoranthenic PAH such as obtained with the nitromethane quenchofluorometric system and the filter fluorimeter.

ACKNOWLEDGMENT

Partial financial support from the Office of Energy, Minerals, and Industry within the Office of Research and Development of the U. S. Environmental Protection Agency under the Interagency Energy/Environment Research and Development Program, is gratefully acknowledged. In order to specify procedures adequately, it has been necessary to identify some commercial materials in this report. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material identified is necessarily the best available for the purpose.

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STUDIES ON STEROIDS CLXIX. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC BEHAVIOR OF SULFATED BILE ACIDS

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ABSTRACT

The elution behavior of sulfated bile acids in high-performance liquid chromatography on the octadecylsilyl bonded column with acetonitrile/0.5% phosphate buffer has been investigated. A significant influence of pH of the mobile phase on the capacity ratio (k') was observed in the higher pH region for bile acid 12-sulfates. Blockage of the 12α-hydroxyl group in sulfated bile acids by acetylation produced a marked decrease in the k' values relative to their parent compounds in the pH range above 6.0. The k' values of dehydrocholate monosulfates and their acetates were measured and the increments exerted by transformation into the acetates were estimated. Remarkable increments were observed for dehydrocholate monosulfates with the 3α-hydroxyl group but not for those with the 7α- or 12α-hydroxyl group. The effect of pH of the mobile phase on chromatographic behavior has been discussed from the stereochemical point of view.

INTRODUCTION

In a previous paper of this series we reported the elution behavior of 3-, 7- and 12-sulfated bile acids in high-performance liquid chromatography (HPLC) on the octadecylsilyl bonded (ODS) column (1). The 7- and 12-sulfates showed behavior similar to

that of the 3-sulfates with mobile phases of varying pH, exhibiting a smaller k' value than the corresponding 3-sulfates with the exception of deoxycholate* 12-sulfates. The glyco- and tauro-deoxycholate 12-sulfates showed larger k' value than the corresponding 3-sulfates in the whole pH range. As for the unconjugated deoxycholate monosulfates, the elution order of the 12- and 3-sulfates was reversed at pH 6.5. A plausible explanation for these phenomena has not yet been offered.

Recently, the occurrence of the 3-sulfates of keto bile acids in human urine was demonstrated by means of gas chromatography-mass spectrometry (2). These sulfates are not so stable under the hydrolysis or solvolysis condition. It is, therefore, desirable to develop a method for the direct analysis of the intact sulfates in biological materials. Previously, the synthesis of the 3-, 7- and 12-sulfates of bile acids having a keto and/or an acetoxyl group in the steroid nucleus was reported (3).

This paper describes the relationship between elution behavior in HPLC and structure of sulfated bile acids. In addition, chromatographic behavior of 3-, 7- and 12-dehydrocholate monosulfates and their acetates has also been investigated and discussed from the stereochemical point of view.

EXPERIMENTAL

Materials

The 3-, 7- and 12-sulfates of bile acids were synthesized in these laboratories by the methods previously reported (3,4). All

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* The following trivial names are used in this paper: deoxycholate, 3α,12α-dihydroxy-5β-cholan-24-oic acid; chenodeoxycholate, 3α,7α-dihydroxy-5β-cholan-24-oic acid; cholate, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; 3-dehydrocholate, 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid; 7-dehydrocholate, 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid; 12-dehydrocholate, 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid.

the reagents used were of analytical-reagent grade. Solvents were purified by distillation prior to use.

Apparatus

The apparatus used for this work was a Waters 6000A solvent delivery system (Waters Assoc., Milford, Mass.) equipped with a Model Uvidec-100 II ultraviolet detector (Japan Spectroscopic Co., Tokyo) monitoring the absorbance at 205 nm. The ODS SC-02 (25 cm x 4.6 mm I.D.) (Japan Spectroscopic Co.) and Radial-Pak A (10 cm x 8 mm I.D.) (Waters Assoc.) columns were employed under ambient conditions. Acetonitrile/0.5% potassium phosphate buffer (pH 3.0--7.5) (8:27-8:17) were used as mobile phases at a flow rate of 2 ml /min.

RESULTS AND DISCUSSION

Initially, the unusual chromatographic behavior of bile acid 12-sulfates was examined in general for dehydrocholate 12-sulfates and their acetates. The effect of pH of the mobile phase on the capacity ratio (Rk') relative to cholate 3-sulfate was investigated on the SC-02 column with the acetonitrile/0.5% phosphate buffer system. The ratio of Rk' value of the 12-sulfate at a certain pH to that of the corresponding 12-sulfate at pH 3.5 was plotted against the pH values (3.5-7.5). As illustrated in Figure 1, the ratio increased with increasing pH, particularly above pH 6.5. The pH effect, however, was not distinctly observed for 3-dehydrocholate 12-acetate 7-sulfate. It is evident from these data that the pH dependence for the capacity ratio is common to bile acid 12-sulfates. The glycine- and taurine-conjugated bile acids have smaller pK values than the unconjugated and are almost completely dissociated in this pH region. Conjugated deoxycholate 12-sulfates exhibited larger k' value than the corresponding 3-sulfates in the whole pH range and somewhat different chromatographic behavior from unconjugated bile acid 12-sulfates.

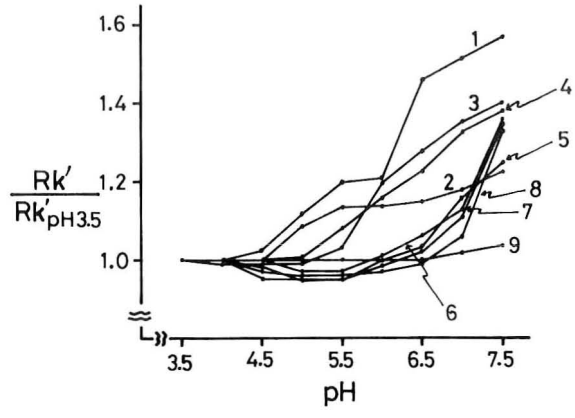

FIGURE 1 Effect of pH of Mobile Phase on Relative k' Values of Sulfated Bile Acids
1, deoxycholate 12-S; 2, cholate 12-S; 3, cholate 3-acetate 12-S; 4, cholate 7-acetate 12-S; 5, 3-dehydrocholate 12-S; 6, 3-dehydrocholate 7-acetate 12-S; 7, 7-dehydrocholate 12-S; 8, 7-dehydrocholate 3-acetate 12-S; 9, 3-dehydrocholate 12-acetate 7-S

Inspection of a Dreiding model indicates that the hydroxyl function at C-12 is sterically close to the carboxylic acid or sulfonic acid residue of the side chain. Therefore, the effect of blockage of the 7α- and 12α-hydroxyl groups by acetylation on chromatographic behavior was investigated with various sulfated cholate derivatives. The ratio of the Rk' values of the acetylated bile acid sulfate to the corresponding non-acetylated bile acid sulfate was plotted against the pH values (see Figure 2). Acetylation of the 12α-hydroxyl group exerted a marked decrease in the Rk' value in the pH region above 6.0. This result strongly implies the presence of steric interaction between the hydroxyl group at C-12 and carboxylic acid moiety at C-24 in the higher pH region.

It has previously been demonstrated that unsulfated deoxycholate and chenodeoxycholate were efficiently resolved under the weakly alkaline condition on the ODS column (5,6). Since it was supposed that a similar steric interaction would exist in deoxy-

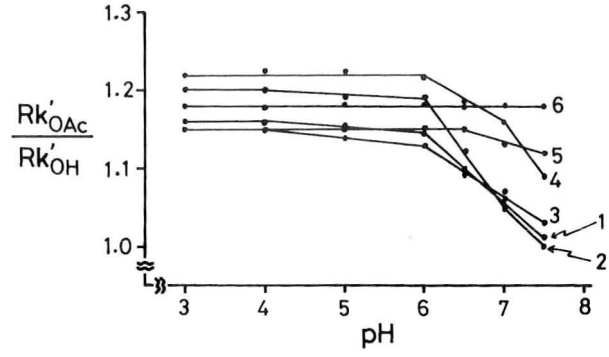

FIGURE 2 Effect of Acetylation on Relative k' Values of Sulfated Bile Acids
1, cholate 12-acetate 3-S; 2, 7-dehydrocholate 12-acetate 3-S; 3, cholate 12-acetate 7-S; 4, 3-dehydrocholate 12-acetate 7-S; 5, cholate 7-acetate 12-S; 6, 3-dehydrocholate 7-acetate 12-S

cholate, the effect of pH of the mobile phase on chromatographic behavior of unsulfated bile acids was also investigated on the Radial-Pak A column. The k' values of unconjugated, glyco-, and taurodeoxycholate relative to the corresponding chenodeoxycholate were plotted against the pH values, respectively (see Figure 3). Unconjugated deoxycholate showed a larger k' value than chenodeoxycholate in the higher pH region, while both gave almost identical values in the lower pH region. On the other hand, glyco- and taurodeoxycholate exhibited larger k' values than conjugated chenodeoxycholate in the whole pH range. These data lend a support on the assumption that the separation of deoxycholate from chenodeoxycholate on the ODS column is ascribable to the steric interaction between the hydroxyl group at C-12 and acidic moiety of the side chain.

Finally, the chromatographic behavior of dehydrocholate sulfates and their acetates were investigated on the SC-02 column with the acetonitrile / 0.5% phosphate buffer system at pH 3.5, 5.0, and 7.0. The k' values of these bile acids relative to cholate

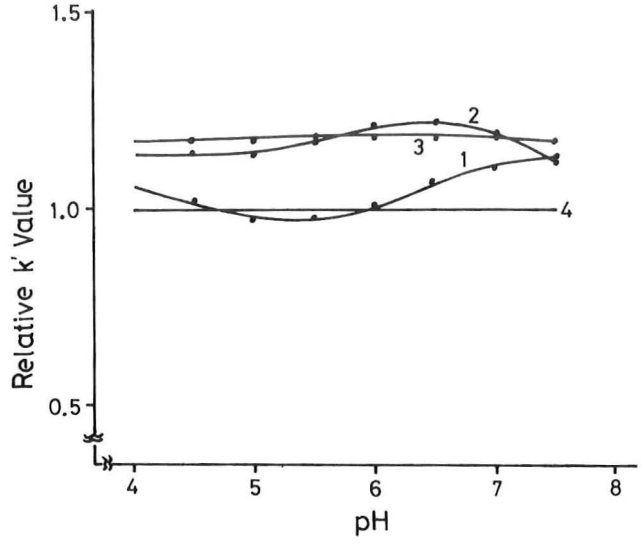

FIGURE 3 Effect of pH of Mobile Phase on k' Values of Bile Acids Relative to Chenodeoxycholate
1, deoxycholate; 2, glycodeoxycholate; 3, taurodeoxycholate; 4, unconjugated, glyco-, and taurochenodeoxycholate

3-sulfate are listed in Table 1. The 3-, 7- and 12-dehydrocholate monosulfates exhibited no significant difference in the Rk' values at each pH, and complete separation was not accomplished. The effect of acetylation on chromatographic behavior was then investigated with six dehydrocholate monosulfates. A marked change in the Rk' values was observed for dehydrocholate monosulfates with the 3α-hydroxyl group but not for those with the 7α- or 12α-hydroxyl group. This result implies that dehydrocholate monosulfates can be efficiently separated by acetylation prior to HPLC. An increment in the Rk' value exerted by acetylation, i.e. ΔRk' value, was estimated (see Table 1). In the lower pH region, the values for the 3α-, 7α-, and 12α-hydroxyl groups were 0.48-0.67, 0.03-0.06, and 0.10-0.14, respectively. At pH 7.5, ΔRk' values were different from those at pH 3.5 and 5.0, indicating the participation of

TABLE 1

Relative Capacity Ratios of Sulfated Dehydrocholates and Their Acetates

Compound	pH 3.5		pH 5.0		pH 7.5	
	Rk'	ΔRk'	Rk'	ΔRk'	Rk'	ΔRk'
7-Dehydrocholate 3-S	0.54		0.50		0.52	
12-Dehydrocholate 3-S	0.47		0.43		0.42	
3-Dehydrocholate 7-S	0.62		0.57		0.69	
12-Dehydrocholate 7-S	0.46		0.39		0.47	
3-Dehydrocholate 12-S	0.64		0.61		0.80	
7-Dehydrocholate 12-S	0.37		0.35		0.50	
7-Dehydrocholate 12-acetate 3-S	0.65	0.11	0.60	0.10	0.52	0.00
12-Dehydrocholate 7-acetate 3-S	0.50	0.03	0.47	0.04	0.44	0.02
3-Dehydrocholate 12-acetate 7-S	0.76	0.14	0.70	0.13	0.76	0.07
12-Dehydrocholate 3-acetate 7-S	1.13	0.67	1.05	0.66	1.26	0.79
3-Dehydrocholate 7-acetate 12-S	0.69	0.05	0.67	0.06	0.94	0.14
7-Dehydrocholate 3-acetate 12-S	0.87	0.50	0.83	0.48	1.17	0.67

steric interaction with the carboxylic acid group of the side chain.

The current methods for the determination of bile acid sulfates involve prior solvolysis and hydrolysis (7,8), but information about the conjugated form and position is thus lost. Having investigated the chromatographic behavior of sulfated bile acids with mobile phases of varying pH, we have now clarified that it depends on the position of the sulfate and hydroxyl groups. The k' values of 12-sulfates increase significantly in the higher pH region, owing to steric interaction with the acidic moiety on the side chain. We have also demonstrated that the effect of acetylation on the k' value depends upon the position of the hydroxyl

function. In particular, the k' values of bile acids having a 3α-hydroxyl group are significantly influenced by acetylation. These findings may be useful in the structural elucidation of sulfated bile acids and in the characterization of sulfotransferases in biological materials. The availability of an excellent method for the analysis may provide more precise knowledge on the metabolic profile of sulfated bile acids in patients with hepatobiliary diseases.

ACKNOWLEDGEMENTS

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EXPERIMENTAL STUDIES WITH A BONDED N-ACETYLAMINOPROPYLSILICA STATIONARY PHASE FOR THE AQUEOUS HIGH PERFORMANCE EXCLUSION CHROMATOGRPAHY OF POLYPEPTIDES AND PROTEINS*

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ABSTRACT

The potential of the micoparticulate, chemically bonded N-acetylaminopropylsilica stationary phase of nominal pore diameter of 100 angstroms in the high speed gel permeation chromatography of polypeptides and small proteins has been further investigated. The influence of ionic strength on the elution behaviour of a selected group of polypeptides and proteins on this bonded hydrophilic support has been examined. The results obtained with this porous, microparticulate bonded 'amide' phase silica support, packed into standard analytical-

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* High Performance Liquid Chromatography of Amino Acids Peptides and Proteins XXXI. For part XXX see ref. [1].

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size stainless steel HPLC columns, indicate that milligram quantities of polypeptides and proteins with molecular mass up to 45,000-50,000 daltons can be efficiently fractionated with excellent recoveries of biological activities. The role of silica-based sorbents in the gel permeation fractionation of polypeptide and protein hormones, including those of pituitary and hypothalamic origin, is discussed.

INTRODUCTION

Gel permeation chromatography of polypeptides and proteins on agaroses, cross-linked dextrans and cross-linked polyacrylamide copolymer gel supports has been extensively used over the last two decades for their fractionation, in desalting experiments as well as for the estimation of the molecular weights of these biopolymers in their native and denatured states. Most conventional xerogels, which have been used as stationary-phases in these open column separations, are unsuited to the chromatographic conditions now commonly employed with high performance liquid chromatographic (HPLC) techniques. For example, variation in eluent composition will result in a change in the degree of swelling of conventional xerogels and this in turn will affect the column bed volume and the pore structure on which the separation depends. Deformation of the gel structure is also commonly found with many of the earlier types of soft hydrophilic organic gels at high flow rates or viscosities of the mobile phase. Recently, attention has focused on the use of pressure stable hydrophilic aerogels of small particle size as stationary phases in the high speed gel permeation HPLC of water soluble biomolecules. Because of its favourable physical and chemical properties rigid, porous silica has been favoured as the support matrix for the preparation of these hydrophilic aerogels via modification of the silica surface with an adsorptively coated or chemically bonded ligand of suitable functionality.

Porous silica itself is generally unsuited for the separation of polypeptides and proteins due to severe ion exclusion and adsorption effects. Although these effects can be partially reduced by adsorptively coating the silica surface with hydrophilic polymers such as polyethylene glycols, the resultant phases exhibit generally poor stability. By chemically bonding hydrophilic phases to accessible silanol groups on the silica surface, many of the limitations arising from the strong ionic and hydrogen bonding interactions between charged or polar groups in the protein or polypeptide and the silica surface can be largely avoided. The 1,2-dihydroxy-3-propoxy-propyl modified silicas ('glycophase'-bonded supports) developed by Regnier and Noel [2] and Becker and Unger [3,4] have been used in several studies [5-10] related to the gel permeation HPLC of native proteins. More recently, the Toya Soda TSK-Gel SW series and the Waters PAC I-series of chemically bonded silica stationary phases have been found to give satisfactory recoveries of native proteins in a number of small scale fractionation studies as well as good correlation between log M.W. and the elution volume for protein polypeptides in the presence of denaturing reagents [11-20]. To date the precise chemical and physical compositions of the bonded phases used in both of these types of commercial support remain proprietary information. The chemical modification of the surface silanol groups of silica with triethoxy-N-acetylaminopropylsilane has been shown by Engelhardt and Mathes [21-23] to yield a hydrophilic 'amide' stationary phase suitable for the exclusion chromatography of water soluble polymers, including proteins.

Most of the previous studies with chemically bonded hydrophilic silicas have been concerned more with the

analytical potential of these supports. In this paper we describe further experimental studies addressed to the small scale preparative exclusion chromatography of polypeptides and proteins using this 'amide' phase bonded to microparticulate, porous silica with a nominal pore diameter of 100 angstroms. The data obtained on the recoveries and elution characteristics of a range of polypeptides and proteins, indicates that solutes in the range 2-40 x 10^3 daltons can be successfully fractionated on this support. The feasibility of using this, and related bonded hydrophilic phase silicas, in the purification of peptide and protein hormones by a combination of gel permeation and reversed phase HPLC techniques is discussed.

MATERIALS AND METHODS

High Performance Liquid Chromatography.

The liquid chromatograph consisted of a M6000A solvent delivery unit, U6K universal injector and a M450 variable wavelength UV monitor (all from Waters Assoc., Milford, Mass., U.S.A.) and a Rikadenki dual channel chart recorder. The bonded 'amide' stationary phase was prepared from LiChrosorb Si100 as previously described [23]. This bonded 'amide' phase silica has an effective surface coverage of bound ligand of 4.4μmol/m^2, whilst the silica matrix used had an average pore diameter of 100 Å, a specific pore volume of 1cm^3/g and a specific surface area of ca. 300m^2/g. The nominal particle diameter is 10μm. All separations were carried out on a standard drilled stainless steel column (25cm x 4.1mm I.D.) slurry packed with the bonded phase. The column volume was 3.3ml. The elution volume (V_e) of each solute was determined from the elution time (V_T) multipled by flow rate, and related to the interstitial volume, V_Z and pore volume, V_P, of the

stationary phase in the column by $V_e = V_Z + \kappa V_P$ where κ is the Wheaton-Baumann distribution coefficient [24]. The sample injections (1-25µl) at protein concentrations in the range 1-20mg/ml were made with Pressure Lok liquid syringes from Precision Sampling Corp., (Baton Rouge, La. U.S.A.). Eluants were degassed and filtered as reported previously [5]. All chromatography was carried out at room temperature (ca. 18°). The mobile phase flow rate was 0.5ml/min unless otherwise indicated in the text.

Chemicals and Reagents.

Tris buffer was obtained from Sigma Chemical Co., (St Louis, Mo., U.S.A.) orthophosphoric acid, and sodium chloride A.R. were obtained from May and Baker (Dagenham, Great Britain). The source of some of the polypeptides and proteins (Table) used in this study has been given previously [25], the remainder were either prepared and purified in this laboratory or purchased from Sigma Chemical Co., Miles Laboratories (Kankakee, Ill., U.S.A.) or Worthington Biochemical Corp., (Freehold, New Jersey, U.S.A.). The ovine anterior pituitary proteins were isolated by a new procedure to be reported elsewhere [18]. The ovine thyroid binding protein preparation was prepared as previously described [26,27] whilst the biological potency of the ovine thyrotrophin and thyroid binding protein preparations were assessed by the McKenzie mouse bioassay [28]. Protein recoveries were determined by the Bradford assay [29] and peak area integration. Detection was at 215nm.

RESULTS AND DISCUSSIONS

Over the past several years, HPLC techniques have attracted considerable attention for the purification of peptides, polypeptides and proteins from tissue homogenates.

TABLE

Polypeptide/Protein	M.W.	pI*	t_e(sec)
1. Phenylalanine	165	6.0	340
2. Tryptophan	204	5.9	367
3. Oxytocin	1,007	~6.6	289
4. Ile^5-Angiotensin II	1,046	~6.8	288
5. Adrenocorticotropin(1-24)	2,930	~8.0	280
6. Bovine Insulin	~5,800	5.3	275
7. Cytochrome C	~12,200	9.3	344
8. Lysozyme	~14,300	11.0	834
9. Myoglobin	~17,200	7.1	251
10. Bovine Growth Hormone	~23,000	7.3	239
11. Bovine Trypsin	~23,300	10.8	297
12. Chymotrypsinogen	~25,700	9.5	309
13. Ovine Thyrotrophin	~32,000	5.5-6.8	220
14. Bovine β-Lactoglobulin	~35,000	5.1	243
15. Ovalbumin	~43,000	4.7	211
16. Catalase	~58,000	6.7	184
17. Bovine Haemoglobin	~64,500	6.8	287
18. Bovine Serum Albumin	~68,000	4.4-4.8	196
19. Lactate Dehydrogenase	~144,000	5.2	194
20. Aldolase	~160,000	9.1-9.7	184
21. Ferritin	~440,000	4.2-4.5	184
22. Thyroglobulin	~660,000	4.5	181

* Data from ref. [18, 40-43].

Recent publications from our and other laboratories have clearly demonstrated that the separation and recovery of bioactive polypeptides and even small proteins up to ca 2×10^4 daltons can be achieved by reversed phase HPLC with appropriately chosen mobile phase conditions, i.e. suitable mole fraction and elutropic characteristics of the organic solvent modifier, pH buffer composition, temperature (for reviews of recent applications and elution strategies see [30-35]). With careful attention to the parameters which control the secondary chemical equilibria established between the solute molecules, the mobile phase and the stationary phase and to the role these effects play in modulating the overall chromatographic distribution process, greatly improved resolution and recoveries can be obtained on microparticulate reversed phase silicas when compared to open column gel or ion-exchange chromatographic separations. With some larger proteins, unsatisfactory resolution and recoveries have been observed with the current generation of reversed phase silicas due in part to inappropriate porosities and surface coverage of the silica support, unfavourable solubility parameter dependencies of the protein solutes on the mobile phase composition, the tendency for little, or no, elution development to occur and to the poor mass transfer kinetics exhibited by many macroglobulins. For these reasons alone, the wider use of chemically stable, non-compressible bonded hydrophilic phase silicas of appropriate pore structure as gel permeation HPLC supports would considerably supplement existing capabilities of reversed phase HPLC methods. A further strong justification for reliable gel permeation HPLC supports comes from the important requirement in most protein purification strategies and particularly with the small- or micro-scale purification of biologically potent or labile proteins, to use two or more

high resolution techniques, preferably based on different separation phenomena, as early and as rapidly in the isolation protocol as possible.

For a bonded phase support to be effective in the gel permeation HPLC separations of biopolymers, the choice of the ligand to be used in the chemical modification of the surface of the porous silica must be based on at least the following four considerations, namely (1) the derivatisation reaction should provide a dense surface coverage of the ligand which acts as a water wettable, non-ionic hydrophilic surface,(2) the ligand neither selectively adsorbs or repels proteins and ideally should be of similar polarity to that of the biopolymers to be separated,(3) the bonded ligand must be chemically stable to the buffered solutions and pH conditions commonly used for peptide or protein isolations and (4) the carbonaceous silane reagent should be easily prepared, form a stable monolayer rather than a polymeric bonded layer and have a small average place requirement. Practical constaints in the coating technology do not currently allow these requirements to be fully met. For example, it is not possible due to steric restrictions and other limitations in the derivatisation reaction to completely modify all the silanols on the silica surface. The presence of residual silanols in bonded hydrophilic supports will lead to ionic interactions most noticable with very basic (i.e. pI > 9) or very acidic (i.e. pI < 5) proteins. These electrostatic interactions can be minimised by the appropriate choice of mobile phase ionic strength or by the use of acidic amine buffers [19,31,32].

The contribution of ionic interactions between the bonded 'amide' phase and the polypeptides and proteins shown in the Table was assessed by comparing the elution volumes as a function of ionic strength at pH 7.5. Representative

results are shown in Figure 1. It was apparent from these experiments that the more basic proteins, e.g. lysozyme, cytochrome c, showed greater dependency of their elution volumes on ionic strength than the weakly basic or acidic proteins and polypeptides. At low ionic strength, negatively charged polypeptides would be expected to be partially excluded from the negatively charge pores of the stationary phase. This will lead to smaller elution volumes than expected on the basis of their molecular weights. Experimentally, this has been observed [9,22] with both the 'amide'

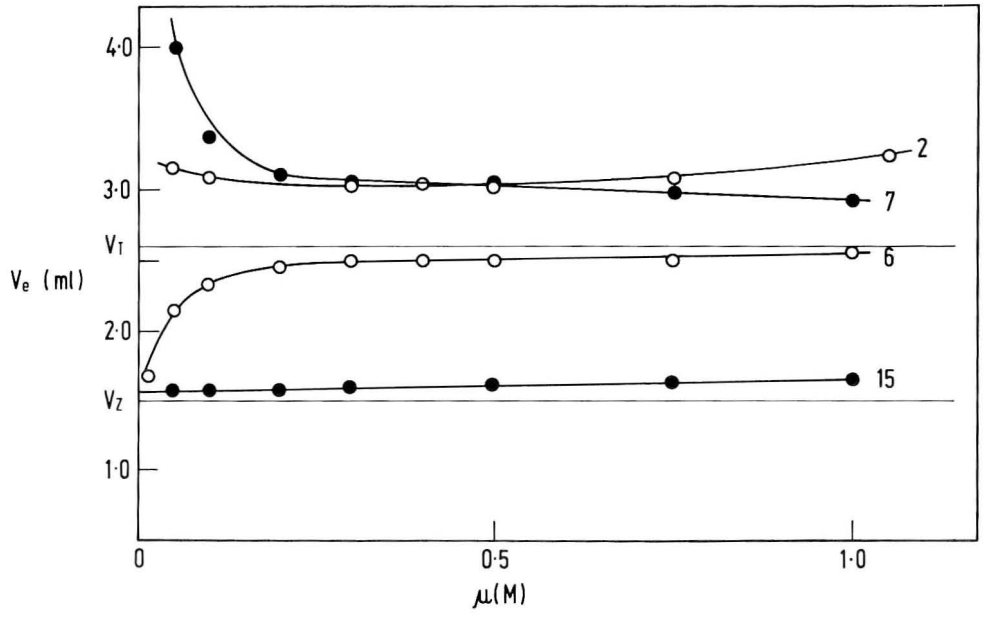

Figure 1.

Retention behaviour of several polypeptides and proteins on the bonded 'amide' phase LiChrosorb Si 100 support. The plots show the variation of the elution volume of different solutes as a function of ionic strength. See the Table for the solute key.

and the 'glyco-phase' supports. Weakly basic and weakly acidic proteins would be expected to be the least affected by changes in the ionic strength and this was generally found in the present study. For most of the peptides and proteins examined the elution volumes were essentially independent of ionic strength above μ=0.3M. For assay convenience, a 25mM Tris-HCl/125mM NaCl (pH 7.5) eluent was found most effective in several subsequent protein isolations. The same eluent was used for the investigation of the log M.W. depending on elution volume. Shown in Figure 2 and 3 are the plots of

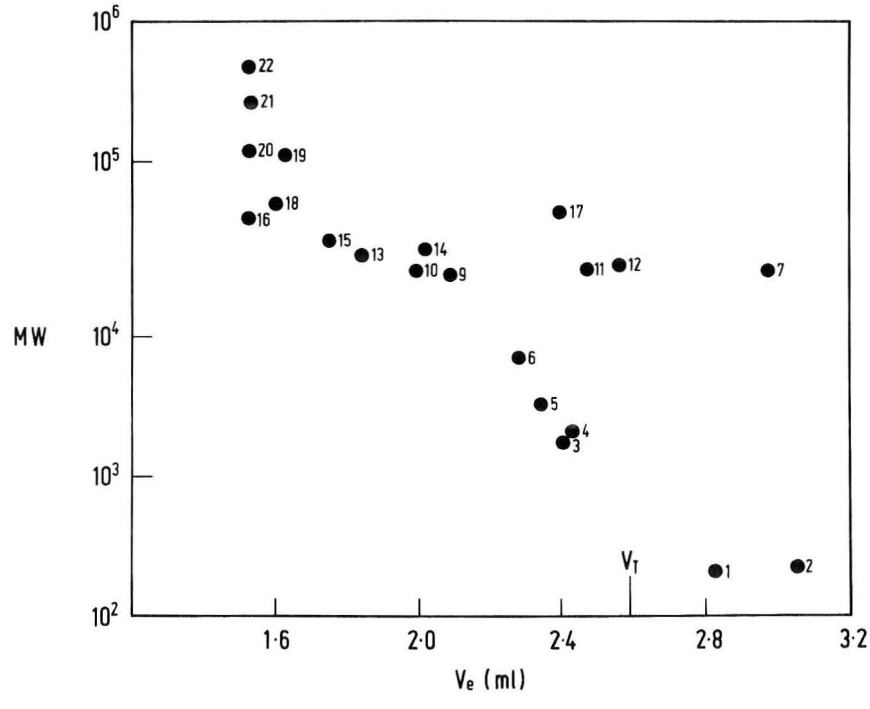

Figure 2.

Plot the log M.W. versus elution volume using a 25mM Tris-HCl/125mM NaCl (pH 7.5) eluent. The legend for the solutes is given in the Table.

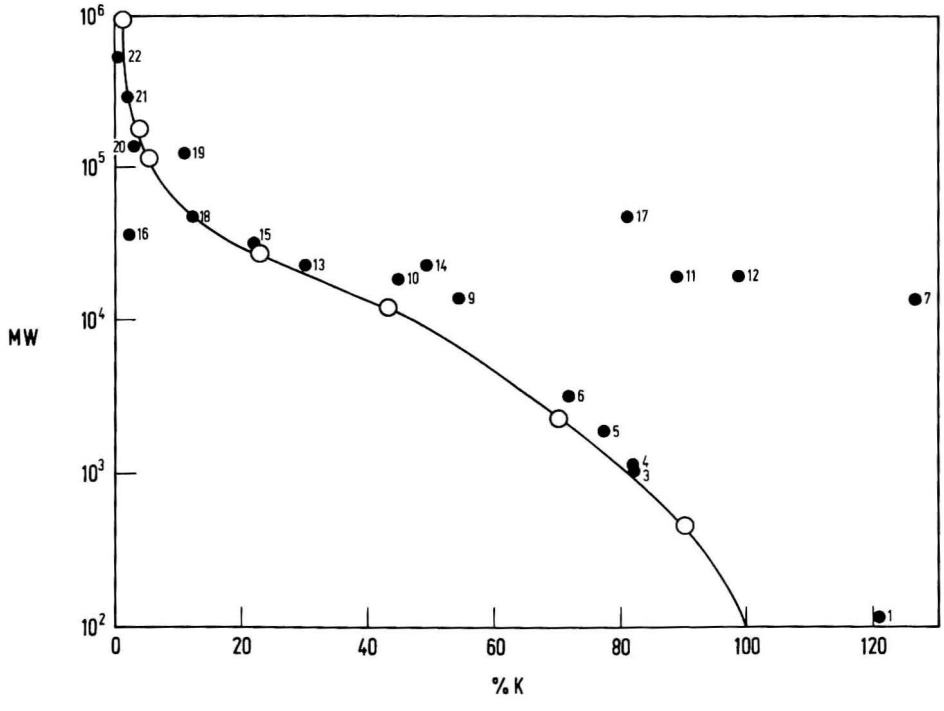

Figure 3.

Plot of the log M.W. versus percentage K of representative polypeptides and proteins. The calibration curve of polystyrene standards eluted with CH_2Cl_2 from the same hydrophilic support is also shown (-O-). Conditions as in Figure 2.

log M.W. versus V_e and log M.W. versus % K for a range of peptides and proteins determined under these conditions. Proteins with a molecular mass greater than 68,000 daltons behave as though they are essentially excluded, i.e. K < 0.1, whilst some low molecular weight peptides and hydrophobic proteins e.g. lysozyme, are retained beyond the theoretical internal volume (as determined with D_2O). Under high ionic strength conditions the divergencies observed in elution behaviour from those

anticipated on the basis solely of a non-ionic gel permeation phenomenon presumably reflect the participation of hydrophobic, rather than electrostatic, interactions between the stationary phase and the solutes. Similar observations have been made by Schmidt et al. [9] in their study on the chromatographic behaviour of proteins on LiChrosorb DIOL and parallel previous findings [36,37] on the relative hydrophobicities of a number of proteins on n-alkyl substituted agaroses and Spheron-300. It is noteworthy that the carbonaceous coverage of the 'amide' phase used in the present study ($4.4\mu mol/m^2$), is approximately twice the value reported [9] for the LiChrosorb DIOL phase, ($2.1\text{-}2.5\mu mol/m^2$). Additional studies have shown [21, 22,38] that improved correlation for the plot of log M.W. versus elution volume can be obtained by using the weight average molecular weight as derived from the Mark-Houwink equation [39] which relates the molecular weight of a protein to its effective Stokes radius in solution. At pH 7.5 and $\mu>0.3M$, the recoveries for the proteins and enzymes used in this study were in the range 85-95% with no significant changes in elution behaviour with sample loadings ranging from 10-500µg on an analytical size column.

Based on the above observations with the selected range of polypeptides and proteins shown in the Table, the use of this surface modified silica as a stationary phase for the gel permeation HPLC fractionation of crude protein mixtures from tissue sources was examined. Many of the polypeptides and proteins currently of interest in, for example, endocrine and neuroendocrine research fall into the molecular weight inclusion range of this stationary phase, i.e. between 2-40 $x10^3$. In addition, most of these substances are available only in minute amounts, e.g. 1µg per gram tissue. Rapid high resolution micromethodologies are obviously needed for their

analysis and purification. When only small amounts of crude polypeptide samples, e.g. less than 10mg, are available, it should be generally feasible to now base their fractionation solely on HPLC methods. A combination of conventional open column chromatographic and HPLC techniques are usually required for reasons of economy when large amounts of crude protein preparations are available. For example, as a routine procedure in use in our laboratory with salt or solvent fractionated tissue extracts, including crude pituitary preparations, we have found that gel permeation and reversed phase HPLC methods under several elution conditions will satisfactorally fractionate ca. 1-5mg of material whilst with amounts above 50mg a sequential combination of open column gel permeation chromatography, a lectin affinity chromatography separation if the substances of interest are glycoproteins, gel permeation HPLC on this bonded 'amide'-, or alternatively the bonded glycerylpropyl- [18], support and a reversed phase HPLC separation on a 100 Å or a 500 Å alkylsilica, most efficient in terms of time, cost, homogeneity and final recovery yield of a specific bioactive polypeptide or protein. In some cases, a normal phase separation on a bonded hydrophilic phase silica using a decreasing gradient of an organic solvent modifier may also prove advantageous. Rubinstein et al. [10] have also employed this approach for the fractionation of human leucocyte interferon on a LiChrosorb DIOL support. Typical of the use of the bonded 'amide' phase as a gel permeation support are the chromatographic profiles shown in Figure 4. These chromatograms represent the high speed exclusion separation of a crude ovine thyrotrophin preparation ex the 1.4M ammonium sulphate (pH 4.0) cut and different fractions containing this glycoprotein hormone sequentially rechromatographed on the same column. After

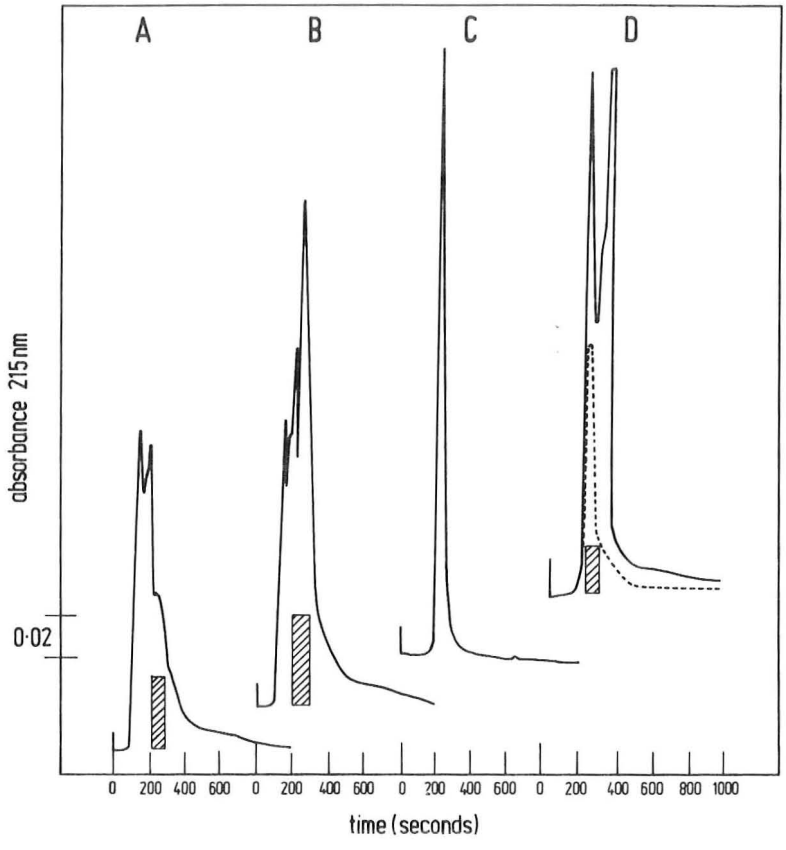

Figure 4.

Chromatography of (A) a crude ovine thyrotrophin preparation on the bonded amide phase, (B) a Sephadex G100 fractionated ovine thyrotrophin preparation, and (C) a sample of this protein after three gel permeation HPLC cycles on the bonded 'amide' phase column, flow rate, 0.5ml/min., eluent 100mM Tris HCl-500mM NaCl, pH 7.4. The regions indicated by hatched zones contained the thyrotrophic activity as assessed in the mouse bioassay. In (D) is shown the elution profiles of a 1 x chromatographed ovine thyrotrophin preparation before and after affinity chromatography on Concanavalin A-Sepharose CL4B, flow rate 0.5ml/min., eluent 25mM Tris, HCl-125mM NaCl, pH 7.4.

three elution-, collection-, desalting- and concentration-cycles, the overall recovery of bioactive ovine thyrotrophin, as assessed by the McKenzie mouse bioassay, was 83% with a total elution time for each chromatographic injection/elution/collection step of ca. 10mins. As we have shown elsewhere [18] this preparation can be used either directly, or following further purification on a 500 Å bonded octylsilica with a triethylammonium formate-acetonitrile eluent, in binding studies with thyroid plasma membrane components. Even though the sample capacity of the analytical size column used in the present study was relatively low, i.e. maximum 250-500µg/protein injection depending on the complexity of the protein mixture, when compared to conventional Sephadex G100 columns of bed volume ca 300ml previously employed for the gel filtration of crude glycoprotein hormones, including crude thyrotrophin preparations, the short elution times, excellent recoveries and improved resolution still allowed milligram quantities of this pituitary protein to be quickly fractionated. Clearly, larger sample loadings, e.g. 10-100mg/injection could be accomodated if desired, with HPLC semi-preparative or preparative columns packed with this bonded 'amide' phase support.

The bonded 'amide' phase support has also been successfully employed as the fractionation of the solubilised sheep thyroid binding protein specific for human thyroid stimulating auto-antibodies present in patients with Graves' disease (Fig.5). After 13 consecutive injections of 300µg protein/injection, no change in the resolution was evident, with a total protein recovery after desalting and lyophilisation of 3.8mg,(97%).

In conclusion, the bonded N-acetylaminopropyl-silica stationary phase of 100 Å nominal pore diameter is a useful

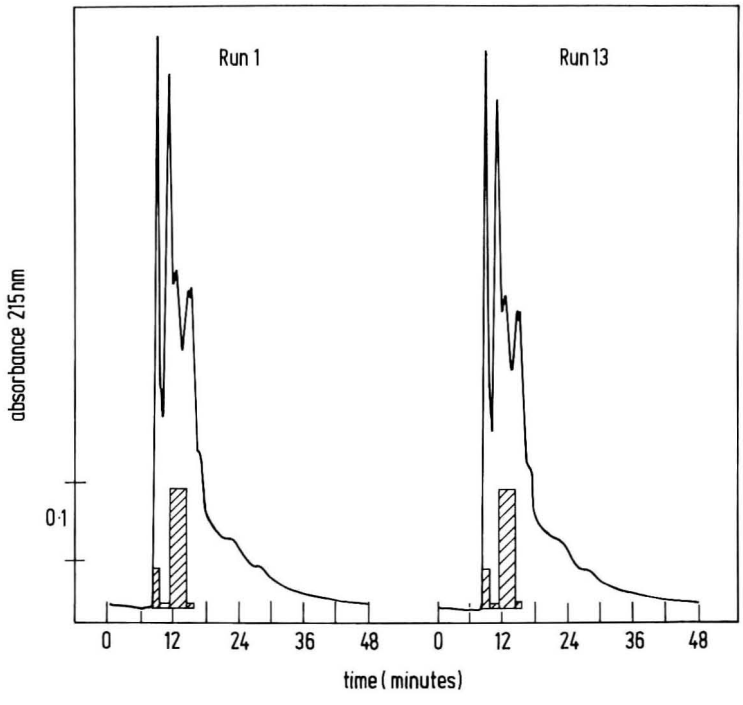

Figure 5.

Fractionation of the crude solubilised sheep thyroid binding protein components specific for human thyroid stimulating autoantibodies on the bonded 'amide'phase support. The profiles correspond to the initial loading (A) and the thirteenth consecutive loading (B) of 300µg of protein/injection. Flow rate 0.2ml/min; eluent, 100mM Tris/HCl-500mM NaCl, pH7.4. The hatched zones correspond to regions of autoantibody binding activity.

support for the aqueous size exclusion HPLC separation of polypeptides and small proteins. Similar supports of larger pore diameter, e.g. 500 Å, would be expected to extend the molecular weight range for exclusion chromatography to larger macroglobulins. Preliminary studies [38] on the chromatography of several common proteins on these large pore bonded 'amide'

phase silicas have confirmed this expectation. It is anticipated that these and related, bonded hydrophilic phase silicas will gain increasing popularity for the rapid fractionation of biologically active polypeptides and proteins by size exclusion HPLC.

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FURTHER EXPERIMENTS IN THE SEPARATION OF GLOBIN CHAINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The applicability of various column packings, solvents, and gradients for the separation of globin chains by high performance liquid chromatography has been examined. Although numerous combinations may be applied to advantage in special situations, a particularly effective system uses a Waters μBondepak C_{18} packing with a slight gradient of perchlorate-phosphate-methanol-acetonitrile mixtures. A chromatogram with human β, α, $A_\gamma T$, G_γ, and $A_\gamma I$ chains is finished in 80 min, and quantitation of the various chains is possible.

High performance liquid chromatography (HPLC) may be applied to various tasks in the study of hemoglobin to provide analytical data, separations, and preparations with much facility. Tryptic peptides of hemoglobin may be separated easily by HPLC on several types of column packing and with a variety of solvent systems (reviewed in (1)). These parameters in various combi-

*Contribution No. 6353

nations provide many dimensions to accomplish difficult separations, and the methods should be equally applicable to other types of cleavage products. The application of HPLC to the study of hemoglobin is not limited to the separation of peptides. Thus, Congote et al. (2) and Shelton et al. (3) describe the separation of the α, β, $^{G}\gamma$, and $^{A}\gamma^{I}$ globin chains by HPLC with the same type of column packing but with different solvent systems. Huisman and collaborators have modified the system of Shelton et al. (3) so that the $^{A}\gamma^{T}$ chain may be separated from the $^{G}\gamma$ and $^{A}\gamma^{I}$ chains and have applied their procedure to various problems (4-7). Here we report an investigation of the separation of globin chains which has considered the effects of varied combinations of column packing and solvents. Particular emphasis was placed on devising methods for the separation of the three γ chains.

MATERIALS AND METHODS

The HPLC equipment consisted of two Altex Model 110A Solvent Metering Pumps, an Altex Mixer 400-02, an Altex 420 Microprocessor, a Waters U6K Universal Injector, an Altex-Hitachi Model 155-10 UV-Vis Variable Wavelength Detector, and a Linear Instruments Corpn. (Irvine, CA) single channel recorder.

The various column packings were: Altex Ultrasphere ODS and CN, DuPont Zorbax C_8, CN, and TMS, and Water μBondepak C_{18}. Dimensions were 4.6 x 250 mm for all except the Waters column (3.9 x 300 mm).

The solvents were various mixtures of a phosphate buffer [49 mM KH_2PO_4 (6.66 g per liter) and 5.4 mM H_3PO_4 (0.37 ml 85% H_3PO_4 per liter)], a perchlorate solution [0.15 M $NaClO_4$

(18.3 g per liter)], 85% H_3PO_4, methanol (MCB OmniSolv), and acetonitrile (Baker's HPLC). Water was doubly deionized and distilled in glass. Solvents were not filtered or degassed. Neither pre-columns nor guard columns were used.

The most effective separations were made with a gradient between Solvent A which was 80:5:15:0.1 (*v/v/v/v*) of perchlorate solution - methanol-acetonitrile-H_3PO_4 and Solution B which was a 20:5:75:0.1 mixture. These solvents with the exception of the added methanol are patterned after Meek (8). The gradient was begun with 66% B and was increased to only 70% B in 80 min at a flow rate of 1.5 ml per min at room temperature. At the completion of the gradient, the column was purged with 100% B and then reequilibrated with 66% B. These were the conditions for all figures below.

Hemoglobin solutions were prepared from saline-washed cells by hemolysis for 30 min at room temperature with water and carbon tetrachloride equal to 1 and 0.4 times, respectively, the packed cell volume. After double centrifugation at 4° and 30,000 g for 30 min, the solution was diluted with at least 10 times its volume of water and filtered through an 0.5 μm cellulose type filter (Rainin Instrument Co., Woburn, MA). A volume (20 to 100 μl) which contained 0.2-0.3 mg of hemoglobin was injected on to the chromatographic column.

Percentages were calculated by planimetry.

Identification of peaks was made by comparing the appearance, disappearance, and proportions of peaks in mixtures of known hemoglobin composition.

RESULTS AND DISCUSSION

Figures 1 and 2 depict separations of chains in the hemoglobins of representative adult and cord blood samples. In this

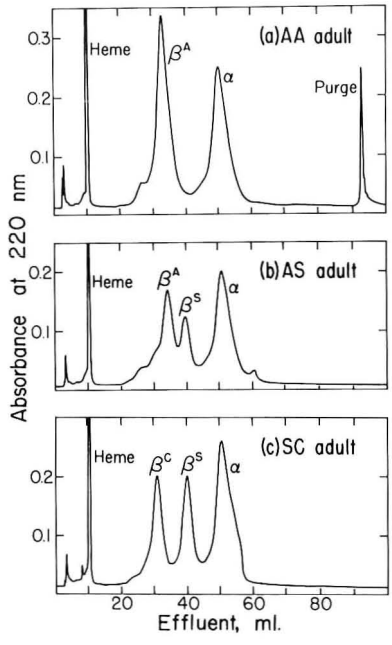

FIGURE 1

Separation of chains of hemoglobins from adults with AA, AS, and AC hemoglobins on a Waters μBondepak C_{18} column. The gradient is described in the text.

perchlorate-phosphate-methanol-acetonitrile system (PPMA), the β chain precedes the α chain as it does in the trifluoroacetic acid-acetonitrile system of Congote et al. (2). Conversely, in the phosphate-methanol-acetonitrile (PMA) system which we initially described, the α chain emerges ahead of the β chain (3). Huisman et al. (4) simplified these separations by using hemoglobin instead of globin as the sample. With PMA developers, conditions may be altered so that the heme emerges either before or after the chains (4, 9); the retention of heme in this system is apparently relatively unaltered by conditions, but the retention of

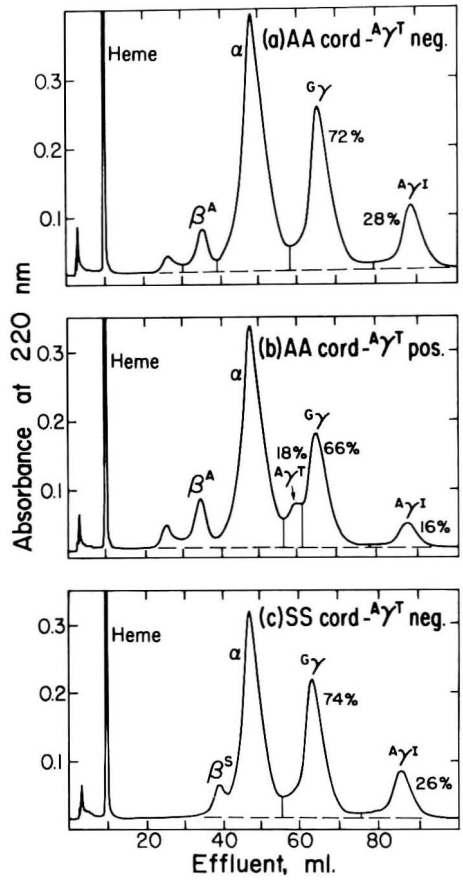

FIGURE 2

Separation of chains of hemoglobins in the cord bloods of infants with AA hemoglobin with and without $^{A}\gamma^{T}$ chains and of an SS infant. Conditions as in Fig. 1.

the chains is changed. The present PPMA system has the advantage of a rapid and effective elution of heme. Although Meek's solvents have 0.1M perchlorate, there seems to be a slight advantage in the use of 0.15M perchlorate for the chain separations.

The α and β^{A} chains separate well. A separation of β^{A}, β^{S}, and β^{C} chains can be achieved, although for no pair is the

separation complete. Although numerous variations in elution pattern and solvents have been tried, the depicted separation of β^A, β^S, and β^C chains is the best that has been achieved. Perhaps, a reduction in the amount of sample might improve the separation.

If γ chains are absent as in the samples in Fig. 1, the chromatograms are complete in about 45 min (70 ml). In Fig. 1(a), the gradient was terminated early and the developer shifted to Solvent B to purge the column; the final peak is material that was removed by the purge. In the other panels of Figs. 1 and 2, the purge is not shown, but was done at 120 ml when the gradient was complete.

The $^G\gamma$ and the $^A\gamma^I$ chains separate well (Fig. 2(a) and (c)). The $^A\gamma^T$ chain is qualitatively detectable, but variation in conditions has not produced a separation that is more satisfactory for quantitative determination. Despite the inadequate separation of the $^A\gamma^T$ chain, the calculated quantity on the basis of the dashed baseline is exactly the average of 39 $^A\gamma^T$ positive samples of cord hemoglobin (10). The present PPMA method has the advantage over the extended PMA modification of Huisman et al. (5) in that the $^A\gamma^T$ chain can be detected in an 80-min instead of a 160-min chromatogram. If the $^A\gamma^T$ chain is detected by the PPMA method, its quantitation could then be substantiated in a duplicate by the modified PMA method, if necessary (5). If the $^A\gamma^T$ chain is absent, the $^G\gamma$ to $^A\gamma$ ratio may be calculated readily. The percentages of $^G\gamma$ and $^A\gamma$ chains as given in Figs. 2(a) and (c) are in excellent agreement with the frequently quoted 3:1 ratio in the newborn. The identity of the small peak that precedes the β^A peak in Figs. 2(a) and (b) is unknown. It may be identical with the incompletely separated material at the leading edge of the β peak in Figs. 1(a) and (b).

In calculating the ratio of γ chains, it is reasonably assumed that their molecular extinction coefficients are identical. However, the quantitation of various hemoglobins in a mixture should be possible by this method, if the relative molecular extinction coefficients of the non-α chains are used. The relative coefficients which are known for 280 nm and commonly applied in chain separations by the Clegg-Naughton-Weatherall procedure, cannot be used here because absorbance at 220 nm is determined. Nevertheless the relative coefficients should be calculable from present data. Because α and non-α chains are present in molar equivalents, an equation such as $\alpha = A\beta + B\gamma$ should apply where α, β, and γ represent the integrated absorbance of a peak and A and B are constants. Likewise, it follows that the percentage of any one hemoglobin (for example, Hb-A) would be $\text{Hb-A} = \frac{A\beta \times 100}{\alpha}$. In Fig. 1(a), the $B\gamma$ term is zero, and the ratio of the areas of the α and β peaks gives a value of 0.87 for A. When this value of A is used with the data from Fig. 2(a), B equals 1.06. The exact value and accuracy of these constants needs to be established by a series of replicate determinations with chromatograms like that in Fig. 1(a) and with others like that in Fig. 2(a) but with a varied ratio of β and γ chains.

When a sample of isolated Hb-A_2 was chromatographed by the present procedure, the δ chain emerged at the position of the β^S chain. The quantity of Hb-A_2 in the normal adult is too small for the δ chain to produce a definite peak between the β and α peaks (Fig. 1(a)), but the presence of the δ chain may be responsible for the fact that the valley between the β and α peaks does not return to baseline.

Although the present procedure should prove useful in some aspects of the study of hemoglobin, there are certain undesirable

phases: it is relatively time-consuming (although less so than the modified PMA method and monumentally less than the Clegg-Naughton-Weatherall separation), the peaks are relatively broad and generally somewhat asymmetrical (hence, valleys may not return to baseline), and the separation of the $^{A}\gamma^{T}$ chain is minimal. Yet, at present, it is the best of the many modifications that were tried and that would permit, at least, the qualitative detection of the three types of γ chain. On the other hand, other conditions might be useful in specific instances. For example, a better separation of α and β^{S} chains might be achieved if the separation of γ chains were inconsequential. Only brief mention can be made of the many variables that were tried and their corresponding effects.

The gradient is so slight that development is almost isocratic, although no isocratic procedure was satisfactory: the percentage of perchlorate solution in the 80-min period of development decreases from 40.4 to 38 while that of acetonitrile increases from 54.6 to 57, and methanol and H_3PO_4 are constant.

Of the six column packings that were tested, the Waters μBondepak C_{18} is the most satisfactory and is followed by Altex Ultrasphere ODS, and DuPont Zorbax CN and C_8 in that order. The Altex Ultrasphere CN and DuPont Zorbax TMS packings could not be made to provide worthwhile separations with PPMA under varied conditions; PMA developers were not investigated.

Although PMA developers clearly produce desired separations as witnessed by the modifications of Huisman et al. (5), unfortunately, in this method, one is forced into 40 min of isocratic development to remove the heme before the gradient is introduced. If conditions are altered (as they can be) to separate the chains before the emergence of heme, the peaks are relatively

sharp, but the chromatogram must be completed in about 45 min and there is little room for maneuvering to improve separations or to provide space for the detection of the $^{A}\gamma^{T}$ chain. The PPMA system with its rapid removal of heme permits the gradient to be altered and extended as necessary.

As already mentioned, the β chain precedes the α chain in the PPMA system as well as in the trifluoroacetic acid-acetonitrile system of Congote et al. (2) whereas the reverse occurs with the PMA developers. However, there is an exception in the PPMA system with one of the acceptable packings. When the PPMA developers are used with a DuPont Zorbax CN column, the α chain emerges first. This system may have advantages although it was not studied extensively. Thus, if the gradient was from 25 to 50% B in A over 40 min at 1.5 ml per min, the peaks were sharper than in Figs. 1 and 2, and separation of β^{A} and β^{S} chains was about as good. When Solution A had the composition 60:5:35:0.1 PPMA, B was 40:15:45:0.1, and the perchlorate concentration was 0.1 M, a gradient from 42 to 55% B in A over 60 min did separate α, β, $^{G}\gamma$, and $^{A}\gamma$ chains in that order. However, the valleys did not return to baseline. Therefore, further study was not made because an effective separation of the $^{A}\gamma^{T}$ chain seemed unlikely.

Methanol was initially included in the developing system in order to increase the retention of the chains. In some experiments, n-propanol or isopropanol was substituted for methanol, the quantity was varied from 0-10%, and/or gradients were employed. None of these variations was more advantageous than the use of methanol itself.

The hemoglobin chains of other species are amenable to separation by this method. The separation of the chains in the

hemoglobin of a 33-day-old baboon (Papio cynocephalus) is seen in Fig. 3. The γ chains of the baboon do not differ at position 136 as do the human γ chains nor at position 135 as do those of some other primates (11). However, they do have either valine ($^{V}\gamma$) or isoleucine ($^{I}\gamma$) at position 75 (12, 13) as does the orangutan (14). Although the baboon $^{V}\gamma$ and $^{I}\gamma$ chains do not separate as completely as the human $^{G}\gamma$ and $^{A}\gamma$ chains, quantitation is possible. The $^{V}\gamma$ chain approximates 40% at birth, but the percentage increases postnatally (15). The identities of the small peak that precedes the β chain and of the two that follow the α chain are unknown.

Numerous systems, therefore, are available for the HPLC separation of hemoglobin chains. The conditions that are described here were designed to achieve the maximum separation of the common human hemoglobin chains with relative speed. On the other hand, with less complex mixtures or for special

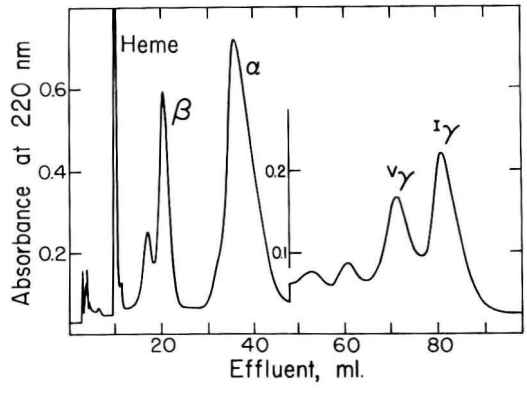

FIGURE 3

Separation of the chains of hemoglobins from a 33-day-old baboon. Conditions as in Fig. 1. At approximately 48 ml, the absorbance setting was changed.

separations, modification of column packings, solvents, or gradients should permit almost any desired separation.

ACKNOWLEDGEMENTS

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A SIMPLE AND ECONOMICAL APPARATUS FOR DEVELOPING THIN-LAYER CHROMATOGRAPHY PLATES IN THE ANTICIRCULAR MODE

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ABSTRACT

An improved method for developing thin layer chromatography plates in the anticircular mode is described. The apparatus consists of two glass dishes arranged concentrically. The outer dish has a diameter of 98 mm x 15 mm deep, and the inner one a diameter of 92 mm x 13 mm deep. The inner dish rests on four small pieces of glass (5 x 5 x 2 mm). A paper wick, (16 mm wide x 1.5 mm thick) sits between the two dishes and transfers the solvent from the outer dish to the thin layer plates. The plate sits on the paper wick with the adsorbent facing down. To ensure an even solvent flow, the dishes are situated on a platform which has adjustable legs, and an horizontal level. Up to 50 samples can be analyzed on one 10 x 10 cm plate in 5 minutes. Less than 10 ml of solvent are needed.

INTRODUCTION

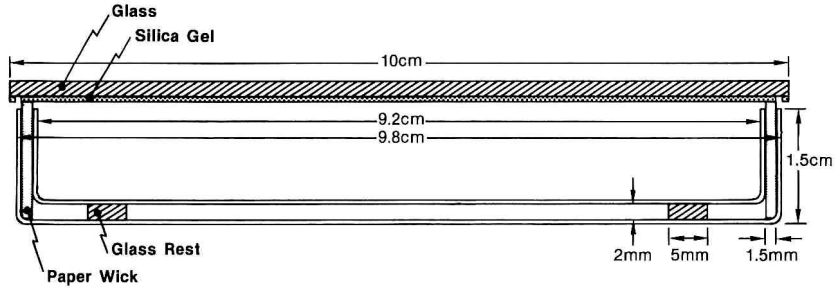
Anticircular thin-layer chromatography (TLC) was introduced in 1970 by Van Dyk (1). In this method, the sample is applied at the circumference of a circular plate and elution proceeds towards the center. Later, a simple apparatus was developed consisting of a turntable on which the plate was placed while the solvent was introduced from a stationary syringe to a felt ribbon surrounding the plate (2). The system needed to be protected from drafts to prevent the development of irregular circles. In 1978 Kaiser (3) introduced a high performance anticircular TLC system, consisting of a modified U-chamber. The solvent

was fed on to the plate by capillary action from a narrow channel. Kariko and Tomasz (4) used a system in which the solvent was placed in a petri dish. The solvent flowed on to the round plate through a cylindrical filter paper strip with feathered edges. This system, although quite simple, gave reasonably good results, but they were not as reproducible as those obtained with the modified U-chamber. An apparatus was developed which is simple, reliable, and easy-to-construct and operate. This apparatus combines the speed and reproducibility of the modified U-chamber with the economy of the petri dish approach.

EXPERIMENTAL

TLC silica gel plates (10 x 10 cm) were purchased from Whatman, Inc. Solvents were "glass distilled" (Burdick & Jackson). A blunt head 10 µl Hamilton syringe with a special dispensing device was used for spotting 0.5 µl of solution. Hexane:ethyl acetate (2:1) was used as the mobile phase.

Our apparatus (Fig 1) consists of two glass dishes arranged concentrically. The outer dish has a diameter of 98 mm x 15 mm deep, and the inner one a diameter of 92 mm x 13 mm deep. The inner dish rests on four small pieces of glass (5 x 5 x 2 mm). A paper wick, (16 mm wide x 1.5 mm thick), sits

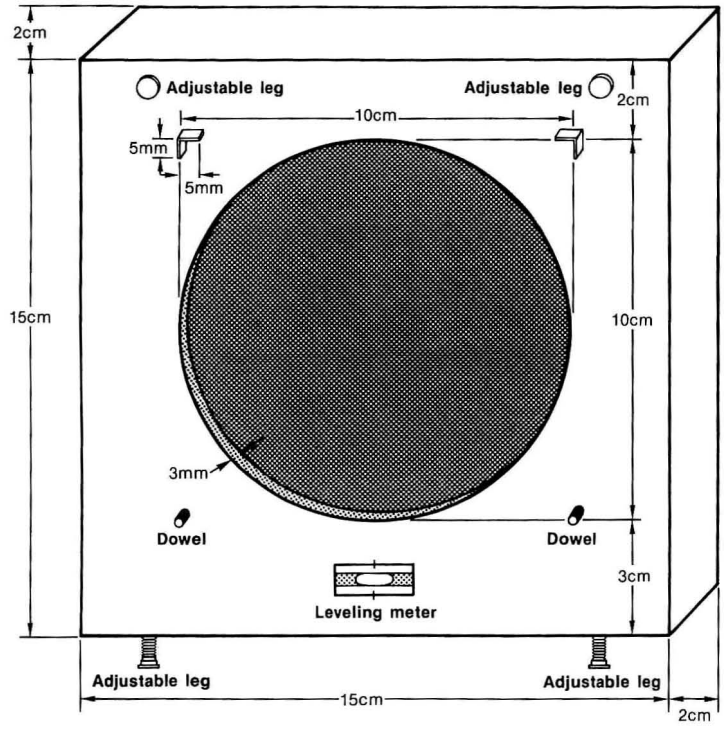


Anticircular Developing Apparatus
Chamber Assembly

Figure 1. Schematic of anticircular apparatus.

between the two dishes and transfers the solvent system from the outer dish to the thin layer plates. The plate sits on the paper wick with the adsorbent facing down. A circle 10 cm in diameter is scored on the 10 x 10 cm plate with a sharp spatula. The sample is spotted on the plate at a distance 5 mm inside the scored circle. The eluting solvent (10 ml) is placed in the outer dish; the inner dish contains either 2 ml of the eluting solvent for equilibration or saturation, or any conditioning reagent required. When the plate is positioned absorbent down on the paper wick, development is initiated.

To ensure an even flow of the solvent the dishes are placed on a special platform which has adjustable legs and a horizontal leveling meter (Fig 2).



Platform for Anticircular Developing Apparatus

Figure 2. Schematic of platform used with the anticircular apparatus.

The platform is equipped with two corner pieces 10 cm apart on one side of the platform (Fig 2). Opposite the corner pieces, at a distance of 10 cm, two dowels are situated. The platform has a circular groove 10 cm in diameter x 3 mm deep into which the outside dish fits. The corner pieces, the dowels, and the groove, ensure that the plate always occupies the same position on the platform. A glass or plastic lidless box 11 x 11 x 2 cm covers the dishes to eliminate drafts. When development is complete, the plate is removed and a dish with the same dimensions as the outside dish is used to cover the system to retain the saturated atmosphere. When 20 x 20 cm plates are developed, larger sized dishes are used.

RESULTS AND DISCUSSION

Thin layer chromatography is a simple and economical analytical technique which gives reasonably reproducible results if plate-to-plate variations are eliminated, and solvent and vapor effects are controlled. Anticircular TLC is the most efficient mode for the simultaneous development of large numbers of samples. It has been shown (3) that the anticircular mode is superior to both circular and linear modes in terms of sensitivity, speed of analysis, and amount of solvent required.

The anticircular U-chamber (3) gives reproducible results at high cost. The unit described by Kariko and Tomasz (4), on the other hand, while cheap lacks certain features which would make it more versatile. The feathered strips do not always give uniform transfer of the solvent because the filter paper is not held firmly in place, and there are gaps between the feathered strips. With neither of these systems is it possible to condition the plates during development with a second solvent system or other reagents, such as sulfuric acid (to control humidity), or ammonia (to control streaking).

The apparatus we have developed (Fig 3) overcomes these disadvantages. The solvent flows from the dish to the plate via capillary action from the paper wick which is held firmly in place by the inner dish. The paper wick is

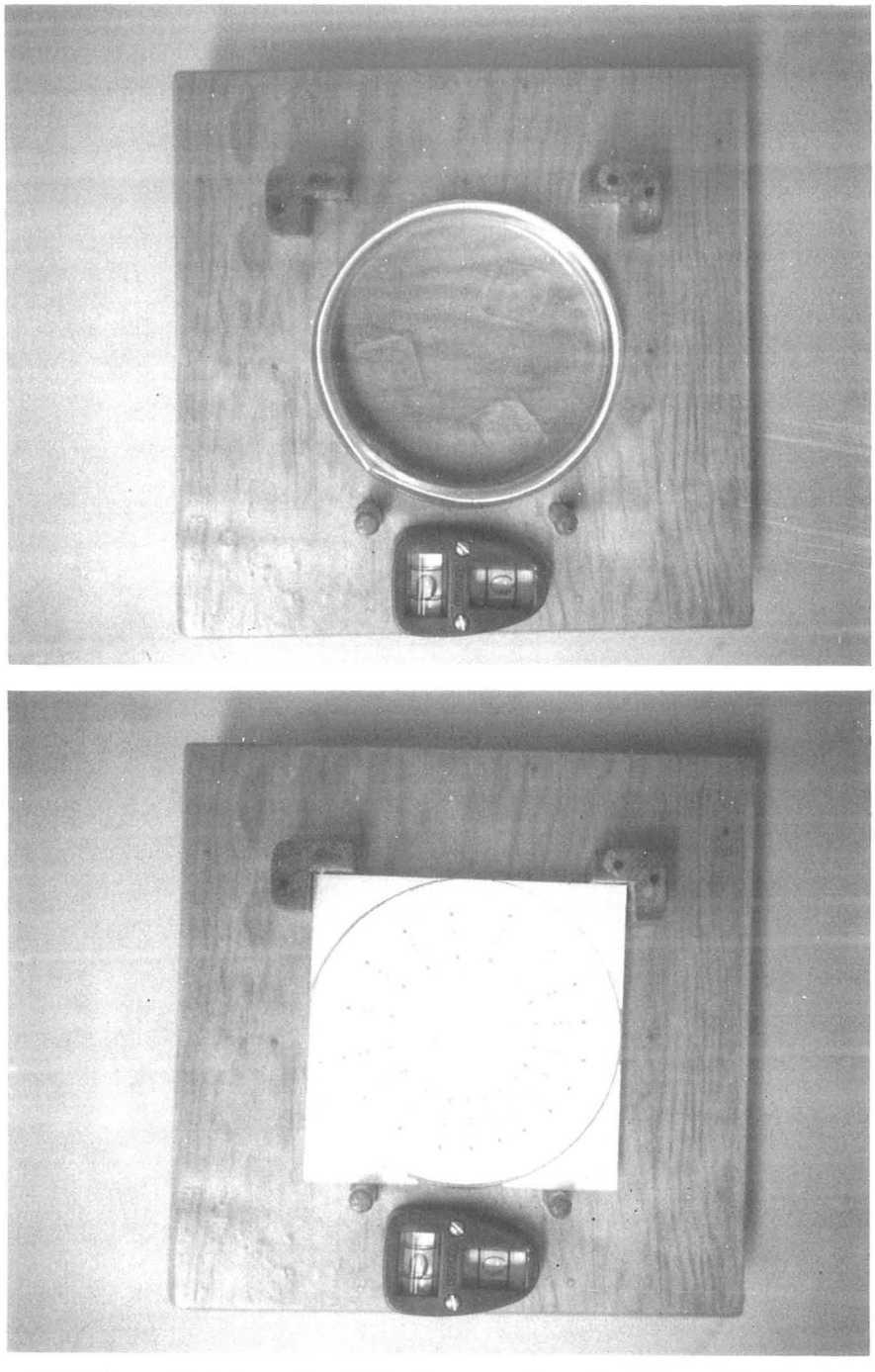


Figure 3. Prototype of anticircular apparatus and platform (a) without plate and (b) with plate in place.

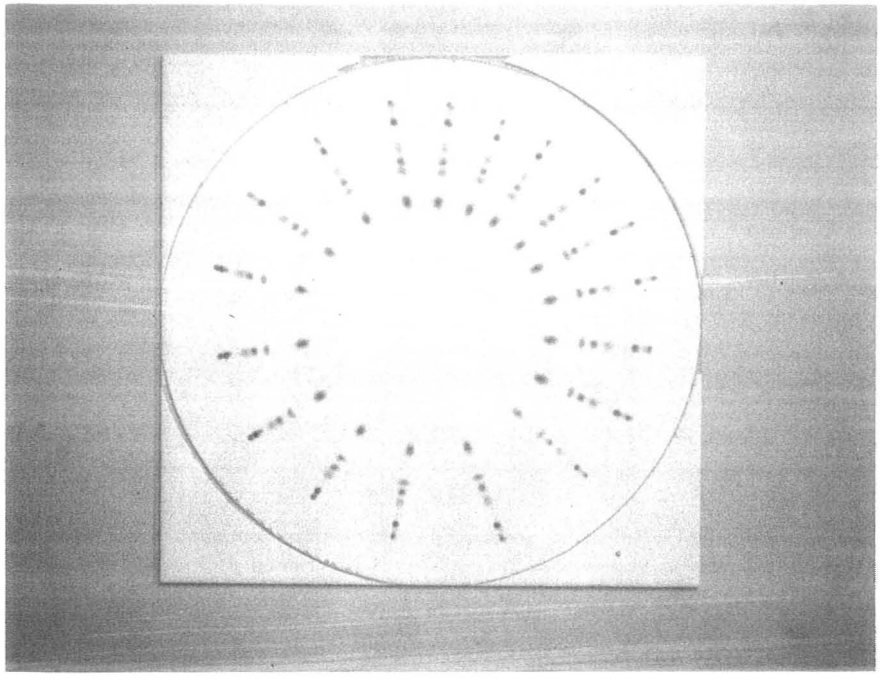


Figure 4. Separation of test dye mixture using the anticircular apparatus.

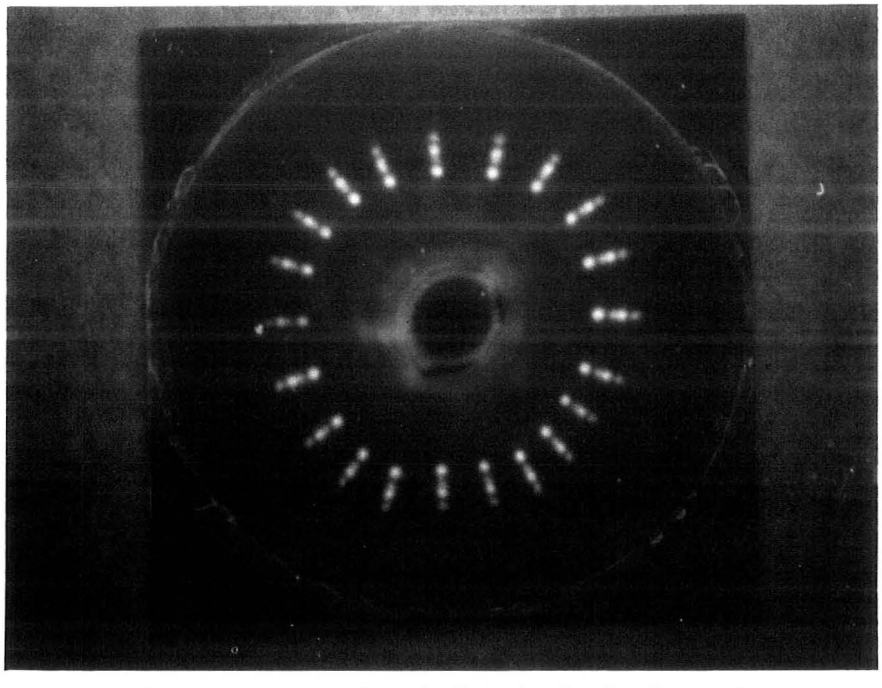


Figure 5. Separation of aflatoxins B_1, B_2, G_1, and G_2.

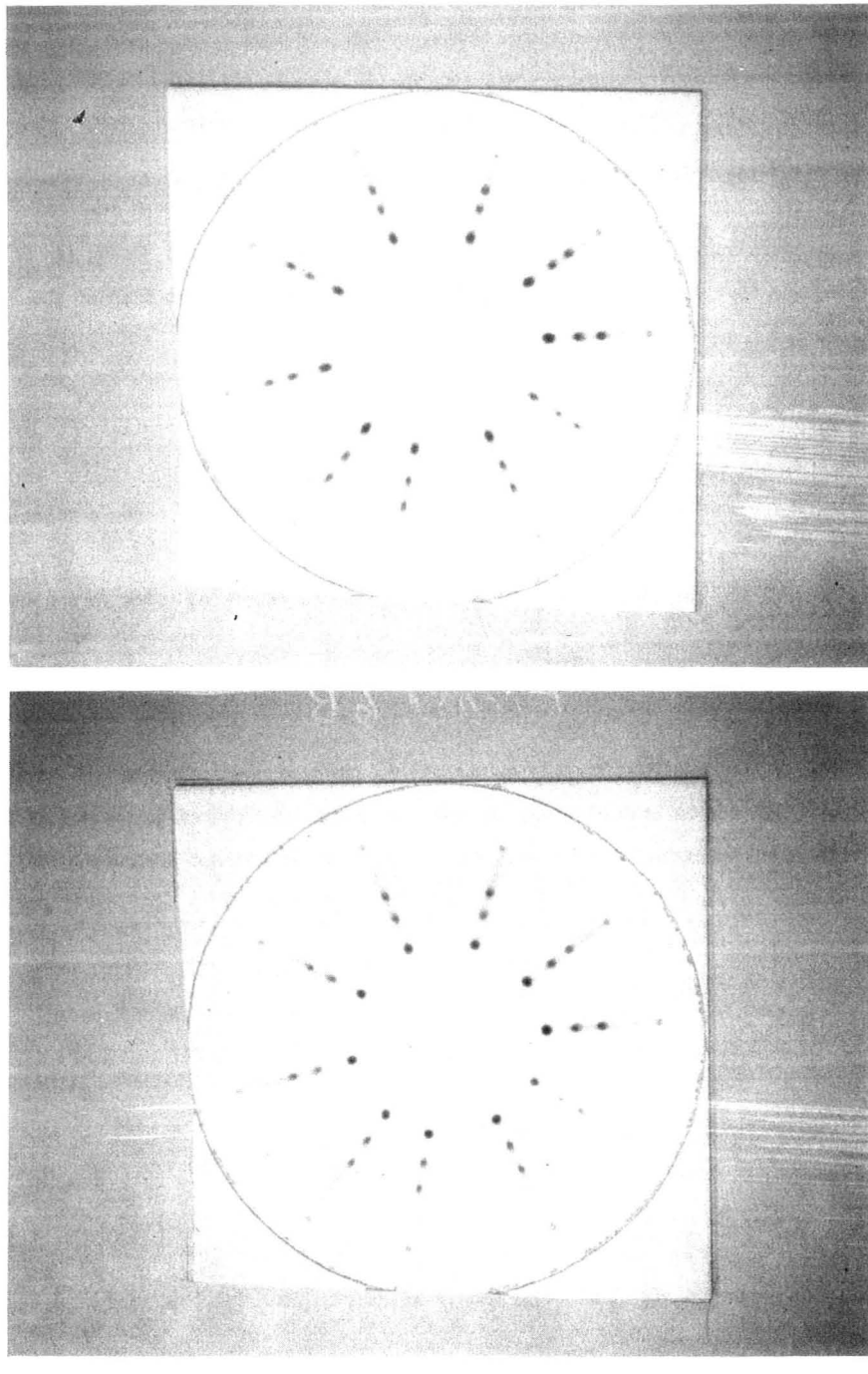


Figure 6. Test dye mixture developed (a) once and (b) twice in the same solvent system.

strong enough to provide good support for the plate while remaining in contact with the solvent. Any conditioning solution is placed in the inner dish, and the eluting solvent in the outer. If a conditioning solution is not needed, it is replaced by 2 ml of the developing solvent to aid in saturation of the atmosphere, reduce solvent evaporation off the plate, and assist in the production of compact and reproducible spots. The platform ensures even distribution of the solvent and reagent in the dishes, which results in regular and even development. The results of the development of a dye mixture on a 10 x 10 cm plate are shown in Figure 4. The apparatus was also employed in the separation of aflatoxins B_1, B_2, G_1 and G_2 (Fig 5).

Anticircular development tends to produce elongated spots at high R_f values. This is especially true when large sample volumes are spotted, and resolution can be affected. To overcome these problems, the plate is developed twice in the same same solvent system (Fig 6).

CONCLUSION

The system described is simple, versatile, and gives reproducible results at low cost. It can be assembled easily, and its operation requires no special skill. The two compartment apparatus allows the use of reagents without distrubing the the eluting solvent.

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ENZYMATIC ESTIMATION AND QUANTITATIVE HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF FRUCTOSE, GLUCOSE AND SUCROSE IN POWDERS FROM ROSE PETALS

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ABSTRACT

There was no significant difference between mean mass fractions of fructose, glucose and sucrose measured enzymatically and by liquid chromatography. So the chromatographic method can be used on powders from rose petals as the peaks of the chromatograms are really caused by the sugars.

A method is described for clean-up of the rose extract before injecting it into the high-pressure liquid chromatograph.

INTRODUCTION

The purpose of this work was to find a way of estimating soluble sugars in cut flowers by high-pressure liquid chromatography.

As yet, we have used an enzymatic method (10). However, the enzymatic analysis had 3 disadvantages:

1. Methanol must be removed from the extract; otherwise commercial enzyme preparations are inactivated.
2. For estimation of sucrose, when the mass ratio of glucose to sucrose is higher than 3:1, glucose must be removed with glucose oxidase and catalase (11).

3 Chromatography offers more options for automation than the enzymatic method.

If the two methods gave similar results for roses, we could assume that peaks for the sugars were not due to artefacts.

EXPERIMENTAL

The roses were cut flowers of the cultivar Sonia.

The powder was prepared from petals of roses by a similar method to onion powder (8). Each sample was bulked from petals of six flowers. In total, there were 20 samples. The samples represented 6 stages of development: 1, 2, 3, 4, 5 and 6 (6) picked on 7 July 1980 (Samples 1-6 of Table 2). Stages 1 and 3 were picked on 13 July 1980 (Samples 7 and 14 of Table 2). The chemical analyses formed part of a study on longevity of the blooms on the plant and after cutting:

Stage 1: 3 days on the plant (Samples 11, 12 and 13); 3 days in a vase (Samples 8, 9 and 10) .

Stage 3: 3 days on the plant (Samples 18, 9, 10); 3 days in a vase (Samples 15, 16 and 17).

Fructose, glucose and sucrose in powders were estimated as described (10) with 80% buffered methanol (0.2 mol/l acetate buffer pH 6.5) and using 0.1 g powder plus 10 ml of buffered methanol for extraction of sugars. For chromatography, the ratio of powder to the methanol was 10 times as great, but extraction of sugars was not affected.

The NADP+ solution was prepared freshly every 3 days (3) and not every month(4).

For chromatography, the buffered methanol was prepared from 20 mL of triethanolamine hydrochloride buffer (pH 7.0) of concentration 0.1 mol/L (4) and 80 mL of absolute methanol. The final concentration of the buffer in the 80% methanol was 0.02 mol/L. The pH, measured with an indicator strip, was

TABLE 1. Enzymatic analysis of soluble sugars in rose powder with or without clean-up of the extract. Values are mass fraction of sugar (mg per 100 mg).

SAMPLE NO.	GLUCOSE				FRUCTOSE			
	Without Clean-up		With Clean-up		Without Clean-up		With Clean-up	
	$\bar{x}_1$	s_1	$\bar{x}_2$	s_2	$\bar{x}_1$	s_1	$\bar{x}_2$	s_2
7	1.17	0.04	1.27	0.03	1.05	0.03	1.16	0.01
8	0.89	0.03	0.82	0.02	0.93	0.02	0.94	0.02
9	2.07	0.01	1.95	0.03	2.21	0.04	2.17	0.06
10	2.85	0.05	2.96	0.05	3.01	0.07	3.10	0.08
11	1.29	0.03	1.29	0.02	1.41	0.02	1.46	0.02
12	2.33	0.07	2.42	0.07	2.67	0.08	2.50	0.05
13	6.63	0.13	6.40	0.12	5.74	0.15	5.99	0.10

$\bar{x}_1$ and $\bar{x}_2$ are averages of triplicate analysis. $s_1 = s_2 = (\Sigma\, d^2/n-1)^{\frac{1}{2}}$

TABLE 2. Mass fraction of glucose, fructose and sucrose in rose powder (mg per 100mg) as estimated enzymatically and by HPLC.

SAMPLE NO.	GLUCOSE				FRUCTOSE				SUCROSE			
	Enzymatic		Chromatographic		Enzymatic		Chromatographic		Enzymatic		Chromatographic	
	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
1	0.82	0.02	0.81	0.01	0.71	0.02	0.81	0.01	0.57	0.02	0.51	0.01
2	1.31	0.02	1.22	0.01	1.58	0.02	1.58	0.01	0.64	0.03	0.65	0.01
3	0.91	0.01	0.91	0.01	1.17	0.03	1.23	0.01	0.43	0.02	0.45	0.01
4	1.58	0.03	1.55	0.01	1.20	0.04	1.31	0.01	0.71	0.02	0.80	0.01
5	5.27	0.10	5.18	0.05	7.61	0.08	7.39	0.06	2.91	0.08	2.75	0.03
6	8.38	0.10	8.17	0.06	10.88	0.10	10.64	0.07	0.79	0.03	0.82	0.02
7	1.17	0.04	1.22	0.03	1.05	0.03	1.05	0.02	0.81	0.02	0.67	0.04
8	0.89	0.03	0.92	0.02	0.93	0.02	0.90	0.02	0.20	0.01	ND[2]	
9	2.07	0.01	2.15	0.05	2.21	0.04	2.17	0.03	ND[1]		ND[2]	
10	2.85	0.05	2.80	0.02	3.01	0.07	3.01	0.03	ND[1]		ND[2]	
11	1.29	0.03	1.24	0.02	1.41	0.02	1.48	0.02	0.41	0.01	0.43	0.04
12	2.33	0.07	2.47	0.04	2.67	0.08	2.73	0.02	0.57	0.02	0.52	0.04
13	6.63	0.13	6.91	0.05	5.74	0.15	5.68	0.06	0.42	0.01	ND[2]	
14	1.93	0.03	2.00	0.01	2.28	0.05	2.33	0.01	0.40	0.02	0.31	0.02
15	4.16	0.02	4.07	0.04	5.30	0.26	5.20	0.02	0.23	0.01	ND[2]	
16	5.02	0.08	5.07	0.03	5.30	0.06	5.48	0.04	ND[1]		ND[2]	
17	6.23	0.09	6.10	0.04	7.01	0.15	7.22	0.04	ND[1]		ND[2]	
18	3.64	0.15	3.72	0.04	4.68	0.17	4.65	0.03	0.48	0.02	0.49	0.02
19	7.97	0.25	8.14	0.18	10.73	0.26	10.46	0.15	0.33	0.01	ND[2]	
20	13.64	0.30	13.48	0.06	17.62	0.40	17.68	0.17	0.43	0.01	ND[2]	

ND[1] Not detected. Value less than 5/μg sucrose in the cuvette (enzymatic analysis).

ND[2] Not detected. Value below 18-25 μg sucrose in 20 μl soln, when injected into the liquid chromatograph.

$\bar{x}$ = average of triplicate analysis, $s = (\Sigma d^2/n-1)^{\frac{1}{2}}$

between 6.5 and 7.0. This type of buffer did not interfere with peaks for sugars, giving a peak in the chromatogram before those of the sugars.

Buffered methanol with acetate (9) was not suitable because we had to increase the concentration from 0.05 to 0.2 mol/L in order to maintain the pH of the extract between 6.0 and 6.5. When the concentration was increased, a peak of acetate appeared between glucose and sucrose.

A buffer of 80% methanol with β , β'-dimethylglutaric acid-NaOH (pH 6.8) (5) of final concentration 0.05 mol/L held the pH of the extract between 6.0 and 6.5, but produced a concavity in the base line of the chromatogram that interfered with the peak of sucrose.

Rose extract was prepared from 1 g powder and 10 ml buffered methanol (previously kept at 20°C) poured into a Pyrex centrifuge tube and placed in a water bath at 55°C.

After 15 min., the suspension was spun for 40 min. at 1600 g at 6°C. The supernatant was the rose extract; its pH was kept between 6.0 and 6.5 (to avoid hydrolysis of sucrose), as checked with a strip indicator.

To clean up the rose extract (whose weight was estimated and of volume about 9mL), it was suspended together with 100 mg of Polyclar AT (BDH, art. 44201) and vibrated (Vibro-Mixer, Vortex Genie) for 1 min.; active charcoal, Darco G 60 (Fluka, A.G. Buchs SG, art. 05100), was added to a mass ratio to the initial rose extract of 0.01.

The suspension was again vibrated for 1 min. and spun at 1600 g for 30 min. at 6°C. The supernatant was collected and weighed. If the supernatant was still colored, a new portion of active charcoal was added to a mass ratio of 0.01, shaken and spun.

The supernatant, colorless at first sight, was passed through a minicolumn Sep-Pak C18, (Waters, art. 51910), and a minicolumn of Al_2O_3 (9).

The cleaned extract was filtered through Millipore filter FHLP 01300 to remove any particles of glass wool and was treated in an ultrasonicator to remove dissolved air.

A reference solution containing fructose, glucose and sucrose was submitted to the same clean-up procedure. Peaks were the same as without clean-up. Recovery was 97 ± 1% for fructose, 100 ± 1% for glucose and 98 ± 1% for sucrose.

Under ultraviolet radiation (wavelength 360 nm), the Al_2O_3 minicolumn with reference solution was uniform in violet color whereas those with samples contained bands of several colors.

Cleaned extracts poured into an Al_2O_3 minicolumn were also uniform, showing that extraneous materials had been removed.

Chromatography was as described by Gorin and Heidema (9) but the cleaned extract was injected automatically (20 µL instead of 10 µl) with the WISP (Waters Intelligent Sampling Processor 710A), instead of manually.

As spiking procedure, fructose (Merck, art. 5323) (5.00 mg), glucose (Merck, art. 8342) (5.01 mg) and sucrose (Merck, art. 7651) (5.00 mg) were added separately to 100 mg of powder and measured by the procedures. The recoveries were 102 ± 2% for glucose, 101 ± 1% for fructose and 99 ± 1% for sucrose.

Fructose and glucose were also measured enzymatically in several powders with and without clean-up.

Sucrose was not measured because if the sample had a mass ratio of glucose to sucrose >3:1, natural glucose had to be destroyed by a tedious procedure.

Both types of extracts were diluted to a tenth with distilled water and poured into the cuvette (where there was a further dilution by a factor 30). A total dilution of 300 times "removed" methanol and also the pigments of the samples without clean-up (8).

The extract could be diluted 10 times, because they originated from 1.0 g powder in this series of experiments instead of 0.1 g and so provided sufficient sugars for the sensitivity of the method.

Moisture in powders was estimated by the method of Gorin (7).

RESULTS AND DISCUSSION

Response of Detector

There was a rectilinear response between peak height (cm) and mass of soluble sugars of 25, 50, 75 and 100 ug in 20 uL. The equation ($y=a_o+a_1x$) of the 3 lines are:

for fructose $a_o = -0.400$, $a_1 = 0.251$, r= 1.000

for glucose $a_o = -0.315$, $a_1 = 0.202$, r= 1.000

for sucrose $a_o = -0.215$, $a_1 = 0.190$, r= 1.000

Enzymatic Comparison With and Without Clean-up

A sign test for paired comparisons (12) showed no significant difference between mean mass fractions of the respective sugars with and without clean-up (Table 1). So the clean-up procedure did not retain the sugars of the samples.

Comparison of Enzymatic and Chromatographic Method

The sign test for paired comparisons (12) demonstrated that there was no significant difference between the mean mass fractions of the respective sugars by the two methods.

So peaks of the chromatograms for the powders were due to fructose, glucose, and sucrose. Other compounds did not interfere.

The mass ratio of powder to methanol buffer (1:10) did not constitute a problem for extraction.

The moisture contents (between 0.5 and 3.0%) of the powders were ignored and not corrected for.

ACKNOWLEDGEMENT

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DETERMINATION OF THE COMPONENTS OF THE BOLL WEEVIL PHEROMONE WITH A HIGH PRESSURE LIQUID CHROMOTOGRAPHIC METHOD

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ABSTRACT

A high pressure liquid chromatographic method was developed to separate the 4 components of the pheromone of the boll weevil. Minimum detectable amounts with 3.5% tetrahydrofuran in hexane was 10 ng for (+)-cis-2-isopropenyl-1-methyl-cyclobutaneethanol, 1 ng for (Z)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethenol, and 2.5 ng each for (Z)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde and (E)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde at 214 nm.

INTRODUCTION

The pheromone produced by the male boll weevil, Anthonomus grandis Boheman, is found in the frass of the feeding male and is attractive to females.

Tumlinson et al. (1) first described the pheromone of the boll weevil as 4 components; compound I is (+)-cis-2-isopropenyl-1-methyl-cyclobutaneethanol; II, (Z)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III, (Z)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; and IV, (E)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde. Several studies have since used GLC analysis of the pheromone from the frass of the male boll weevil. The limit of detection in these studies was about 20 ng of each compound by GLC.

The clean-up procedures used for the GLC method consisted of steam-distillation or extraction with microsoxhlets, which are quite tedious and time consuming. Because of our interest in the pheromone as an indicator of the quality of mass-reared insects, we needed a quick method for analysis with a sensitivity at least equivalent to that of the GLC method. We report in this paper the development of a sensitive high pressure liquid chromatographic procedure that requires a minimum of clean-up preparation and also permits the analysis of the four components of the boll weevil pheromone with a single injection by use of dual wavelength detection at 214 and 254 nm.

APPARATUS

A high performance liquid chromatographic system (Waters Associates, Inc., Milford, Mass.) was used with an M6000-A solvent pump, a U6K injector coupled through a column to a Waters model 440 fixed-wavelength detector @ 254 nm and a Laboratory Data Control (Riviera Beach, Fla.) fixed-wavelength detector @ 214 nm. For preliminary investigations, a Waters model 450 variable-wavelenth detector @ 214 nm and a Waters model R401 refractive index detector were used. A Texas Instruments Omniscribe (Houston, Tex.) dual pen chart recorder was used. A Waters microporasil column (3.9 mm ID x 300 mm L.) with a 10μ particle size was used in the system. Hamilton (Reno, Nev.) 10 and 25 μℓ syringes (#701 and 702) were used for injections.

Puffer-Hubbard Calumet (Grand Haven, Mich.) incubators with envirotrol microcomputer programmers were used to hold the boll weevils at 55 $\pm$ 5% relative humidity, 29 $\pm$ 1°C; and were programmed for the 16L:8D, 2L:22D and 0L:24D photoperiods used. A Vortex Jr. Mixer from Scientific Industries (Queens Village, N.Y.) was used to stir mixtures in the test tubes.

CHEMICALS

All HPLC solvents were obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). The tetrahydrofuran was held in containers flushed with N_2 gas and kept in the dark to prevent peroxide formation. Solvents for extraction of the samples were obtained from Fisher Scientific Co. (Norcross, Ga.). The pheromone standards were obtained from Chem-samp Co., Inc. (Columbus, Ohio) and had a ratio of 30:40:15:15 of compounds I:II:III:IV. Individual standards were available for compounds I and II but not III and IV which were mixed.

METHODS

All analyses were conducted at 25°C. The eluting solvent consisted of 3.5% tetrahydrofuran in hexane degassed under reduced pressure with stirring. Samples were isocratically eluted from the microporasil at a flow rate of 2 ml/min. We used a dual detection system consisting of single wavelength detectors at 214 nm and 254 nm. The microporasil column was washed weekly with 100 ml methanol, 100 ml methylene chloride, and then 500 ml hexane.

Frass (feces) was collected from 100 male boll weevils. The newly emerged adults were fed for 5 days on slabs of artificial rearing diet (3) in plastic trays held under three different photoperiods: 16L:8D, 2L:22D and OL:24D (total darkness). After the five day feeding on slabs, all weevils were fed cotton squares (flower buds) and held in a photoperiod of 16:8 for 3 days. This procedure is similar to that used for mass-reared weevils to be released in the field. The frass was collected on day 3 and stored below 0°C in a closed container.

Samples of boll weevil frass were placed in graduated test tubes, hexane or pentane (5 ml) added, and then the tubes were stoppered and vortexed vigorously for 30 seconds. The suspension

was allowed to settle for about five min, and then a 10-to 25-µℓ aliquot of the clear supernatant was injected into the chromatograph.

RESULTS

The final choice of solvent for the assay was 3.5% tetrahydrofuran in hexane after methylene chloride and ethyl acetate were found in preliminary experiments to be inappropriate. A Waters refractive index detector was sensitive to µg quantities, but a Waters variable wavelength detector set at 214 nm would not maintain a stable baseline and was an order of magnitude less sensitive than the single-wavelength 214 nm detector chosen for the assay. Detection at 254 nm was also used, and although it did not detect compounds I and II, it was nearly as sensitive as the 214 nm detector for compounds III and IV. The advantages of dual detection were the ability to determine the pheromone peaks III and IV when obscuring peaks were present and the accurate quantitation of compounds III and IV relative to the differences in sensitivity at the two wavelengths. The isocratic 2 ml/min. flow rate was chosen because it was simple and because gradient methods with either solvent concentration or flow rate did not increase resolution.

A separation of standards with 3.5% tetrahydrofuran in hexane demonstrated the minimum detectable amounts of the pheromone compounds to be 10 ng of compound I, 1 ng of compound II, and 2.5 ng each of compounds III and IV at 214 nm. At 254 nm, 5 ng of compounds III and IV were detectable. In comparison, Bull et al. (3) stated that in their GLC method 20 ng of each component standard was needed for accurate quantitation, and McKibben et al. (4) found that 1 µg of total pheromone was necessary for quantitation because of interfering peaks in actual frass samples.

Figure I is a chromatograph of a typical frass sample. Elution time for good resolution was approximately 20 min. with 3.5% tetrahydrofuran in hexane. The α-terpineol used in GLC assays as

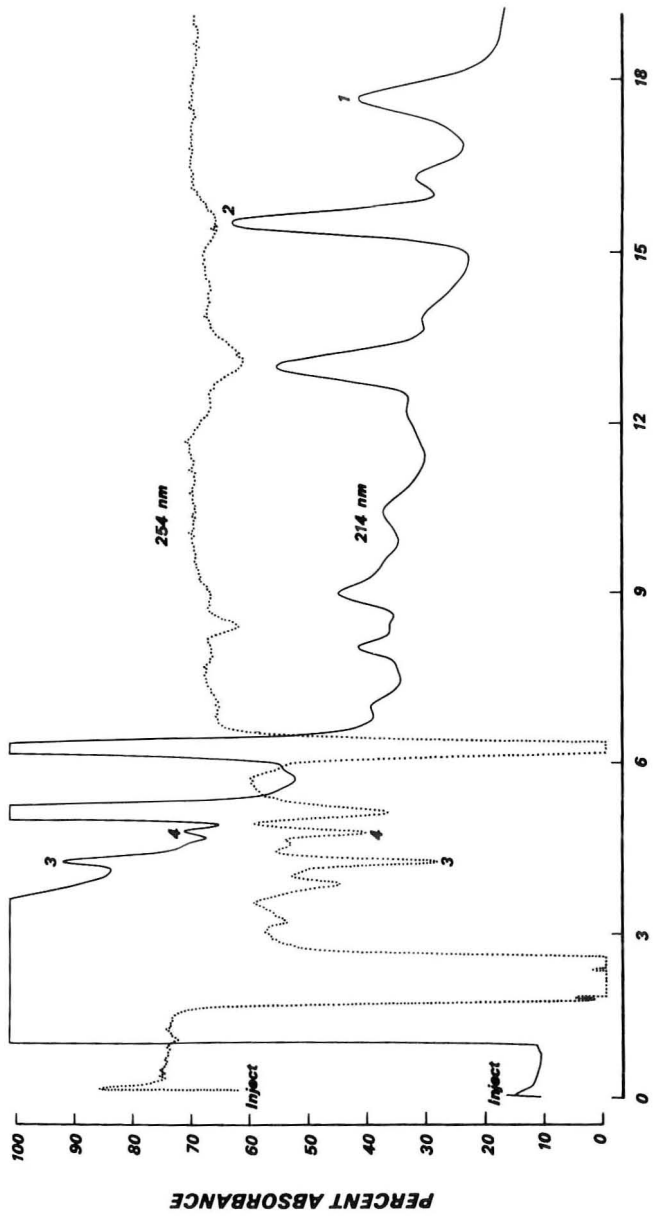

FIGURE 1. Typical HPLC chromatographic separation of 4 components of the boll weevil pheromone from the frass of males with 3.5% tetrahydrofuran in hexane.

an internal standard was found to elute before compound II and was impure, as 3 peaks were identified on the HPLC, although only one peak was found on the GLC. Therefore, we did not use α-terpineol as an internal standard for quantitation. Other peaks were present in the analysis besides those of the pheromone components; in most cases, these did not interfere with analysis. The relatively simple and quick extraction method used gave 100% recovery from frass spiked with pheromone. The extraction method for GLC required 1 hour or more for a single sample, whereas 5 min. or less was required for 10 samples with our method.

The results in Table I were from 3 separate experiments with 100 male boll weevils each. Three injections were made of each sample from each experiment. While the weevils were fed on diet slabs no pheromone was found in their frass, but when they were later fed squares in the 16L:8D photoperiod pheromone was produced. The weevils held in the dark and then fed squares in 16L:8D produced more pheromone than the weevils maintained in the other photoperiods. This was significant since the 24L:0D photoperiod, not total darkness, is used in mass rearing the boll weevil. Gueldner and Wiygul (9) found low levels of pheromone in frass of weevils fed on squares and reared in total darkness, but

TABLE I

Effects of Photoperiod on Pheromone Production.

Photoperiod	Pheromone Compounds (ng/Weevil ± SD)			
(light:dark)	I	II	III+IV	Total[a/]
16:8	14± 8	10± 8	8± 9	32 a
2:22	26±25	11± 7	20±10	51 a
0:24	96±22	56±17	69±22	187 b

a/ Means not followed by a common letter differ significantly at the 0.01 level of probability according to Duncan's multiple range test.

because they did not later feed these weevils in the 16L:8D photoperiod a direct comparison is not possible. Our weevils fed only in darkness on slabs of diet produced no pheromone. The weevils in the dark produced a pheromone compound ratio of approximately 2:1:1 (I:II:III+IV).

McGovern et al. (6) reported that with the GLC method the ratio of components was 40:18.5:23:18.5, and Hedin et al. (7) reported a ratio of 40:40:14:6 Hardee et al. (8) used a formulation in the field based on the ratio 23:17:30:30.

DISCUSSION

The HPLC method presented in this paper is more sensitive, i.e., 10 ng I, 1 ng II, 2.5 ng III, and 2.5 ng IV, than the reported GLC methods that require about 20 ng for each of the compounds. The methods of McKibben et al. (3), Bull et al. (2), Hedin et al. (6), and Gueldner and Wiygul (9) depend on crucial sample preparation and have not been extended to assay the pheromone produced by an individual weevil as this HPLC method has. Boll weevils are reared in large numbers (millions) for release as sterile insects, and it is necessary to quantitate pheromone production as a measure of the quality of the released adults. This HPLC method now gives us a rapid and sensitive assay in a minimum of time.

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SEPARATIONS OF THREO-ERYTHRO AMINOALCOHOLS BY PREPARATIVE HPLC

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ABSTRACT

A liquid chromatographic method which enables the separation of the threo/erythro diastereoisomers obtained from the reduction of Wellbutrin™ brand bupropion using a ternary eluent system is described. This has been achieved on a preparative scale.

INTRODUCTION

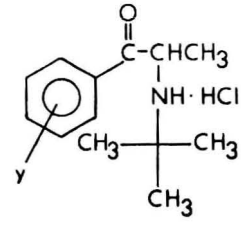
The rapid and facile separation of a mixture of isomers has always been the hope of the bench chemist. The separation of a mixture of threo and erythro isomers, particularly aminoalcohols, has not been easy (1). The major obstacle has been the great similarity in physico-chemical properties such as polarity, melting points, and solubility. Thin-layer chromatography (TLC) using silica gel does not give complete separation. A study was therefore initiated to explore the possibility of using preparative HPLC to bring about a quantitative separation. This has now been

achieved. Our primary interest, as reported herein, was to obtain the pure threo isomer as usually it is not easily accessible by chemical separations.

The aminoketone, 3-chlorophenyl-2-tert-butylaminopropiophenone, 1, (Wellbutrin™ brand bupropion, y=Cl) on reduction gave a mixture of diasteriomeric threo/erythro aminoalcohols, 2, in varying proportions depending upon the reducing agent and conditions employed. Reduction with Adam's catalyst, known to give pure erythro isomer, could not be employed because of reductive dehalogenation of the aromatic ring (1a). Of the several reducing agents attempted, it was found that sodium borohydride in aqueous ethanol at room temperature gave predominately the erythro (ca 80%) isomer. Reduction with diborane in THF resulted in a threo/erythro isomer ratio of ca 80:20 respectively.

Initially, efforts at establishing a TLC system which could effectively separate the two isomeric aminoalcohols using acetonitrile/toluene or chloroform/ethyl acetate were not successful. Complete separation was not achieved due to tailing of the spots. The use of ammonia vapors in the TLC chamber was employed (2). This resulted in a definite separation on silica plates giving a ΔR_f value (3) of 0.09. The use of ammonia to deactivate (4) the silica of the prep 500 cartridge was inadvisable; however, the use of 0.1% diethylamine in the mobile phases, mentioned above, gave desirable conditions for the effective separation of the two isomers. Triethylamine appeared to be equally useful.

In the course of these studies, it was observed that when large amounts (50 g) of the isomeric mixture were to be separated, the first load of 10 g on the fresh cartridge eluted slowly (long retention times) and showed a long tailing effect for the second



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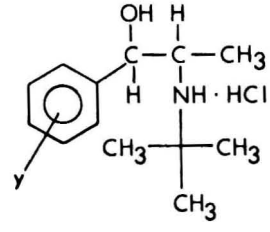

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component, the erythro isomer (see Fig. 1). However, if the new silica cartridge was initially equilibrated with a different eluent solution having a higher concentration (1.0%) of the amine, deactivation was sufficient to make the subsequent separation of the two isomers very facile and the tailing effect, observed earlier, minimized.

For reasons obvious from Figure 1, recycling was not practical as the first component, the threo isomer, would be out of phase in

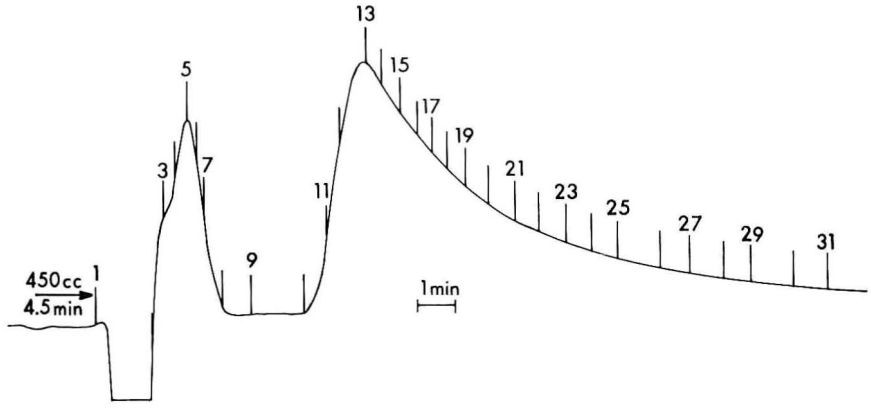


Figure 1. Preparative HPLC chromatogram of threo/erythro (79:21) Separation.

the eluting process and would precede the tail of the erythro isomer. However, the intermediate fractions between the pure threo and pure erythro fractions could be concentrated and rechromatographed. Using this approach, it was possible to obtain in a single run 47.2% of pure threo, 13.9% of a middle fraction having a threo/erythro ratio of 64:36, respectively and 2.8% of a final fraction which was 85% erythro/15% threo. This accounted for a total recovery (5) of 63.9%.

Subsequently, several other examples of the threo/erythro aminoalcohols of this class have been separated using essentially the same techniques. It should be mentioned that for the large scale separations, regular reagent grade solvents were satisfactory. Also, the availability of a continuous feeding type syringe (6) could allow the loading of 30 to 40 g of the mixture without disengaging the needle from the injector plate.

EXPERIMENTAL

Apparatus

a) Chromatographic equipment: (i) preparative LC - The apparatus consisted of a Waters Associates Liquid Chromatography Preparative 500 system equipped with a differential refractive index detector. (ii) Analytical LC-Water Associates Model 244 equipped with a Model 6000A pump, U6K universal injector and a R401 differential refractometer. (iii) RCSS-LC-Waters Radial Compression Separation System (RCSS) equipped with a Model 6000A pump, U6K injector and a R401 differential refractometer.

b) General equipment: The NMR spectra were recorded on a Perkin-Elmer R24A or a Varian XL 100 spectrometer. Results are reported on the δ scale in parts per million (ppm) downfield from TMS internal standard.

The gas chromatographic analyses were performed on a Varian 1800 chromatograph with a flame-ionization detector.

Reagents

The following solvents were used without further purification (7): Fisher HPLC grade acetonitrile and ethyl acetate, Mallinckrodt analytical reagent grade toluene and chloroform, diethylamine purchased from Aldrich Chemical Co.

The aminoalcohols were synthesized by procedures published earlier (8a-c). The isomeric mixtures of 3-10 g of threo (T)/ erythro (E) isomers were dissolved in 10 milliliters of the mobile phase (S1).

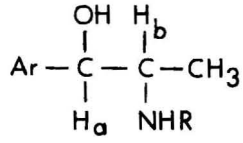
Procedures

The solvent reservoirs, injection port and the columns were maintained at ambient temperature. The mobile phase (S1) consisted of acetonitrile, toluene and diethylamine 20:80:0.1 (V:V:V). For the preparative LC separations, the "Prep-Pack"® cartridge was equilibrated using two different procedures. The first procedure (A) consisted of flushing the new cartridge with one liter (two column volumes) of the mobile phase (S1) which was discarded. The mobile phase (S1) was then recirculated for one-half hour prior to the separation. In the second procedure (B), the new cartridge was pretreated with one liter of a different solvent system (S2) consisting of acetonitrile, toluene and diethylamine 20:80:1.0 (V:V:V), that is, enhanced ten fold in diethylamine concentration. This latter eluent (S2) was discarded after which the original mobile phase (S1) was recirculated as before for one-half hour prior to the separation. The flow rate for the preparative separations was 100 mL per minute and the relative response of the refractive index detector was ten. Normally, the fractions collected were between 40 and 100 mL of eluent.

For the analytical and RCSS separations the columns were equilibrated to the mobile phase (S1) for approximately one-half hour prior to the separation.

Results and Discussion

The degree of purity of the threo and erythro isomers obtained by preparative high performance liquid chromatography was verified by other methods. The identification and purity of the two isomers



was accomplished by NMR spectroscopy (9). The NMR spectrum of the threo isomer had a doublet centered at 3.9 ppm (J_{HaHb} = 8.2) as shown in Figure 2. The erythro isomer had a doublet at 4.6 ppm (J_{HaHb} = 3.5). There was also a definite difference in the chemical shifts for the doublets attributed to

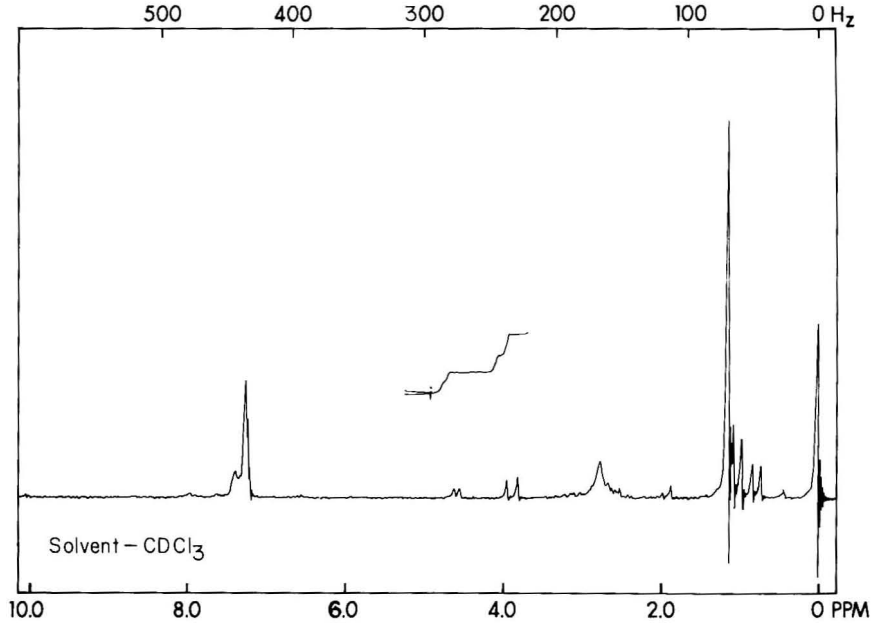


Figure 2. NMR spectrum of threo/erythro mixture (combined fractions 31-33).

the methyl protons. The threo-CH_3 doublet was found at 1.09 ppm and the erythro-CH_3 doublet at 0.8 ppm.

The threo and erythro isomers were also characterized by gas chromatography as shown in Figure 3. Using an OV-11 on Supelcoport column, the retention times for the threo and erythro isomers were 7.5 min. and 7.25 min. respectively for the conditions shown in Table 1.

The thin-layer chromatography of the threo/erythro mixture on silica gel using acetonitrile/toluene (20:80) in an ammonia atmosphere gave an R_f(T) of 0.29 and an R_f(E) of 0.20. (See Figure 4) (10). From the TLC data, the ΔR_f value (11) for this mixture was 0.09. This indicated that a load in the range of three to five

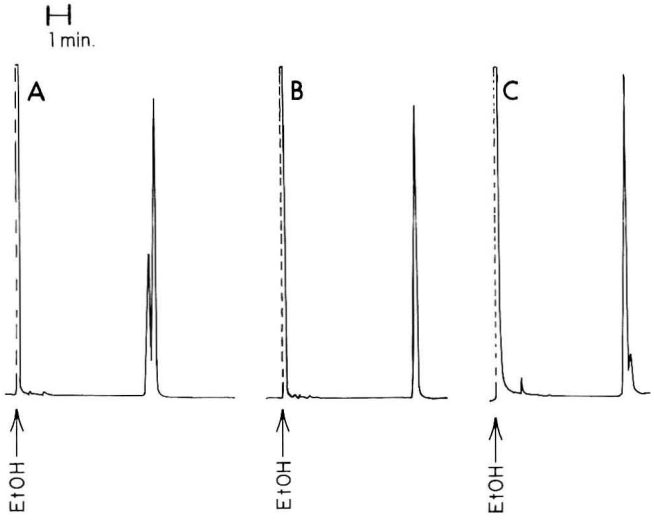


Figure 3. GC Analysis of threo/erythro mixture (A) Combined fractions 31-33; (B) combined fractions 11-30; (C) fraction 34 (See Table I for GC conditions).

Table 1

Conditions for Gas Chromatography

instrument:	Varian 1800 GC with FID
column:	10% OV-11 on 100/120 supelcoport (6 ft. x 2 mm glass)
column temp:	180-260°C at 8°/min
injector temp:	240°C
detector temp:	275°C
carrier gas:	Helium at 40 cc/min
chart speed:	0.5 inch/min
solvent:	ethanol

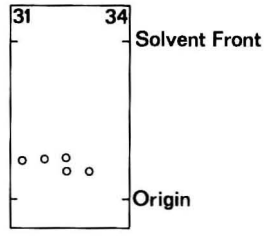


Figure 4. TLC of threo/erythro mixture on silica gel using acetonitrile/toluene (20:80) in an ammonia atmosphere (Fractions 31-34)

grams could be statisfactorily separated by a single pass using one Prep-Pak® cartridge (3).

The mixture prior to separation, as determined by NMR, consisted of the threo and erythro isomers in a ratio of 79:21 respectively. Three and six-tenths grams of the mixture was dissolved in 10 mL of the previously described eluent to load the cartridge. Figure 1 shows the preparative chromatogram for this separation. The individual fractions were scanned by TLC. Similar fractions were combined and concentrated. These were analyzed by NMR and gas

chromatography. Fractions 1-10 consisting of approximately 1100 mL of eluent were discarded since thin-layer chromatography showed no fluorescent material (12). The chromatogram asymptotically returns to the baseline, therefore fractions 31 to 34 are not shown. Fraction 32 contained 500 mL of eluent while fractions 31, 33 and 34 were approximately one liter each. Fractions 11 to 30 gave 1.7 grams of pure threo isomer as shown by NMR (see Figure 5). Fractions 31 to 33 gave 0.5 grams of a mixture consisting of 36% erythro and 64% threo (Figure 2). The last fraction gave 0.1 grams of a mixture which was 85% erythro and 15% threo (Figure 6).

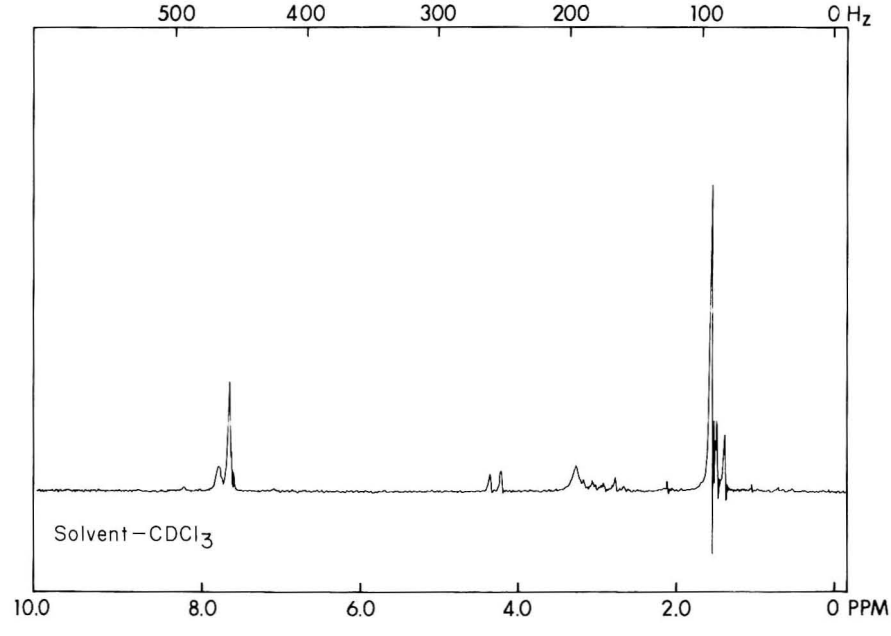


Figure 5. NMR of combined fractions 11-30 (pure threo isomer).

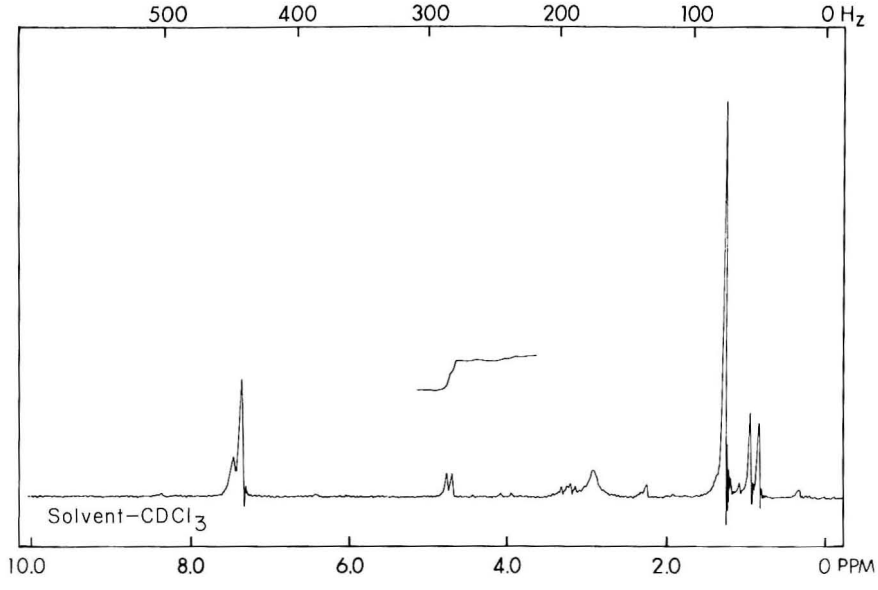
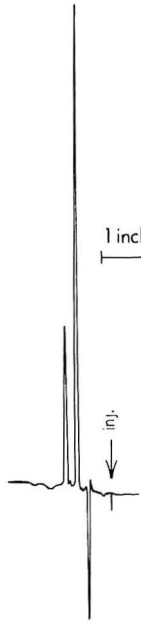


Figure 6. NMR of fraction 34 - erythro/threo mixture - 85%/15% respectively

These studies were essentially directed at obtaining the pure threo isomer. Since the initial mixture was enriched in the threo isomer, the erythro isomer was not obtained in pure form in this specific separation. As shown in Figure 1, the erythro isomer eluted in the tail of the threo isomer (Fractions 31 to 34).

Figures 7 and 8 show the analytical HPLC and the Radial Compression Separation System (RCSS) chromatograms respectively. Table 2 compares the chromatographic data of the two analytical HPLC's with the data obtained from the preparative LC. It should be noted that overloading the column as in the preparative mode



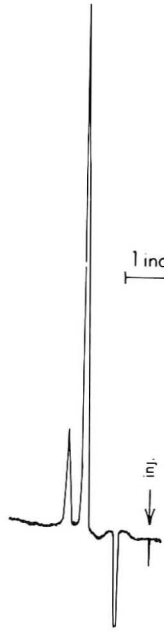
Column: Waters μ-porasil

Mobile phase: Acetonitrile/Toluene/ diethylamine - 20:80: 0.1 (V:V:V).

Chart speed: 0.2 inch/min.

Flow Rate: 2 cc/min

Figure 7 - Analytical HPLC chromatogram



Column: Waters Radial-PAK SI®

Mobile phase: Acetonitrile/Toluene/ diethylamine - 20:80: 0.1 (V:V:V)

Chart speed: 0.4 inch/min.

Flow Rate: 3 cc/min

Figure 8 - RCSS chromatogram

Table 2

Comparison of Analytical and Preparative LC Data

	Analytical HPLC	RCSS	Prep LC
k'(T)	0.6	1.0	1.7
k'(E)	1.2	1.5	4.8

reduces the k' ($k' = (V_1-V_o)/V_o$, where V_1 = the retention volume of a particular compound and V_o = one column volume) of the threo isomer relative to the erythro isomer.

Several other mixtures which differed from 85% threo/15% erythro to 10% threo/90% erythro have been separated. These studies in varying percentage composition are shown in Table 3 and a discussion of their results follows.

When the threo isomer predominated, as in examples 1 and 2, it was obtained in excellent yield by a single pass through one "Prep-Pak"® cartridge using the standard eluent (S1) acetonitrile, toluene and diethylamine 20:80:0.1 (V:V:V). The remaining fractions were enriched in the erythro isomer. These enriched fractions from examples 1 and 2 could presumably be rechromatographed to obtain the pure erythro isomer as illustrated in example 4.

It has been our observation that when the erythro isomer predominates in the original mixture, it can be obtained in a high degree (>99%) of purity as illustrated by example 4. However, the yield is rather poor. Several factors could be attributed to this low yield. First, since the erythro (i.e., the second elution component) isomer was predominant, the cartridge was overloaded

Table 3

Examples of Mixtures of Varying Compositions

Example	Amount of Mixture, g	% Composition[a]	Equilibration Method[b]	Results[a,c]
1	12.5 g[d]	85%T/15%E	A	8.9 g T 1.16 g 46%T/54%E 1.12 g 16%T/84%E
2	9.9 g	85%T/15%E	B	6.8 g T 1.9 g 61%T/39%E 0.8 g 5%T/95%E
3	5.9 g	13%T/87%E	A	3.6 g E(>97%) 2.3 g 29%T/71%E
4	8.2 g	10%T/90%E	B	2.6 g E(>99%) 4.5 g 20%T/80%E
5	3.4 g	59%T/41%E	A	1.5 g T 0.5 g 43%T/57%E 0.6 g 9%T/91%E
6	2.0 g[e]	43%T/57%E	A	0.5 g T 0.4 g 37%T/63%E 0.7 g 6%T/94%E

[a] % composition determined by NMR.
[b] See procedure section for description of method A and B.
[c] Single pass through one "Prep-Pack"® cartridge. Results are given for various combined fractions.
[d] Two separations of 6.25 g each (second separation was performed on same column as the first).
[e] This separation was done using the mobile phase (S3) ethyl acetate, chloroform and diethylamine 20:80:0.1 (V:V:V).

with respect to this isomer which caused it to elute faster than normal. At the same time, the cartridge was not overloaded with respect to the threo (the primary elution component) isomer which continued to come off the cartridge at its normal elution rate. This explains why the erythro isomer "had caught up with" the threo

isomer giving a poor yield of the second component - the highly pure erythro isomer.

Also, deactivation of the column through pretreatment with an amine, makes both isomers elute faster which could enhance this effect. The tailing of the threo (i.e. the first component) isomer was also a factor which reduced the amount of the pure erythro product obtained.

Example 3 demonstrated that the use of a non-pretreated cartridge gave a higher yield of the erythro isomer but with a lower degree of purity (>97%). This again reflects the tailing of the threo component which is more pronounced when a non-pretreated cartridge is used. Rechromatography of the combined first fraction in example 3 could give the pure erythro isomer.

The fifth example in Table 3 illustrates that when the percent composition was nearly equal, a fair separation can be obtained. The threo isomer was obtained pure, while the erythro isomer was obtained having about 91% purity. Again, rechromatography of the remaining fractions could give the pure erythro isomer. It is important to note that the reversal of the composition of the threo/erythro mixture does not alter the order of elution of the two isomers. The threo isomer is always followed by the erythro component.

Other eluents can also be employed advantageously for this separation. For example, ethyl acetate, chloroform and diethylamine 20:80:0.1 (V:V:V) (S3) gave similar results as shown in

example 6. This solvent system gave higher R_f values of 0.52 and 0.39 for the threo and erythro isomers respectively in the TLC (10) as compared with the earlier eluent system (S1). Although the higher R_f values would imply that the isomers would not be retained as long on the cartridge, the actual observation was that the threo isomer was retained longer relative to the erythro isomer. However, the order of elution remained unchanged. This resulted in a lower yield of the pure threo component. Consequently, almost one and one-half as much of the nearly pure (94%) erythro isomer was realized than was obtained by the eluent system (S1). Ethyl acetate/dichloromethane 20:80 (V:V) (10) gave similar results by TLC (R_f (T) = 0.59 and R_f (E) = 0.46). While this eluent system (S4) was not employed for the preparative LC separations, it would be expected to give results similar to those obtained using eluent system (S3).

Pretreatment of the fresh cartridge with a different solvent system (S2) containing 1% diethylamine as described earlier in the procedure section, resulted in a duration of separation of one-half to one-fourth the time required for a non-pretreated cartridge. Also, the solvent consumption, as would be expected, was reduced proportionately.

Using the same set of conditions as described above for the 3.6 g sample, separations of large amounts (ca 50 g) of the isomeric aminoalcohols of this class using five to ten gram portions per injection, have been successful. For a given set of conditions, the separation was repeatable.

These studies in separations of the two diastereoisomers seem to indicate that it would be more advantageous to use two different sets of solvent systems if the recovery of both of the isomers, in high yield, is desired. The fractions enriched in the erythro isomer after chromatography with eluent (S1) could be rechromatographed using the latter two solvent systems (S3 or S4).

Conclusion

Preparative HPLC has been found to be a satisfactory method for the separation of threo/erythro mixtures resulting in high yields of the individual isomers having excellent purity.

Acknowledgment

The authors are indebted to Suzette R. Medlin for her excellent technical assistance. Also thanks are due to Dr. B. Stuart Hurlbert for analytical and spectral data and its interpretation and to Mr. James Cichetti for the gas chromatography. Appreciation is also expressed to Mr. Don Harris and Dr. Warren Beverung of Waters Associates for their excellent technical suggestions.

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2. Uchytil, B. Thin-layer and High-Speed Liquid Chromatography of the Derivatives of 1,4-Phenylenediamine, Journal of Chromatography, 93, 447-455 (1974).

3. Waters Associates "TLC to Prep" manual copyright 1979 (Part No. 82185) page 8.

4. Deactivation of the "Prep-Pak"® cartridge was accomplished using diethyl or triethylamine. Aqueous ammonium hydroxide dissolves the silica gel and shortens the column life.

5. No systematic study to optimize the recovery has been attempted. Several parameters such as activity of the silica gel, concentration of the diethylamine, etc. could effect the total recovery.

6. A three-way valve which can be placed between the syringe and the needle is available from Popper and Sons, Newhyde Park, New York, N.Y. (Part No. 6017).

7. For the analytical LC, the solvents were filtered through a 0.45 μM millipore filter and degassed by a steady stream of helium.

8 a) See Reference 1a.
b) Angiolini, L. and Tramontini, M. Stereochemistry of Amino Carbonyl Compounds. IX. Lithium Aluminum Hydride and Lithium Trialkoxy Aluminum Hydride Reduction of α-Asymmetric β-Amino-propiophenones, J. Org. Chem., 39, 2056 (1974).
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b) The threo-erythro compounds shown in Table V Reference 1a were studied in cooperation with Dr. S. Hurlbert using 100 MHz NMR to determine the NMR shifts.

10. The TLC plates used were purchased from MC/B Manufacturing and were silica gel 60 F_{254} on aluminum support. Layer thickness was 0.2 mm. The ammonia atmosphere was accomplished by placing a beaker of concentrated aqueous ammonia in the TLC chamber and lining the walls of the chamber with filter paper.

11. The ΔR_f value can be corelated to the load which can be separated by one Prep-Pak® cartridge on a single pass through the column.

12. The negative deflection could possibly be due to some soluent carried over from the preparation of the aminoalcohols or from displacement of the amine modifier, diethylamine, by the aminoalcohols.

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RETENTION IN REVERSED-PHASE ION-PAIR CHROMATOGRAPHY OF AMINES ON ALKYL-BONDED PHASES.

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ABSTRACT

Reversed phase ion-pair chromatography of phenethylamine derivatives (noradrenaline, adrenaline, dopamine, synephrine, tyramine and pholedrine) and lower alkylamines has been performed with octyl sulfate as counter ion in an aqueous eluent with a low content of 1-pentanol. LiChrosorb RP-18 was used as the solid phase. The retention of lithium and potassium in the system has also been studied.

On the basis of adsorption and retention studies a model for the chromatographic behaviour of the amines is proposed that includes interaction with two sites with different binding ability in the stationary phase. The adsorption capacity of the sites has been calculated as well as adsorption constants for the octyl sulfate ion pairs.

INTRODUCTION

Reversed phase ion-pair chromatography has been widely used during the last years for separation of cationic and anionic compounds (e.g. [1-4]). Most separations are performed on a

n-alkyl bonded phase with a solution of the counter ion in mixtures of aqueous buffers and an organic solvent as eluents.

Different models have been proposed for the chromatographic process in ion-pair chromatographic systems with a retaining solid phase. Horvath and co-workers have used a retention model based on the so called solvophobic theory [5,6]. Others have measured the adsorption of hydrophobic counter ions like cetrimide [7], octyl sulfonate [8] and n-alkyl sulfonates [9] on the solid phase and claimed that the chromatographic system can be characterized as ion exhange chromatography with the adsorbed counter ions acting as the stationary phase. The retention mechanism has also been discussed by Bidlingmeyer et al. in a study of sulfonates both as samples and counter ions [10] and Cantwell et al. have applied the Stern-Gouy-Chapman theory of electrical double layer to explain the retention [11].

Tilly-Melin et al. have studied the influence of tetrabutyl-ammonium on the retention of anionic and basic compounds and proposed a retention model based on competition for sites on the adsorbing solid phase between ion pairs formed by components in the eluent and the sample [12,13]. This model have also been used in studies of hydrophobic amines where ions with the same charge as the sample was used to regulate the retention on alkyl-bonded solid phases [14,15], as well as on underivatized silica [16].

In the present study the model of Tilly-Melin et al. has been applied on the chromatographic behaviour of hydrophilic amines as ion pairs with octyl sulfate in a system with 1.15% 1-pentanol in the eluent.

It has been found that the retaining phase is composed of two kinds of sites with different binding ability for the ion pairs. The adsorption of octyl sulfate on the solid phase has been measured and the adsorbing capacity of the solid phase has been calculated as well as constants for the adsorption of the ion-pairs.

EXPERIMENTAL

Apparatus

The liquid chromatograph comprised an Altex Model 100 solvent metering pump, an LDC UV-detector Model 1205 with 8 µl cell volume, measuring at 254 nm wavelength and a Valco high pressure valve injector with a sample loop of 30 µl. The chromatographic equipment was thermostated in a water bath, model HETO 02 PT 923 TC (Birkeröd, Denmark). The chromatographic columns were made of 316 stainless steel with a polished inner surface. They were equipped with modified Swagelok connectors and 2 µm stainless steel frits from Altex. The dimensions of the separation columns were (150 x 4.5) mm.

The equipment for post-column reaction with phthaldialdehyd included a second Altex Model 100 solvent metering pump and an LDC fluorometric detector Model 1311 with 30 µl cell volume, excitation wavelength at 340-380 nm and emission cut off at 418 nm. The reactor column, (300 x 4.6) mm, was drypacked with glass beads 16 $\pm$ 2.3 µm.

A Corning 400 Flame Photometer (Halstead, Essex, England) was used as detector in the chromatographic studies on lithium and potassium using the wavelengths 671 nm and 767 nm respectively.

The determination of 1-pentanol was performed with a Varian 1400 gas chromatograh equipped with a flame ionization detector and pH was measured with an Orion 801 Research pH meter equipped with an Ingold combined electrode.

Chemical and Reagents

Sodium octyl sulfate was obtained from E. Merck (Darmstadt, GFR) and from Eastman Kodak (Rochester, N.Y., USA). 1-Pentanol was

of A.C.S. quality from Fisher Scientific (Pittsburg, Pa., USA). Phthaldialdehyd (für die Fluoreszenzanalyse) were obtained from E. Merck and 2-mercapto-ethanol from Serva (Heidelberg, GFR). The cation exchanger was Amberlite^{R} IR-120 p.a.

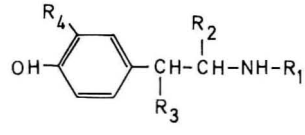
All amines used as chromatographic samples were of pharmacopeial or equivalent grade. The structures of the phenethylamine derivatives are given in Table 1.

All other substances used were of analytical or reagent grade and used without further purification.

Phosphate buffers were prepared from phosphoric acid, sodium dihydrogen phosphate and disodium hydrogen phosphate while borate buffers were prepared from boric acid and sodium hydroxide. All buffers were prepared with an ionic strength of 0.1 using water, purified by ion-exchange and filtered through a Milli-Q Reagent-Grade Water system.

TABLE 1

List of Phenethylamine Derivatives



Name	R_1	R_2	R_3	R_4
Pholedrine	CH_3	CH_3	H	H
Tyramine	H	H	H	H
Synephrine	CH_3	H	OH	H
Dopamine	H	H	H	OH
Adrenaline	CH_3	H	OH	OH
Noradrenaline	H	H	OH	OH

Column Preparation

LiChrosorb RP-18, 10 µm, obtained from E. Merck was used as solid phase. The same batch was used through the study.

The separation columns were packed at 400 bar by a balanced-density slurry technique according to Majors [17]. The solid phase was suspended in tetrachloroethane 0.1 g/ml and treated in ultrasonic bath for 30 sec immediately before packing. After packing 300 ml hexane and 80 ml acetone was passed through the column.

The quality of the columns was tested with methanol-water (6+4) as eluent. A test solution of phenol, 2-phenylethanol, 2,6-dimethylphenol and 2,3,5-trimethylphenol was injected. The accepted columns had a reduced plate height of less than 6 for capacity ratios above 2. The flow rate was 0.6 mm/s.

Preparation of Eluents

Sodium octyl sulfate was freed from lower alkyl sulfates by the following procedure. An aqueous solution of sodium octyl sulfate was passed through a cation exchanger in hydrogen form. Phosphoric acid was added to the eluate to a content of 0.1 M. The octyl sulfuric acid was then extracted to pentanol using equal phase volumes and re-extracted to aqueous phase by repeated extractions with phosphate buffer pH 3. The concentration of octyl sulfate in the aqueous phase was measured with the extraction method given in [18].

The eluents containing 1.15% pentanol were prepared by mixing solutions equilibrated with pentanol at 25.0°C with equal volumes of pentanol-free buffers.

Chromatographic Technique

The separation column, the reservoir and the injector were thermostated at 25.0 $\pm$ 0.1°C by immersion in a water-bath.

The columns were conditioned by passing 50 ml of buffer with the same content of pentanol as the eluent [19,20] followed by eluent until test samples had a constant retention. The hold-up volume of the column, V_m, was determined by the peak obtained when potassium nitrate was injected. The determination was made before the passage of eluent had started.

All samples were injected dissolved in the eluent. All chromatographic results reported are the means of triplicate injections.

Post-column Derivatisation

Primary amines without UV-adsorbance were detected fluorometrically after on-line post-column derivatisation with phthaldialdehyd [21-23].

The reagent was prepared by mixing 0.4 g phthaldialdehyd, 1 ml 2-mercapto-ethanol, 5 ml ethanol and boric buffer pH 10.4 to 500 ml. The solution was protected from light.

The post-column reactor system with a packed bed reactor described by Deelder et al was used [23]. The flow rate in the separation column was set to 0.6 ml/min and the reagent was pumped at a flow rate of 1.4 ml/min which gave a reaction time of about 1 min.

Flame Photometer as Detector

The separation column and the ordinary intake of the flame photometer was connected by a capillary tube, 500 mm in length and with an inner diameter of 0.25 mm. The flame photometer was adjusted to aspirate 1.3 ml/min which was equal to the flow rate used in the separation column.

Determination of Octyl Sulfate and 1-Pentanol Adsorbed on the Solid Phase

Octyl sulfate and 1-pentanol were stripped off by eluting the column with 5 ml ethanol-water (1+4) followed by 10 ml

ethanol-water (4+1) and ethanol to 100 ml. The amount of adsorbed species was obtained as the difference between the amount found in the eluate and in the mobile phase.

The amount of pentanol in the eluate was determined by gas chromatography on a 1.5 meter silanized glass column with an inner diameter of 2 mm packed with 15% OV 225 on Gas Chrom Q 100/200 mesh using 1-butanol as internal standard.

Octyl sulfate in the eluate was determined by the extraction method given in [18]. Control by further elution with water showed that > 99% of adsorbed octyl sulfate had been eluted.

At concentrations of octyl sulfate in the eluent lower than 2×10^{-3} M the adsorption of octyl sulfate was measured by break through curves [24].

RESULT AND DISCUSSION

Purification of Octyl Sulfate

Commercially available sodium octyl sulfate contains an ionic impurity, that can be detected and quantified in a straight phase chromatographic system with N-metylprotriptyline as counter ion [25]. A chromatogram is shown in Fig. 1. The quantitation was based on peak height measurements and external standardisation.

The degree of impurity was found to vary between 1 and 20 % in different batches of sodium octyl sulfate. The exact nature of the impurity is not known but it has the same retention in the chromatographic system in Fig.1 as propyl sulfate and hexyl sulfonate.

Sodium octyl sulfate was purified by a procedure that included a transformation to octyl sulfuric acid by ion exchange followed by an extraction of the acid from an aqueous phase of pH 2 (i.e. 0.1 M phosphoric acid) into pentanol. Octyl sulfuric acid has in this system a distribution ratio of more than 10 while the

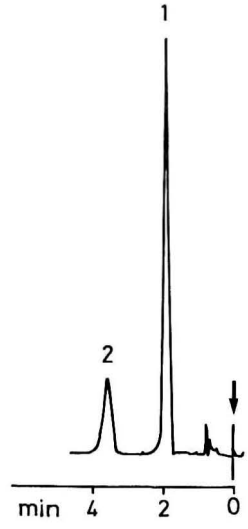


FIGURE 1. Chromatogram of octyl sulfate. Stationary phase: 2.7×10^{-2} M N-metylprotriptyline (MPT) in phosphate buffer pH 8.0 on LiChrosorb-diol, 5 µm; Eluent: chloroform + 1-propanol (92+8); Flow rate: 1.1 ml/min; Peaks: 1 = octyl sulfate; 2 = impurity.

extraction of the impurity was insignificant as controlled by the chromatographic system given in Fig. 1. The octyl sulfate was re-extracted to an aqueous buffer of pH 3.0. Repeated extractions were required [18].

Ion-pair Chromatography with Adsorbing Stationary Phase

Previous studies with aqueous eluents containing 1-pentanol and LiChrosorb RP-18 as solid phase have indicated that adsorption has a decisive influence on the retention of octyl sulfate ion pairs when the content of 1-pentanol is as low as 1.15% [20].

Studies of Tilly-Melin et al and others [12-15] have shown that the retention of ion pairs in adsorbing systems can be

expressed by a model based on competition for a limited number of sites on the adsorbing surface. The principe can be illustrated by the following example.

The distribution of a cation, Q^+, to an adsorbing surface as ion pair with the counter ion, X^-, can be expressed by the formula

$$Q^+_m + X^-_m + A_s = QX.A_s$$

where Q^+_m and X^-_m represent the ions in the mobile phase, A_s the available adsorption sites and $QX.A_s$ the adsorbed ion pair. A quantitative expression is given by the adsorption constant, K_{QX}, defined by

$$K_{QX} = \frac{[QX.A]_s}{[Q^+]_m \cdot [X^-]_m \cdot [A]_s} \qquad (1)$$

The brackets signify concentrations in mol/l in the mobile phase and in mol/g in the solid phase. Analogous equilibrium expressions are valid for each adsorbed ion pair.

The surface can accomodate a limited number of mol of adsorbed species per g. If the ion pair, QX, is the only adsorbed species, the capacity of the surface, K_0, is given by the expression

$$K_0 = [A]_s + [QX.A]_s \qquad (2)$$

The concentration of the adsorbed ion pair in the stationary phase is then given by the equation

$$[QX.A]_s = \frac{K_0 \cdot K_{QX} \cdot [Q^+]_m \cdot [X^-]_m}{1 + K_{QX} \cdot [Q^+]_m \cdot [X^-]_m} \qquad (3)$$

obtained by combination of eqs. (1) and (2).

If a sample ion, B^+, is injected on the column and Q^+ and X^- are components of the mobile phase, the ion pairs BX and QX will compete for the available adsorption sites.
The expression of the adsorption capacity is then

$$K_0 = [A]_s + [QX.A]_s + [BX.A]_s \qquad (4)$$

An expression for the capacity ratio of B^+, k'_{BX}, can be obtained by combining eq. (4) with the equations for adsorption of the two ion pairs (eq. (1))

$$k'_{BX} = \frac{q.[BX.A]_s}{[B^+]_m} = \frac{q.K_0.K_{BX}.[X^-]_m}{1 + K_{QX}.[Q^+]_m.[X^-]_m + K_{BX}.[B^+]_m.[X^-]_m} \qquad (5)$$

where q is the phase volume ratio expressed in g of solid phase per 1 of mobile phase present in the column.

Symmetric chromatographic peaks are obtained under such conditions that the concentration of the sample has an insignificant influence on the retention as shown by the relationship

$$(1 + K_{QX}.[Q^+]_m.[X^-]_m) >> K_{BX}.[B^+]_m.[X^-]_m \qquad (6)$$

From eq. (5) follows that the retention can be regulated by the nature and the monomeric concentration in the mobile phase of the counter ion, X^-, and the competing ion, Q^+, with the same charge as the sample. The eq. is valid for an adsorbing stationary phase that has one kind of adsorption site. Tilly-Melin et al [12,13], Sokolowski et al [14] and others [15] have, however, given examples of systems where the retention of ion pairs on alkyl-bonded phases can be described by a model with two sites of different binding ability. The equation for the capacity

ratio will then in analogy with eq. (5) have the following form when the retention is independent of the sample concentration.

$$k'_{BX} = \frac{q \cdot K_0 \cdot K_{BX} \cdot [X^-]_m}{1 + K_{QX} \cdot [Q^+]_m \cdot [X^-]_m} + \frac{q \cdot K_0^x \cdot K_{BX}^x \cdot [X^-]_m}{1 + K_{QX}^x \cdot [Q^+]_m \cdot [X^-]_m} \quad (7)$$

K_{QX}^x and K_{BX}^x are the adsorption constants of the ion pairs to the second site while K_0^x is its adsorption capacity. The total concentration of QX adsorbed to these two sites in the stationary phase is given by

$$C_{QX,s} = \frac{K_0 \cdot K_{QX} \cdot [Q^+]_m \cdot [X^-]_m}{1 + K_{QX} \cdot [Q^+]_m \cdot [X^-]_m} + \frac{K_0^x \cdot K_{QX}^x \cdot [Q^+]_m \cdot [X^-]_m}{1 + K_{QX}^x \cdot [Q^+]_m \cdot [X^-]_m} \quad (8)$$

The influence of a third species, e.g. 1-pentanol adsorbed to the same sites as QX and BX, can be expressed by eqs. analogous to (3), (5), (7) and (8) by introducing a term in the denominator that expresses the concentration of 1-pentanol in the stationary phase (cf. [15,16]). The magnitude of this term will be constant if the concentration of 1-pentanol in the mobile phase is constant as in the present study.

Adsorption of Mobile Phase Components

In a previous study it was established that a LiChrosorb RP-18 column in equilibrium with a mobile phase of phosphate buffer pH 3 with 1.15% 1-pentanol was coated with a monolayer of 1-pentanol [20].

If octyl sulfate is added to the mobile phase there is a change of the composition of the adsorbed layer as demonstrated in Fig. 2. The amount of 1-pentanol on the solid phase decreases with

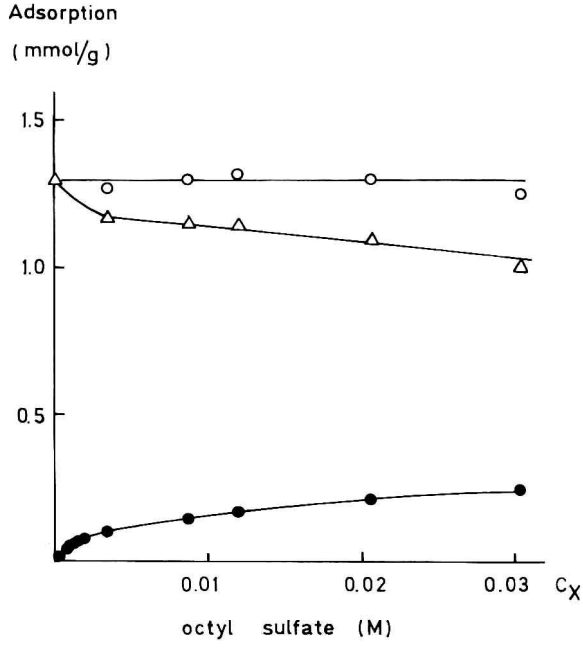


FIGURE 2. Adsorption of 1-pentanol and octyl sulfate to the solid phase. Solid phase: LiChrosorb RP-18, 10 µm; Eluent: octyl sulfate in phosphate buffer pH 3.0 with 1.15% 1-pentanol; ● = octyl sulfate; △ = 1-pentanol; ○ = total adsorption.

increasing adsorption of octyl sulfate, but the total number of moles of the two species is almost constant. This indicates that the two species compete for the same binding sites on the solid phase.

Octyl sulfate can be adsorbed both as ion pair with sodium and in acidic form but it has not been possible to distinguish between the two forms of adsorption by the experimental technique used. The eluent used in the adsorption studies had pH 3.0 and a concentration of sodium between 0.10 and 0.13 mol/l. Distribution studies of octyl sulfate between this aqueous phase and 1-pentanol in batch have shown that the extraction as ion pair with sodium dominates [18], and all the calculations below have been based on

the simplified assumption that octyl sulfate is distributed to the stationary phase as ion pair with sodium only.

The adsorption of sodium octyl sulfate could not be described by a single site model as indicated by an inversed plot based on eq. (3). The two site model given in eq. (8) was then applied as follows.

The two sites were assumed to have highly different binding ability, K_0 and K_{QX} being the constants of the stronger site and K_0^x and K_{QX}^x those of the weaker binding site. A computation of the constants was based on eq. (8) transformed to

$$\frac{b}{C_{QX,s} - \frac{K_0^x \cdot K_{QX}^x \cdot b}{1 + K_{QX}^x \cdot b}} = \frac{1}{K_0 \cdot K_{QX}} + \frac{b}{K_0} \qquad (9)$$

where $b = [Q^+]_m \cdot [X^-]_m$

A preliminary estimation was made with values of $C_{QX,s}$ obtained at low concentrations of octyl sulfate, $[X^-] < 2 \times 10^{-3}$ M, where the weak adsorption site, A_s, should be only slightly covered and the approximation $K_{QX}^x \cdot b < 1$ could be applied. A value of $K_0^x \cdot K_{QX}^x$ was found that gave a straight linear relationship between $b/(C_{QX,s} - K_0^x \cdot K_{QX}^x \cdot b)$ and b by way of trial. Preliminary K_0 and K_{QX} were calculated from the slope and the intercept of the line. A small dimerization of octyl sulfate in the aqueous phase was taken into account when calculating the constants [18,20].

The found value of K_{QX} indicated that the strong adsorption site, A_s, should be covered to ca 95% with 8×10^{-3} M octyl sulfate in the mobile phase. The preliminary K_0 and K_{QX} and values of $C_{QX,s}$ obtained at higher concentration of octyl sulfate, $[X^-]_m = (8\text{-}30) \times 10^{-3}$ M, were used in the next computation which was based on the eq.

$$\frac{b}{C_{QX,s} - \frac{K_0 \cdot K_{QX} \cdot b}{1 + K_{QX} \cdot b}} = \frac{1}{K_0^x \cdot K_{QX}^x} + \frac{b}{K_0^x} \qquad (10)$$

where $b = [Q^+]_m \cdot [X^-]_m$

A plot of $b/(C_{QX,s} - K_0 \cdot K_{QX} \cdot b/(1+K_{QX} \cdot b))$ versus b gave a linear relationship. The values of K_{QX}^x and K_0^x obtained from the slope and intercept of the line indicated that the assumption of $K_{QX}^x \cdot b < 1$ was not valid in the entire range it had been applied.

The premliminary values were then used in repeated calculations by successive use of eqs. (9) and (10) until the values of the constants remained unchanged. The results are given in Table 2.

Retention of Alkali Metal Ion

In the calculation of adsorption constants in Table 2 it was assumed that octyl sulfat is retarded as ion pair with sodium. If the

TABLE 2

Equilibrium Constants for Octyl Sulfate

Solid phase: LiChrosorb RP-18
Eluent: octyl sulfate, X^-, in phosphate buffer pH 3.0 with 1.15% 1-pentanol

Site	$C_X \times 10^3$ (M)	$K_0 \times 10^4$ (mol/g)	$K_{QX} \times 10^{-3}$	site coverage A_s (%)	site coverage A_s^x (%)
A_s	0.23- 1.9	0.52	25	36-83	1- 9
A_s^x	8.6 -31	3.1	0.50	96-99	30-62

assumption is valid, it should also be possible to retain other alkali metals, e.g. potassium and lithium, as ion pairs with octyl sulfate.

The experiments were performed with on line flame photometric detection (see EXPERIMENTAL). A typical chromatogram is given in Fig. 3. The relation between the capacity ratio and the octyl sulfate concentration is demonstrated in Fig. 4. The corresponding relationship for sodium, calculated by use of eq. (7) and the constants from Table 2, is included in the Figure.

The results indicate that lithium, as expected, is less hydrophobic than sodium while potassium has a slight higher hydrophobicity. Fig. 4 illustrates that all ionic compounds have a specific influence on the retention in ion-pair chromatographic systems with adsorbing stationary phases. An exchange of sodium for lithium in the eluent would thus give rise to an increase of the retention of other cationic samples.

Retention of Amines as Ion Pair with Octyl Sulfate

All retention studies were performed at pH 3.0 where the alkyl- and arylalkylamines are completely ionized and will be

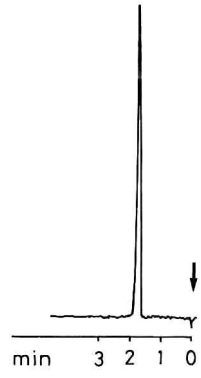


FIGURE 3. Chromatogram of lithium, retained as ion pair with octyl sulfate. Solid phase: LiChrosorb RP-18, 10 µm; Eluent: 0.005 M octyl sulfate in phosphate buffer pH 3.0 with 1.15% 1-pentanol; Flow rate: 1.3 ml/min; Detector: flame photometer; Sample: lithium (0.2 µg).

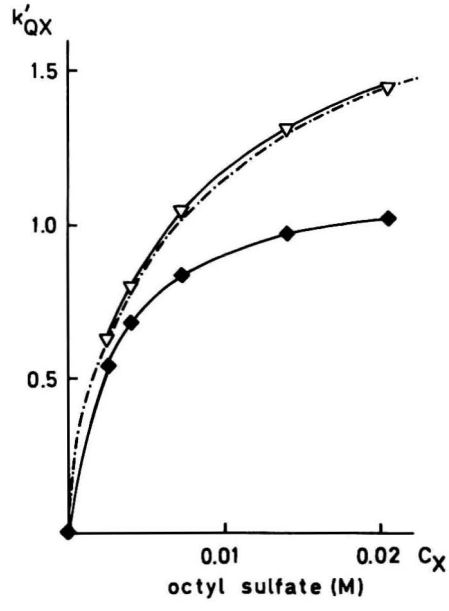


FIGURE 4. Retention of alkali metals as ion pairs with octyl sulfate. Solid phase: LiChrosorb RP-18, 10 µm; Eluent: octyl sulfate in phosphate buffer pH 3.0 with 1.15% 1-pentanol; Sample: ▽ = potassium; ◆ = lithium; -·-·- = sodium, calculated by use of eq. (7), q=822 and constants from Table 2.

retained as ion pairs only. The influence of the concentration of octyl sulfate on the retention of some phenethylamine derivatives (Table 1) is illustrated in Fig. 5.

Since the adsorption of sodium octyl sulfate follows a two site model, it is obvious that an analogous model should be applied to the retention of amines as ion pairs with octyl sulfate. The retention model used is given in eq. (7). The validity of the model was tested by computation of constants for ion pair adsorption and adsorption capacity by successive approximation as demonstrated above.

Eg. (7) can be transformed to

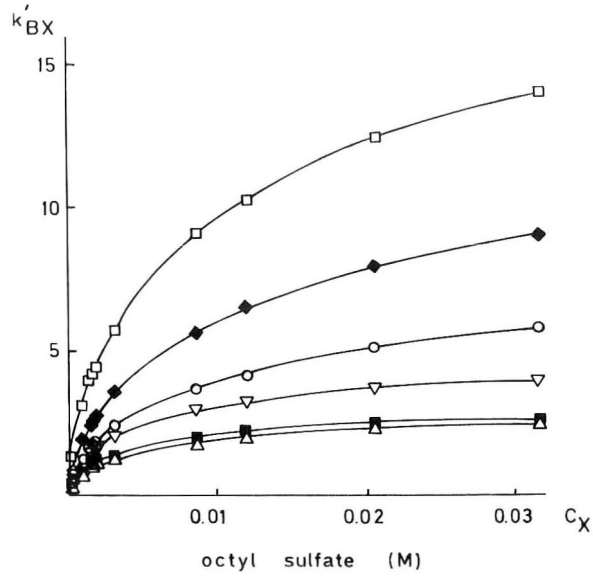


FIGURE 5. Retention of phenethylamine derivatives as ion pairs with octyl sulfate. Conditions as in FIGURE 2.
Samples: △ = noradrenaline; ■ = adrenaline; ▽ = synephrine; ◔ = dopamine; ◆ = tyramine; □ = pholedrine.

$$\frac{[X^-]_m}{k'_{BX} - \dfrac{c}{1 + K^x_{QX}.b}} = \frac{1}{q.K_O.K_{BX}} + \frac{K_{QX}.b}{q.K_O.K_{BX}} \qquad (11)$$

where $b = [Q^+]_m.[X^-]_m$ and $c = q.K^x_O.K^x_{BX}.[X^-]_m$

A preliminary computation of $q.K_O.K_{BX}$ and K_{QX} was made by use of capacity ratios obtained at $[X^-]_m < 2 \times 10^{-3}$ M and the approximation $K^x_{QX}.b < 1$. The found value were used in a graphical computation of $q.K^x_O.K^x_{BX}$ and K^x_{QX} by use of capacity

ratios obtained at higher concentration of octyl sulfate, $[X^-]_m$ = (8-30) x 10^{-3} M, and the eq.

$$\frac{[X^-]_m}{k'_{BX} - \frac{d}{1 + K_{QX}\cdot b}} = \frac{1}{q\cdot K_0^x \cdot K_{BX}^x} + \frac{K_{QX}\cdot b}{q\cdot K_0^x \cdot K_{BX}^x} \qquad (12)$$

where $b = [Q^+]_m \cdot [X^-]_m$ and $d = q\cdot K_0 \cdot K_{BX} \cdot [X^-]_m$

The preliminary values were finally corrected by repeated calculations with successive use of eqs. (11) and (12). It should be notified that the retention of the amines in the absence of octyl sulfate was very low and disregarded in the calculations above. The results are given in Table 3.

TABLE 3

Equilibrium Constants for Phenethylamine Derivatives

Solid phase: LiChrosorb RP-18
Eluent: octyl sulfate, X^-, in phosphate buffer pH 3.0 with 1.15% 1-pentanol

	C_X=(0.23-1.9) x 10^{-3}M		C_X=(8.6-31) x 10^{-3}M	
Sample	K_{BX} x 10^{-3} [a]	K_{QX} x 10^{-3}	K_{BX}^x x 10^{-3} [a]	K_{QX}^x x 10^{-3}
Noradrenaline	39	24	0.89	0.57
Adrenaline	37	18	0.93	0.60
Synephrine	51	15	1.2	0.51
Dopamine	61	20	1.6	0.35
Tyramine	97	22	2.5	0.36
Pholedrine	155	20	4.0	0.38

a) calculated by use of q = 822, K_0 = 5.2 x 10^{-5} and K_0^x = 3.1 x 10^{-4} (Table 2)

The found values of K_{QX} and K^{x}_{QX} are in good agreement with the results from the adsorption studies (Table 2) which confirms the validity of the model. The stronger site has about 40 times higher adsorption constant than the weaker site for all the phenethylamine derivatives studied. The effect of the two sites on the retention is illustrated in Fig. 6 with dopamine as object. The retention by the weaker site dominates at higher concentrations of octyl sulfate because the weaker site has about six times higher capacity than the stronger one.

Retention studies were also performed with some lower alkylamines, using fluorimetric detection after post-column derivatisation with phthaldialdehyd (see EXPERIMENTAL). The relation between the capacity ratio and the concentration of octyl sulfate in the eluent is demonstrated in Fig. 7.

The retention data were applied to the two site model and adsorption constants were calculated by eqs. (11) and (12) using the same method as for the phenethylamine derivatives. Straight

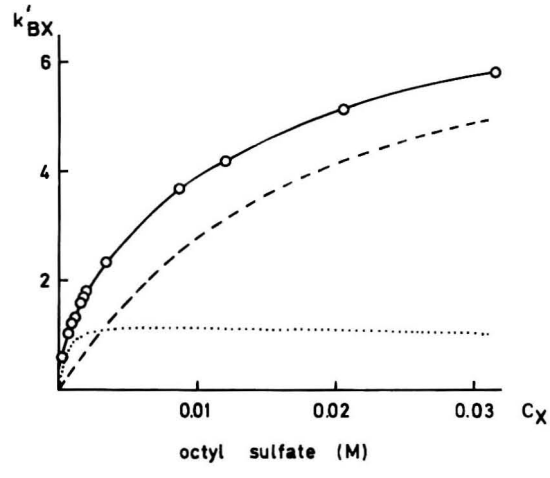


FIGURE 6. Retention of dopamine as ion pair with octyl sulfate. Conditions as in FIGURE 2.; ○ = total retention;
----- = retention to the weaker adsorption site;
..... = retention to the stronger adsorption site.

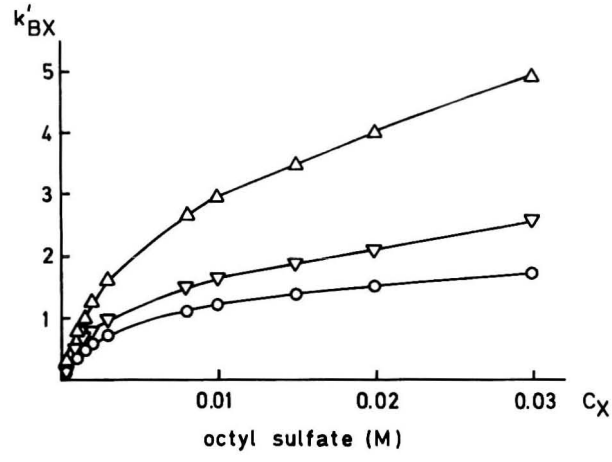


FIGURE 7. Retention of aliphatic amines as ion pairs with octyl sulfate. Conditions as in FIGURE 2 with fluorometric detection after post-column derivatisation with phthaldialdehyd; Samples: ○ = methylamine; ▽ = ethylamine; △ = propylamine.

linear plots were obtained in the graphical computation of the constants and the found values of K_{QX} and K^{x}_{QX} were in good agreement with those found for noradrenaline and dopamine in the same chromatographic system. The alkylamines seems to be adsorbed to the same sites as the phenethylamine derivatives.

The nature of bonded phases has been discussed by several authors (cf. [26-29]). This study has indicated the presence of two sites on the solid phase with different ability to retain ion pairs. It is not possible however to state the nature of the individual site, since the samples used have similar structure and show the same relative binding to both sites.

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USE OF SEP-PAK^{R} C_{18} CARTRIDGES FOR THE COLLECTION & CONCENTRATION OF ENVIRONMENTAL SAMPLES *

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ABSTRACT

High Pressure Liquid Chromatography (HPLC) is being increasingly employed in environmental analysis. However, HPLC is somewhat limited by the sensitivity of the detection systems commercially available. Many are not sensitive enough to cope with the lower levels being demanded by various governmental agencies. The purpose of this study is to show the efficiency of a simple sampling device, a SEP-PAK which not only allows efficient on-site sampling but offers the ability to concentrate samples down to a level where UV or fluorescent detection can determine trace quantities in the ppb range.

INTRODUCTION

It has been previously possible to concentrate large volumes of aqueous samples for analysis by HPLC by use of a technique called trace-enrichment (1). In this technique, the water sample is passed through a column containing non-polar packing such as octadecylsilane bonded onto silica. Because the organics have a much greater affinity

*Based on lectures given at the Pittsburgh Conference (Cleveland, 1979) & Eastern Pesticide Conference (Guelph 1979).

for the packing material than for the aqueous mobile phase they will be adsorbed at the head of the column. If the organic content of the mobile phase is then increased (as in a solvent gradient) the organics will be selectively eluted. In this way, "injections" of large volumes of sample can be made onto the chromatographic column without any of the deleterious effects associated with such large injection volumes. An examples of this is shown in Figure 1 where 175 µl of a mixture of anthracene and pyrene are directly injected (concentrations 0.6 + 0.2 ppm respectively) with a mobile phase of 100% water. If the mobile phase is then stepped to 60% acetonitrile-

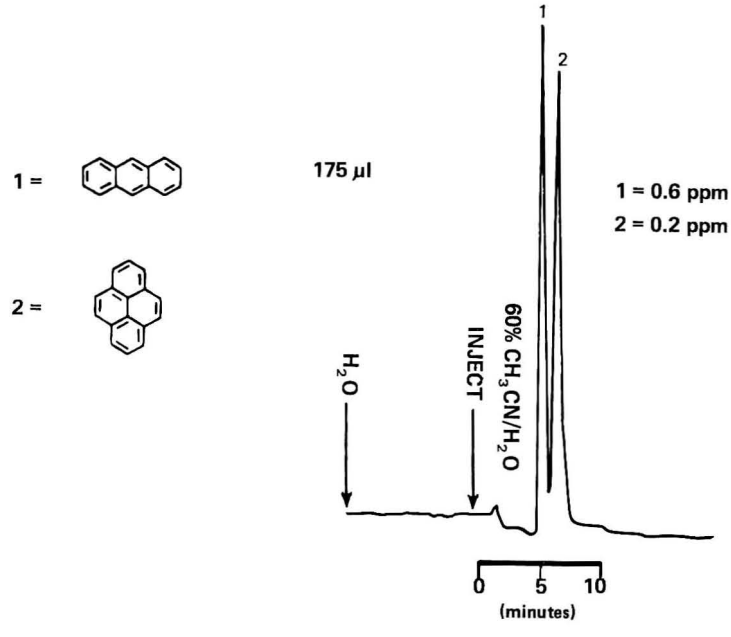

FIGURE 1 Trace enrichment of anthracene and pyrene on C-18. Sample: 175 µl injection of 0.6 ppm anthracene & 0.2 ppm pyrene. Conditions: column-µBondapak C-18 column; solvent-water stepped up to 60% CH_3CN/water after injection' flow rate of 4 ml/min.

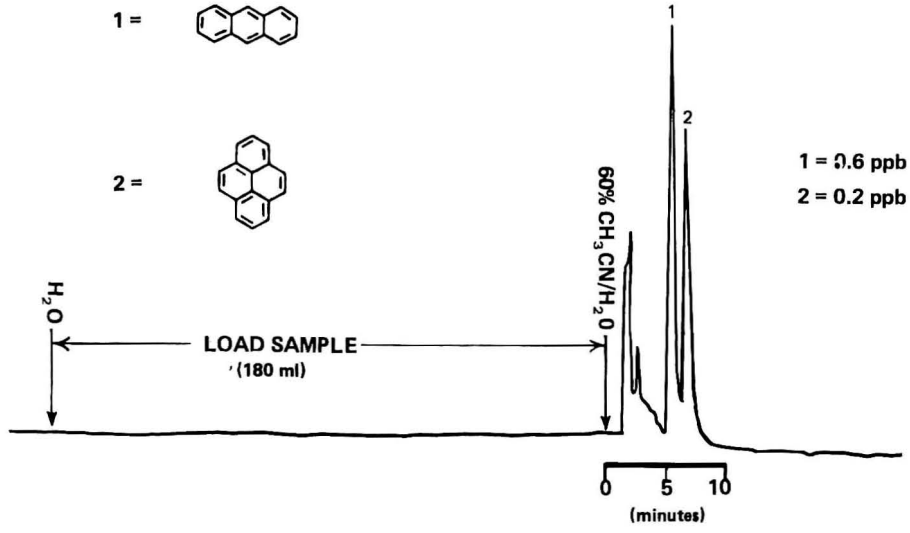

FIGURE 2 Trace enrichment of anthracene and pyrene on C-18. Sample: 180 ml injection of 0.6 ppb anthracene & 0.2 ppb pyrene. Conditions: same as Figure 1

water the organics are eluted. For more dilute solutions, the sample can be pumped onto the column. Figure 2 shows the result of a 180ml sample of 0.6 and 0.2 ppb of anthracene and pyrene respectively, being pumped onto the column and subsequently eluted in the same fashion as above. A comparison of the two chromatograms shows nearly identical resolution for 1000-fold concentration procedure done directly on the column.

An obvious extension of this technique is to prepare a disposable column packed with a non-polar packing material. Since the organics are concentrated at the head of the column, a short column should suffice. This is in fact a SEP-PAK* (Sample Enrichment Purification)

*SEP-PAK, Porasil and Milli-Q are registered trade marks.

which is a small prepacked column containing about 0.4 g of C-18 on Porasil A.

The packing material is contained in a virgin polyethylene sheath which has undergone Radial Compression to form a highly reproducible homogeneous chromatographic bed. The SEP-PAK is also available with a silica packing but for this aqueous work C-18 is the obvious packing of choice.

The use of SEP-PAK offers certain advantages over collection of samples in the usual manner. In most cases, larger volumes of water samples are collected in glass bottles, a preservative being added if necessary. This can lead to problems where compounds of interest are adsorbed onto the glass resulting in erroneous analysis (2). In addition, the transportation of large volumes of samples can be quite costly. Subsequent extraction of these samples can cause difficulties in loss of very volatile components, multi-solvent requirements for multi-residues, cross-contamination of samples and costly solvent requirements. A SEP-PAK can be taken directly to the sampling site and a known quantity of water passed through the SEP-PAK thereby trapping any trace organics. These organics can be eluted in a small volume of organic solvent (usually about 1-2 ml) affording a 500 to 1000 fold increase in concentration (depending on the initial volume samples).

In this work, the efficiency with respect to collection and concentration of environmental samples of the SEP-PAK was studied. The actual study involved passing a quantity of material through the SEP-PAK and checking the SEP-PAK "Effluent" for compounds which were not retained, i.e. checking for "breakthrough." Two studies were done, (a) carbamate pesticides and (b) polynuclear aromatic hydro-

carbons (PNA's). Samples were run at fairly high concentrations so as to look at loading capabilities and to make small percentages of breakthrough easily detectable.

MATERIALS AND METHODS

HPLC

The chromatographic system consisted of a Waters Model 244 Luquid Chromatography equipped with two M6000A pumps and an M660 gradient programmer. Ultraviolet detection was done with a Waters Model 440 UV detector at 254 nm and Fluorescence detection using a Waters Model 420 Fluorometer with excitation and emission filters of 360 nm and 440 nm respectively. Integration was performed using a Shimadzu E1A integrator. For quantitation purposes, standards were injected with a Waters Model 710 WISP.

Columns used were either a μBondapak C-18 (3.0 mm ID x 30 cm) or a Radial-PAK A used with an RCM-100 (Waters Radial Compression Separation System).

The mobile phase used was acetonitrile (Burdick & Jackson-Spectro-grade) and water abtained from a Milli-Q system. This water required further polishing for the gradient work at low sensitivities. This was achieved by putting a Bondapak C-18 on Porasil B column, (7.8 mm ID x 122 cm) on line after the water pump and before the mixing chamber located on the acetonitrile pump. This allows us to obtain an essentially flat baseline for the gradient (Figure 3) while operating at high sensitivity on the UV detector.

Chemicals

Standards were obtained from commercial sources and used without purification. Standard solutions were made up in acetonitrile

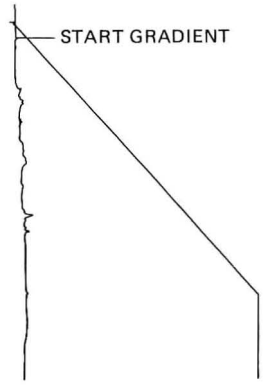

FIGURE 3 Blank Gradient Sample: 50 ml of treated water (see text)
Conditions: column-μ-Bondapak C-18, 3.9mm I.D. x 30 cm; solvent-A-water, B-acetonitrile; gradient: 0.100% B, 30 minutes, linear; flow rate 2.0 ml/min; UV detection at 254 nm at 0.05 AUFS.

and then diluted with the appropriate volume of water for trace enrichment studies.

Methods

For trace enrichment studies on SEP-PAK C-18 the following procedure was employed. The SEP-PAK was activated with 5 ml acetonitrile or methanol, then flushed with 5 ml water. The appropriate volume of the test solution was passed through the SEP-PAK at approximately 10 ml/min The effluent from this step was then trace enriched on the chromatographic column and the sample was eluted off the SEP-PAK with 2 ml of THF.

The response factors of each of the compounds was determined by making multiple automatic injections. Calibration data are given in TABLES 1 & 2. Note the excellent reproducibility of the two pump gradient system on the gradient running from o - 100% acetonitrile.

TABLE 1

Calibration data for carbamates *

PEAK IDENTIFICATION	RETENTION TIME (MIN)	SLOPE ($\times 10^{-2}$)	INTERCEPT	CORRELATION COEFFICIENT
Carbofuran	13.28±0.03	380	115	0.9997
Carbaryl	13.99±0.03	2513	-193	0.9996
IPC	15.06±0.03	947	- 96	0.9995
CIPC	17.13±0.03	2021	-185	0.9997

*for conditions see Figure 4

TABLE 2

Calibration data for PNA's **

PEAK IDENTIFICATION	RETENTION TIME (MIN)	SLOPE ($\times 10^{-2}$)	INTERCEPT	CORRELATION COEFFICIENT
Impurity in Benzanthrone (area vs. µl)	21.65±0.01	21311	33730	0.9999
Benzanthrone	23.59±0.01	20.09	1.31653	1.0000
Fluroanthene	25.61±0.01	21.21	.92885	1.0000
Benzo(a)Pyrene	28.35±0.02	50.02	-2.92475	0.9993
Benzo(ghi) Perylene	29.46±0.02	15.75	- .50435	0.9998

*for conditions see Figure 4
**for conditions see Figure 7

RESULTS AND DISCUSSION

The chromatogram of the carbamate standards used in the study is shown in Figure 4. Figure 5 shows the same standards trace enriched onto a Radial-PAK A column. After passing 45 ml of a standard solution of carbamates, a trace enrichment of the SEP-PAK effluent gave the chromatogram

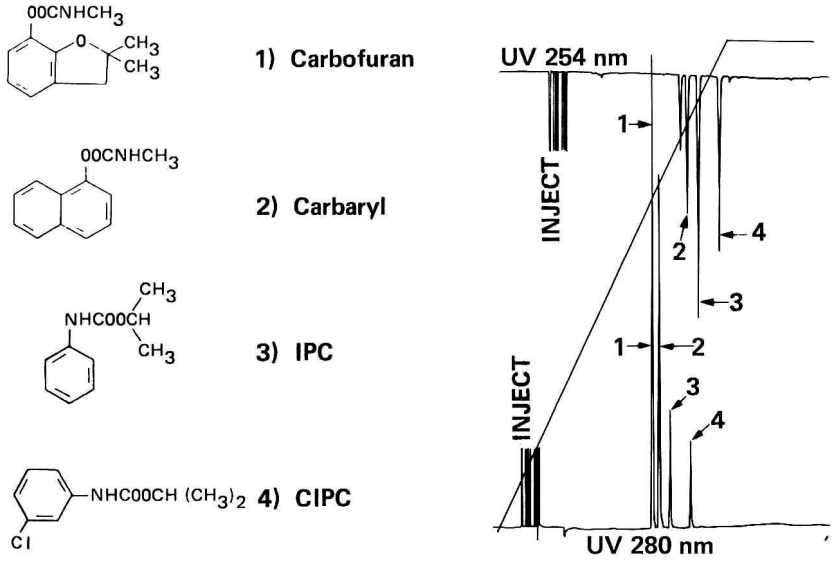

FIGURE 4 Carbamate Standards Conditions: column Radial-PAK A; solvent A-water, B-acetonitrile; gradient 0-100% B, linear, 25 minutes; flow rate 3.0 ml/min. UV detection at 254 & 280 nm, 0.2 AUFS.

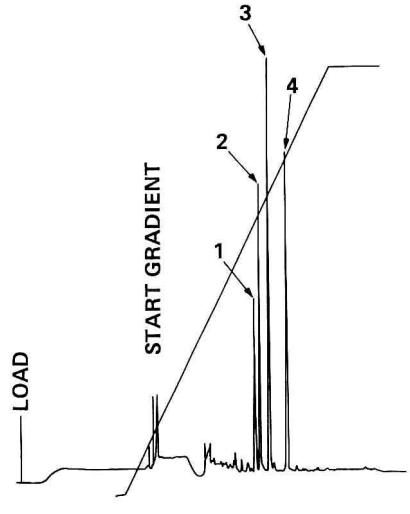

FIGURE 5 Trace Enrichment of Carbamate Standards Sample: 45 ml of diluted standards. Conditions: see Figure 4; UV detection of 0.1 AUFS; 1=carbofuran 157 ppb, 2= carbaryl 39 ppb, 3= IPC 206 ppb, 4= CIPC 70 ppb.

shown in Figure 6. No breakthrough is observable. The quantitation results are summarized in TABLE 3.

[1] Similarly the chromatogram of PNA standards used in the study is given in Figure 7. As above, trace enrichment onto a μBondapak C-18 column gave the chromatogram shown in Figure 8. Trace enrichment on the SEP-PAK effluent (Figure 9) showed breakthrough of two components - fluoranthene and benzo(a)pyrene. Quantitive results are summarized in TABLE 4. These show that breakthrough of benzo(a)pyrene is the highest, somewhere in the

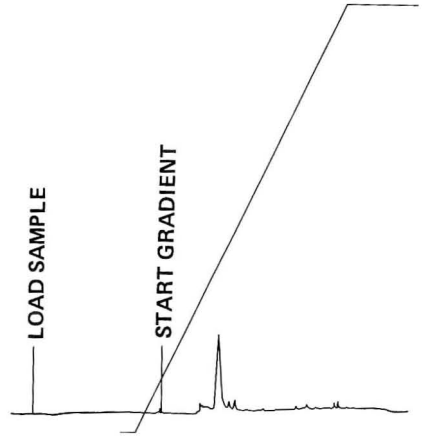

FIGURE 6 SEP-PAK Effluent (45 ml) of carbamate standards. Conditions: same as in Figure 5.

TABLE 3

SEP-PAK TRACE ENRICHMENT

STANDARD	AMOUNT TRACE ENRICHED (ng)	" % BREAKTHROUGH"
Carbofuran	7065	not detectable (minimum amount detectable is approx. 0.05 - 0.3 ng)
Carbaryl	1755	
IPC	9270	
CIPC	3150	

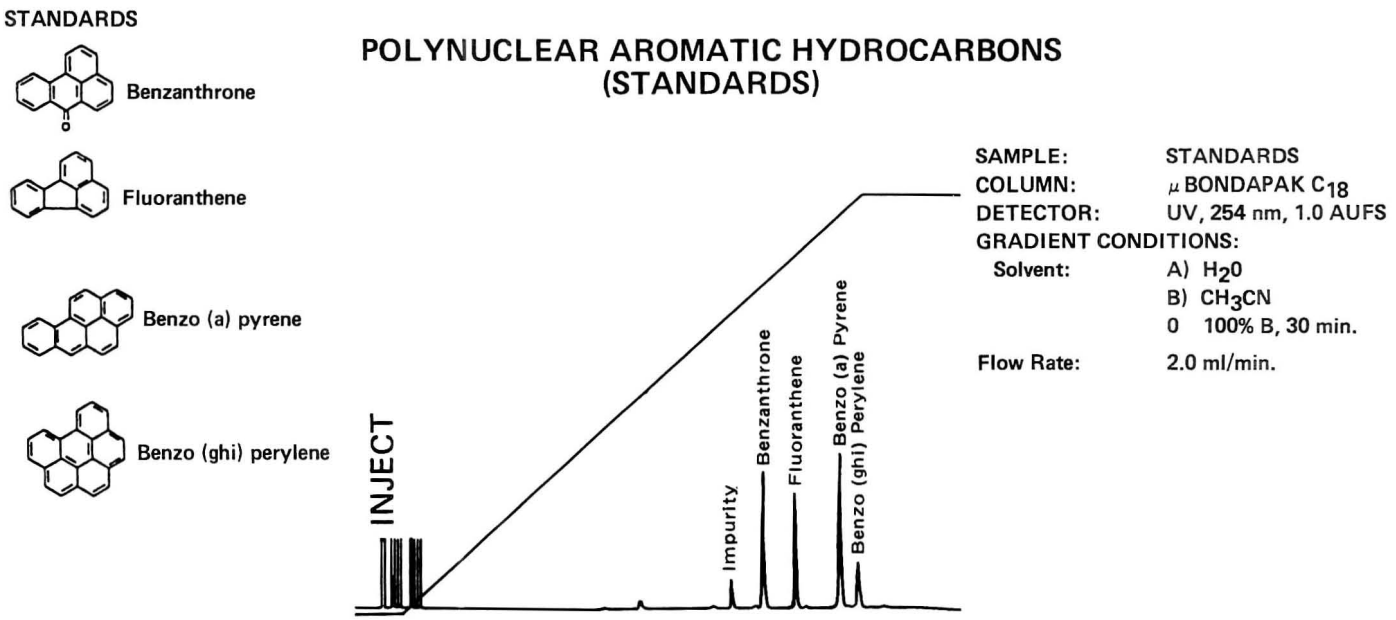

FIGURE 7 Chromatogram of PNA standards used in this study. Conditions same as in Figure 3. UV detection at 254 nm, 1.0 AUFS.

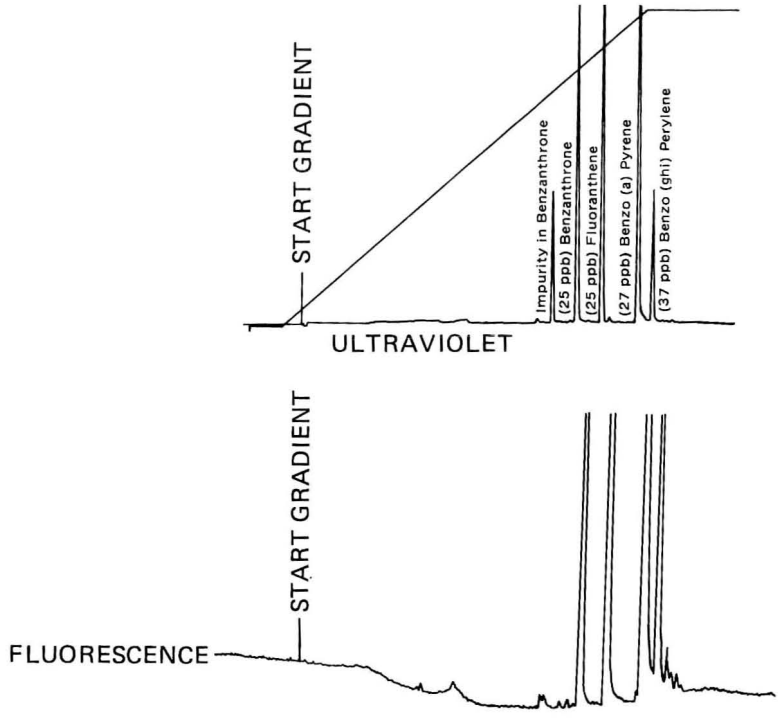

FIGURE 8 Trace enrichment of PNA standards. Sample size - 50ml. Conditions same as Figure 3. UV detection at 254 nm, 0.2 AUFS: Fluorescence detection at 8X.

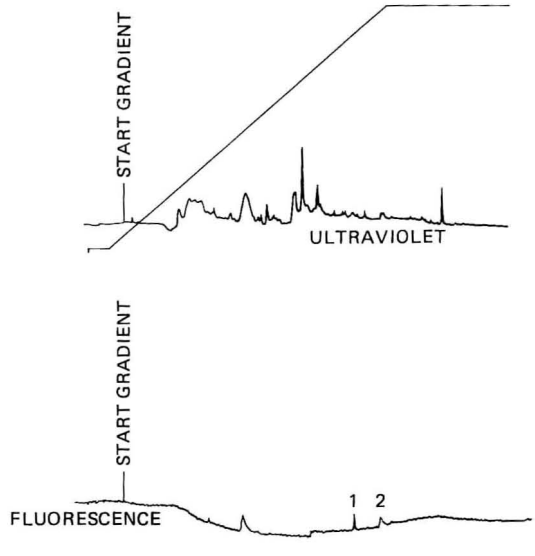

FIGURE 9 SEP-PAK effluent of PNA standards. Sample size 40 ml. Conditions same as in Figure 3. UV detection at 254 nm. 0.02 AUFS, Fluroescence detection at 8X.

CTABLE 4

SEP-PAK TRACE ENRICHMENT

STANDARD	AMOUNT TRACE ENRICHED (ng)	"% BREAKTHROUGH"
Benzanthrone	1000	0.9
	430	1.5
	10	not detectable
Fluoranthene	1468	0.7
	631	1.2
	15	9
Benzo(a)pyrene	1080	3-13
	464	15
	11	9
Benzo(ghi)perylene	1000	1.0
	430	1.1
	10	not detectable

region of 10%. That this compound would breakthrough more easily than the others is unusual since, based on relative retention times, it should have more affinity for the packing than would, say, benzanthrone. The authors have no explanation for this phenomenon as yet, but 90-100% recoveries have been observed in similar systems (3).

No work on environmental subjects would be complete without some study, albeit preliminary, of an actual environmental sample. Figure 10 shows the chromatogram obtained from 50 ml of stream water trace enriched across a μBondapak C-18 column. This water sample was collected in a glass bottle and brought back to the laboratory for analysis. Contrast this with the chromatogram obtained when the same stream was sampled on-site using a SEP-PAK, the material then being eluted with THF and chromatographed (Figure 11). It is here that another of the advantages of employing SEP-PAK can be clearly seen. Obviously compounds have been adsorbed on

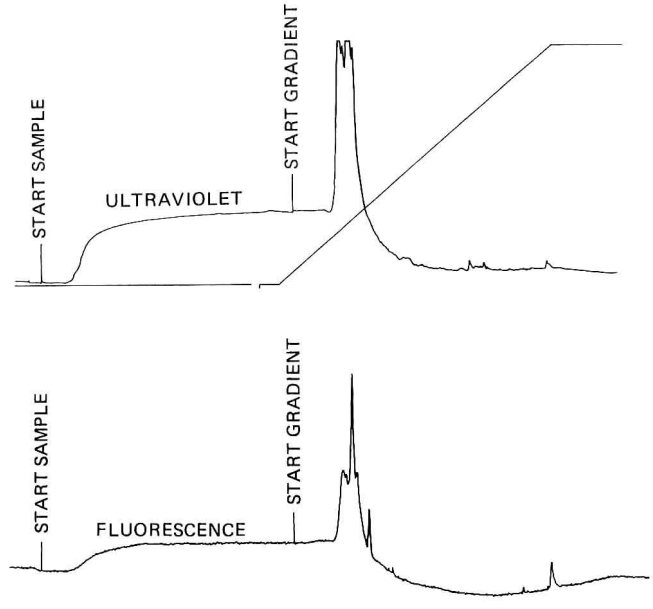

FIGURE 10 Stream water trace enrichment. Sample size 50 ml. Conditions same as in Figure 3. UV detection at 254 nm, 0.05 AUFS. Fluorescence detection at 8X.

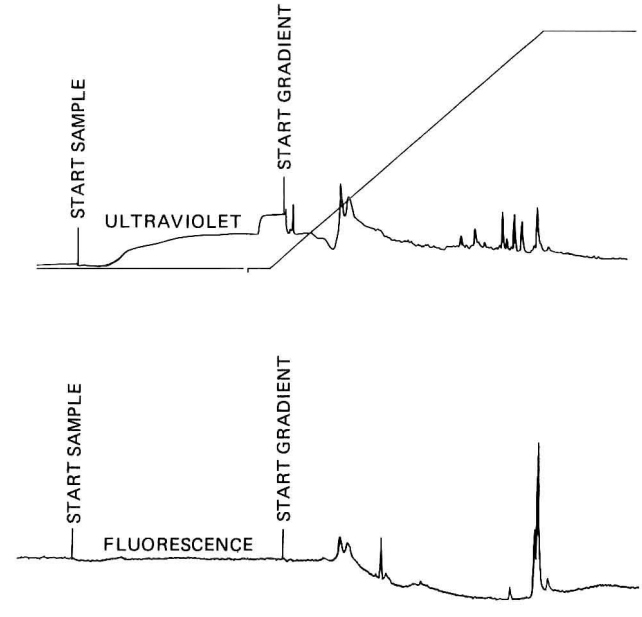

FIGURE 11 Stream water SEP-PAK extract. Conditions same as in Figure 10.

the glass giving an erroneously lower result fro the amount of trace organics in the stream.

Some suggestions to circumvent this problem that have been put forward (1) are pre-silanization of the glass vessels, use of teflon vessels or the addition of 20% methanol to the sample. Obviously none of these are as suitable or easy to use as the methods described above using the SEP-PAK.

The results show that the SEP-PAK is a simple efficient device for the on-site sampling, allowing the dual advantage of simultaneous collection and concentration. It thus reduces the cost of transportation of large water samples, reduces consumption of costly extration solvents, saves man-hours in sample manipulation (cleanups and concentrations) and avoids the serious problems of loss of trace components on the surface of glass collection vessels.

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STUDIES ON ENVIRONMENTAL POLLUTANTS: SELECTIVE SEPARATION AND RECOVERY OF Pb(II) ON FERRIC PHOSPHATE COLUMNS

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ABSTRACT

A new method is described for the recovery and selective separation of Pb(II). The following mixtures have been separated: Hg(II)-Pb(II), ZrO(II)-Pb(II), Cd(II)-Pb(II), Cu(II)-Pb(II), Mn(II)-Pb(II), Zn(II)-Pb(II), Sr(II)-Pb(II) and Ni(II)-Pb(II), on ferric phosphate columns. Cu(II), Cd(II), Ni(II), Hg(II), Mn(II), Zn(II), Sr(II) and ZrO(II) were eluted with 0.01M-NH_4NO_3 and Pb(II) with a mixture of 0.1M-HNO_3 + 1.0M-NH_4NO_3 solution. Proposed studies can be applied to pollution analysis and alloy analysis.

INTRODUCTION

Lead poisoning has long been known and has been exhaustively studied. Although fatal cases of plumbism are now relatively rare, lead and its compounds still constituted one of the most important industrial hazards. Serious lead intoxication is most frequently encountered in persons who have inhaled the vapours, fumes or dust of lead and lead containing compounds. For example, the process of sanding and spraying lead paints are extremely

hazardous with adequate protection. The allowable limits of lead in potable water (1) and air (2) as fixed by U.S. Public Health Service is 0.2-2 μg/1 and 0.15 mg./cubic meter of air respectively. The recovery and selective separation of lead in water and in other various biological samples is necessary with the view point of pollution studies. In continuation of our work on analytical applications of ion exchange materials (3-5), the present communication extends our studies to the application of ferric phosphate, an inorganic ion exchanger, for the selective separation and recovery of lead.

EXPERIMENTAL

Apparatus:

A glass column (i.d. 1.3cm) was used for column operation. 1.0g. of ferric phosphate (H^+-form) was taken in the column and flow rate was initially maintained at 1.5ml/minute.

For pH measurements an ELICO pH meter model LI-10 was used.

Reagents and Chemicals:

Ferric nitrate (BDH) and ammonium dihydrogen phosphate (BDH) were used. All other chemicals were of analytical grade.

Synthesis of Ferric phosphate:

Ferric phosphate was prepared by adding 0.1M aqueous solution of ammonium dihydrogen phosphate to 0.1M-ferric nitrate solution as described earlier(4). The dried product was converted into the H^+-form by treating with 0.1M nitric acid for 24h with ocassional shaking and intermittant changing of the acid. The product thus formed was dried at 40°C in a temperature controlled ovan.

Distribution Studies:

The distribution studies were carried out for 19 metal ions by batch process in the usual manner(4) after attaining equilibrium by shaking the metal ion solution with exchanger beads for 6 hours at room temperature($30^{+}-1^{\circ}C$). The distribution coefficients (K_d) for metal ions were calculated by using the following expression:

$$Kd = \frac{I-F}{F} \times \frac{20}{0.2}$$

where I is the volume of EDTA consumed by the original solution and F is the volume of EDTA consumed after equilibrium. The total volume of solution was 20 ml and the amount of exchanger used was 0.2g.

Elution behaviour:

From preliminary elution studies of Pb(II), Cu(II), Ni(II), Cd(II), Hg(II), Zn(II), Mn(II), Sr(II) and ZrO(II), it is observed that Pb(II) remains strongly adsorbed on ferric phosphate (H^{+}-form) bed in 0.01M-NH_4NO_3 where as Cu(II), Ni(II), Cd(II), Hg(II), Zn(II), Mn(II), Sr(II) and ZrO(II) percolate through the exchanger bed and quantitatively collected in the effluent. The flow rate is kept at 1.5ml/minute. After removal of Cu(II), Ni(II), Cd(II), Hg(II), Zn(II), Mn(II), Sr(II), and ZrO(II), Pb(II) can be eluted quantitatively with a mixture of 0.1M-HNO_3+1.0M-NH_4NO_3 (1:1) solution.

Procedure:

The ion exchanger column (H^{+}-form) was washed with demineralized water before passing the test solution. A known volume of test solution (or synthetic mixture) was taken accurately and was recycled thrice through the exchanger bed at the flow rate of 1.5ml/minute. The column was then washed with 0.01M-NH_4NO_3. Cu(II), Ni(II), Cd(II), Hg(II), Zn(II), Mn(II), Sr(II) and ZrO(II) were percolated out with the effluent and washing liquid, the adsorbed Pb(II) was then eluted quantitatively with a mixture of 0.1M-HNO_3 + 1.0M-NH_4NO_3 (1:1) solution.

RESULTS AND DISCUSSION

The distribution coefficients (K_d) of the cations are reported in TABLE-I. It is obvious from the results that the K_d values of Cu(II), Ni(II), Cd(II), Hg(II), Zn(II), Mn(II), Sr(II) and ZrO(II) are approximately ten times less than Pb(II) hence Pb(II) can be separated from these metals.

Recovery of Pb(II):

TABLE-II shows the successful recoveries of Pb(II) from solutions containing Cu(II), Ni(II), Cd(II), Hg(II), Zn(II), Mn(II), Sr(II) and ZrO(II). In the presence of five fold excess of these metal ions, all of them could be eluted with 0.01M-NH_4NO_3 except Pb(II). The retained Pb(II). The retained Pb(II) was easily eluted with a mixture of 0.1M-HNO_3 + 1.0M-NH_4NO_3 (1:1) solution. This shows the importance of ferric phosphate in the recovery of Pb(II).

Column Separation:

Unlike Pb(II) which was retained by the exchanger, Cu(II), Ni(II), Cd(II), Hg(II), Zn(II), Mn(II), Mn(II), Sr(II) and ZrO(II) passed out unsorbed. Quantitative separations of these metal ions from Pb(II) have been successfully achieved. TABLE-III shows a list of separations successfully achieved on a column of ferric phosphate. The order and the eluents are presented in Figures 1-8.

The minimum number of theoretical plates(N) necessary for separation as tabulated in TABLE-III were calculated using equation

$$N \geqslant 2\pi \left[\frac{\frac{K_{d,Pb} + a}{K_{d,M} + a} + 1}{\frac{K_{d,Pb} + a}{K_{d,M} + a} - 1} \right]^2$$

where $K_{d,Pb}$ and $K_{d,M}$ are distribution coefficients of lead

TAPLE-I

Distribution coefficients of Metal Ions on Ferric Phosphate in Water (pH≅ 5-6)

Metal ions	K_d	Metal ions	K_d	Metal ions	K_d
Mg(II)	18.2	Ca(II)	14.3	Cd(II)	12.5
Zn(II)	11.3	Sr(II)	9.0	Hg(II)	75.0
Mn(II)	9.8	Ba(II)	20.8	Nd(III)	46.4
Ni(II)	15.5	Cu(II)	6.6	Vo(II)	52.0
Co(II)	53.0	Pb(II)	130.0	Al(III)	100.0
ZrO(II)	9.8	La(III)	52.2	Cr(III)	54.6
Th(IV)	T.A.				

Note: T.A. = Totally Adsorbed.

TABLE-II

Recovery of Pb(II) From Solutions Containing 5,000µg Each of Cu(II), Ni(II), Cd(II), Hg(II), Zn(II), Mn(II), Sr(II) and ZrO(II)

Recovery of	Eluents	Volume of effluent(ml)	Amount of Pb(II) loaded(µg)	Amount of Pb(II) Recovered(µg)	Error%
Pb(II) from Impurities	Impurities-0.01M-NH_4NO_3	50	-	-	-
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	10	1,000	1,000	0.0
Pb(II) from Impurities	Impurities-0.01M-NH_4NO_3	60	-	-	-
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	45	10,000	10,000	0.0

TABLE III

Separation of Pb(II) From the Solutions Containing Cu(II), Ni(II) Cd(II) Hg(II), Zn(II) Mn(II), Sr(II) and ZrO(II) on Ferric Phosphate (H^+-Form) Columns.

Separation	Eluents	Vol. of Effluent (ml)	Amount of cation Loaded (µg)	Amount of cation Recovered (µg)	Error %	N[a]	h[b]
Cu(II)-Pb(II)	Cu(II)-0.01M-NH_4NO_3	35	5883	5851	-0.54	7.95	1.01
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	50	7251	7251	0.0		
Ni(II)-Pb(II)	Ni(II)-0.01M-NH_4NO_3	20	2348	2348	0.0	10.20	1.01
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	50	7251	7251	0.0		
Zn(II)-Pb(II)	Zn(II)-0.01M-NH_4NO_3	30	5393	5393	0.0	9.0	0.253
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	50	7251	7251	0.0		
Cd(II)-Pb(II)	Cd(II)-0.01M-NH_4NO_3	25	4720	4777	+1.10	9.30	1.01
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	50	7251	7251	0.0		
Hg(II)-Pb(II)	Hg(II)-0.01M-NH_4NO_3	25	10,932	10,932	0.0	86.0	1.01
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	50	7251	7251	0.0		
Mn(II)-Pb(II)	Mn(II)-0.01M-NH_4NO_3	30	1705	1705	0.0	8.60	1.01
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	50	7251	7251	0.0		
Sr(II)-Pb(II)	Sr(II)-0.01M-NH_4NO_3	25	4381	4381	0.0	8.30	1.01
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	50	7251	7251	0.0		
ZrO(II)-Pb(II)	ZrO(II)-0.01M-NH_4NO_3	25	2144	2144	0.0	8.60	1.01
	Pb(II)-0.1M-HNO_3+1.0M-NH_4NO_3	50	7251	7251	0.0		

Note: a = Minimum number of Theoretical plates.
b = Height equivalent to one theoretical plate.

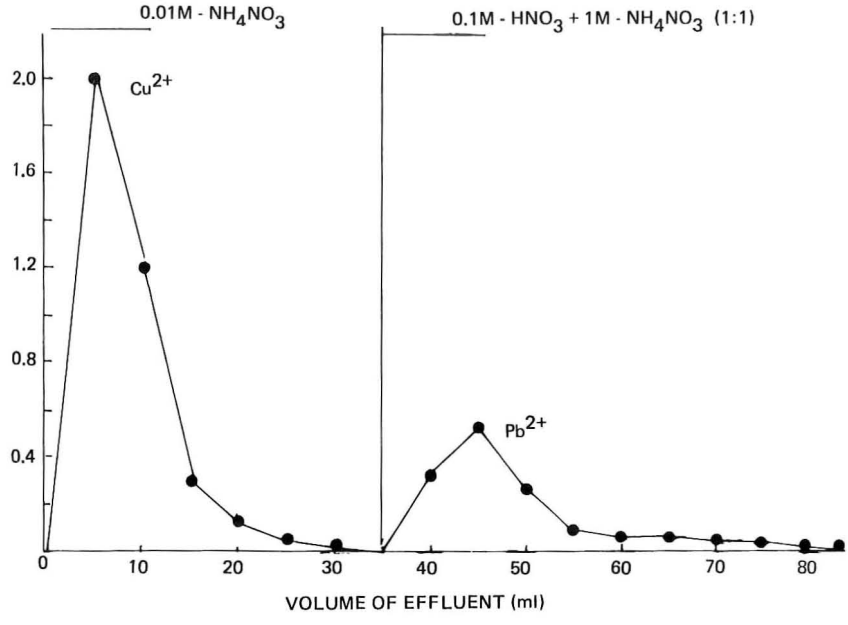

FIG. 1 SEPARATION OF Cu^{2+} FROM Pb^{2+}

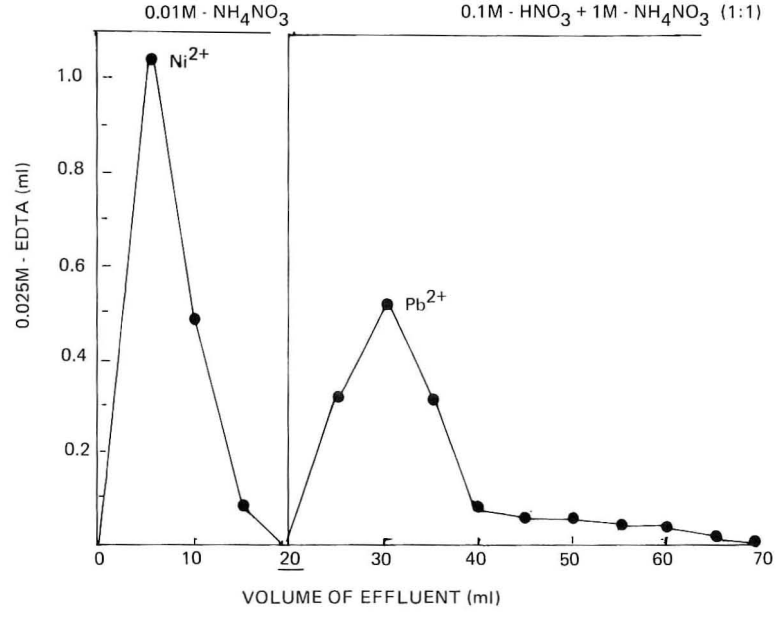

FIG. 2 SEPARATION OF Ni^{2+} FROM Pb^{2+}

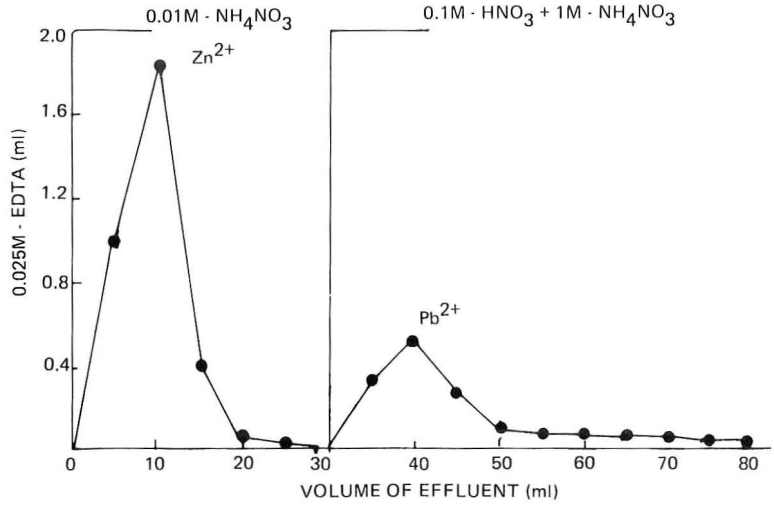

FIG. 3 SEPARATION OF Zn^{2+} FROM Pb^{2+}

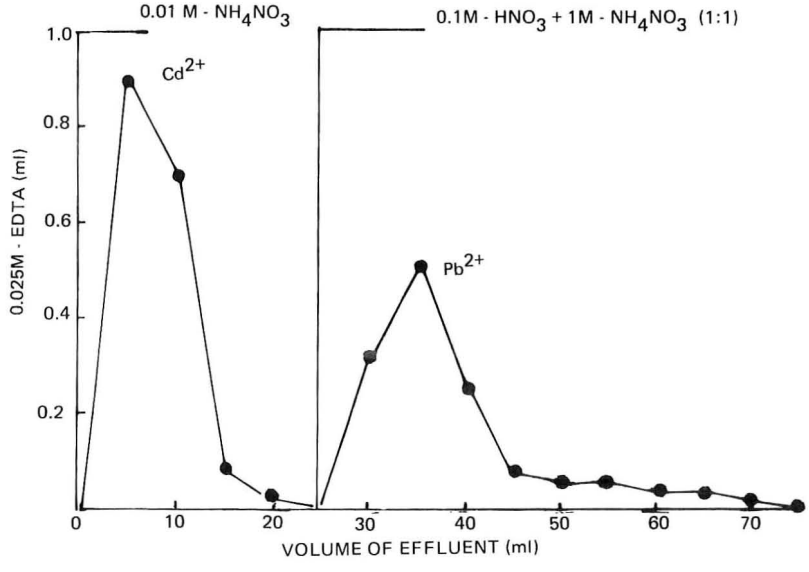

FIG. 4 SEPARATION OF Cd^{2+} FROM Pb^{2+}

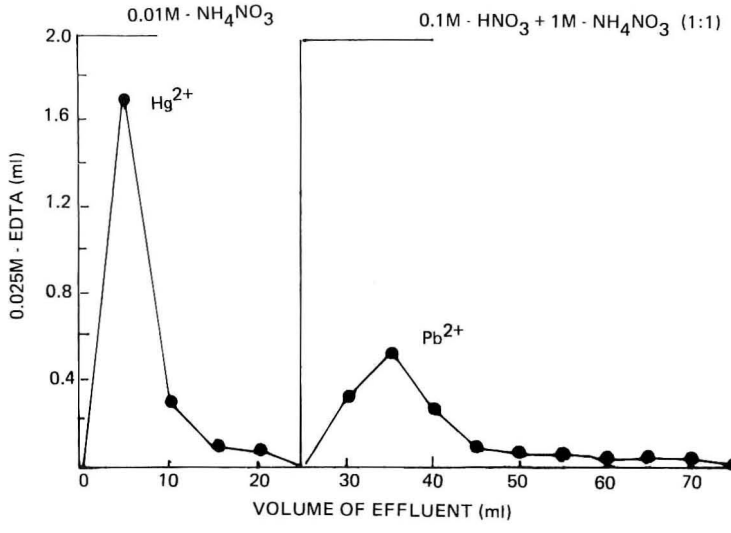

FIG. 5 SEPARATION OF Hg^{2+} FROM Pb^{2+}

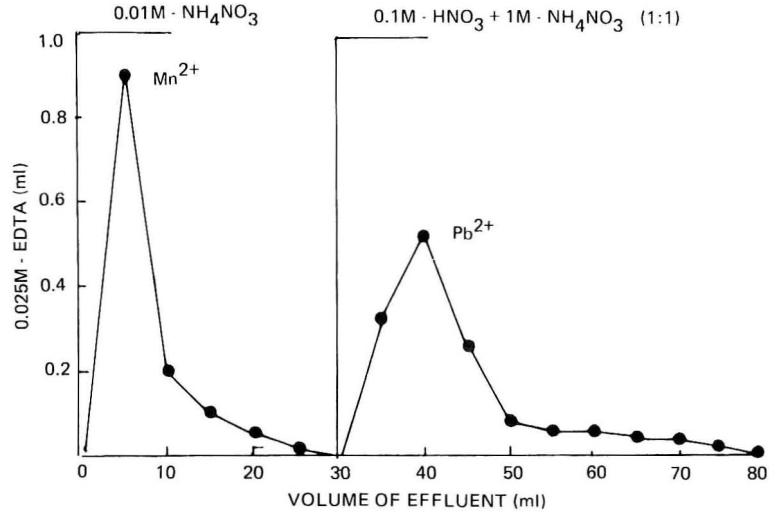

FIG. 6 SEPARATION OF Mn^{2+} FROM Pb^{2+}

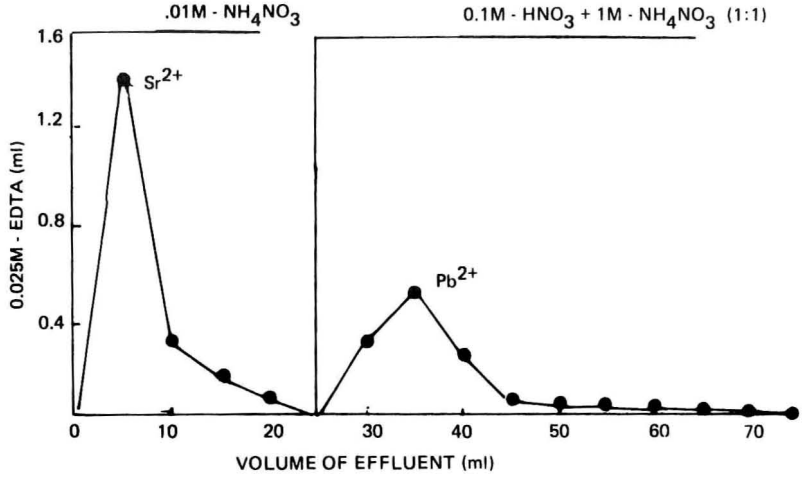

FIG. 7 SEPARATION OF Sr^{2+} FROM Pb^{2+}

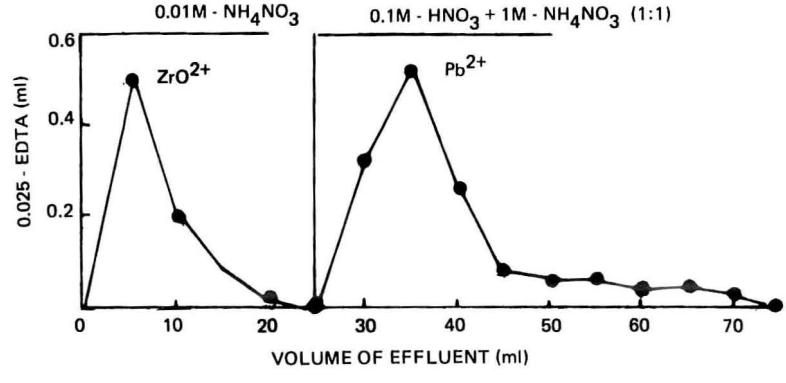

FIG. 8 SEPARATION OF ZrO^{2+} FROM Pb^{2+}

and the other metal separated respectively. a is the Void fraction (~0.4). The values of height equivalent to one theoretical plate (h) as reported in, TABLE-III, can be determined from the data obtained from an elution curve

$$h = \frac{L\, b^2}{8\, v_{max}^2}$$

where L is the length of the ion exchange column (cm), v_{max} is the eluent volume at peak (ml) and b is the peak width (ml) at a height of 0.368 C_{max}.

Application to Pollution Studies:

The main sources of lead pollution are the industries manufacturing storage batteries, cable covering, paints and gasoline. Because Hg(II), Cu(II), Zn(II), Cd(II) and Ni(II) that react with dilhizone are usually present in polluted samples. Hence, the proposed method of selective separation of Pb(II) followed by determination of lead by dilhizone is the better method compared to other time consuming methods.

Application of Analysis of non-ferrous Alloys:

Lead is present as a major constituent of the following alloys: white metal bearing alloys, solders, type metals, leaded brasses and bronzes, various zinc-base die casting alloys and many others. The method of decomposition applied depends, of course, on the composition of the alloy to be analyzed. For this reason, no universally applicable procedure can be cited. Usually gravimetric methods are used for the analysis of alloys but these methods are time consuming. The proposed method is simple and rapid for the estimation of lead in various alloys as these alloys contain Cu,Ni,Cd,Zn,Mn as main constituents that can be selectively separated from Pb(II).

This method can also be used for the concentration of traces of lead in food products e.g. tea, coffee, sugar and butter. The final determination is made with dilhizone spectrophotometrically.

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LC NEWS

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November 19-20 — "1981 International Chromatography Conference", Carillon Hotel, Miami Beach, Florida, USA. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7.

1982

March 28-April 2 — "National American Chem. Soc. Meeting", Las Vegas, NV USA. Contact: A. T. Winstead, Am. Chem. Sco., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

April 14-16 — "12th Annual Symposium on the Anal. Chem. of Pollutants", Amsterdam, The Netherlands. Contact: Prof. R. W. Frei, Congress Office, Vrije Universiteit, P. O. Box 7161, 1007-MC Amsterdam, The Netherlands.

June 28-30 — "Analytical Summer Symposium", Michigan State Univ., East Lansing, MI USA. Contact: A. I. Popov, Chem. Dept., Michigan State Univ., East Lansing, MI 48824, USA.

July 12-16 — "2nd Int'l Symposium on Macromolecules", - IUPAC, University of Massachusetts, Amherst, Massachusetts, USA.

August 15-21 — "12th Int'l Congress of Biochemistry", Perth, Western Australia. Contact: Brian Thorpe, Dept. of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.

September 12-17 — "National American Chem. Soc. Meeting", Kansas City, MO USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

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March 20-25 — "National American Chem. Soc. Meeting", Seattle, WA USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

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