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ION-PAIR CHROMATOGRAPHY IN THE LOW CONCENTRATION RANGE BY USE OF HIGHLY ABSORBING COUNTER IONS

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SUMMARY

High-performance systems for ion-pair chromatography that permit the detection of down to 7 pmole (about 1 ng) of non-UV-absorbing samples have been constructed, the high detector response being obtained by using a stationary phase containing a counter ion of high absorbance.

Amino acids, dipeptides and alkylamines are separated with naphthalene-2-sulphonate as counter ion. The systems have a high selectivity, and a change of the amino acid sequence if often sufficient for a complete chromatographic resolution of dipeptides.

A comparison with batch extraction data showed that the retention of the cations, except the most hydrophobic, is due mainly to liquid–liquid distribution.

The naphthalene-2-sulphonate systems have a very high stability under careful thermostatted conditions.

Systems for the separation of anionic compounds with highly absorbing quaternary ammonium ions in the stationary phase are being investigated.

INTRODUCTION

The isolation of nanogram amounts of organic compounds from complex samples is of rapidly increasing importance in many fields such as biochemical and pharmacological investigations and studies of pollution and occupational exposure. Liquid–liquid chromatography based on ion-pair distribution, *i.e.*, ion-pair chromatography, is a valuable technique in such studies owing to its high selectivity and high sample capacity[1,2].

By ion-pair distribution of an ion, Q^+, a counter ion, X^-, is added to the aqueous phase and the ion pair, QX, is extracted into an organic phase:

$$Q^+_{aq} + X^-_{aq} = QX_{org}$$

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The distribution system is highly versatile and can easily be adapted to different kinds of samples by changing the nature and the concentration of the counter ion.

One of the major advantages of ion-pair chromatography is that a considerable increase in the detector response can be obtained by a proper choice of the counter ion. The photometric detectors used in liquid chromatography have a very high sensitivity and the ion-pair chromatographic technique offers excellent possibilities for a high detector response by using a counter ion of high molar absorptivity at the measuring wavelength. The principle has been applied for determining small amounts of acetylcholine and alkylammonium ions with picrate and aromatic sulphonates as counter ions[3–5]. For anions of carboxylic acids, quaternized cations containing highly conjugated ring systems have been used as counter ions[6].

The amplification procedures mentioned above were used in systems with cellulose as the support for the stationary phase containing the highly absorbing counter ion.

In this paper, methods are given for the preparation of high-performance liquid chromatographic (HPLC) systems containing highly absorbing counter ions that permit the detection and determination of a few picomoles of substances with a low or no absorbance such as dipeptides, amino acids, alkylamines and sulphonates. The stability, separating efficiency and selectivity of the systems is demonstrated.

EXPERIMENTAL

Apparatus

The detector was a Model 440 UV monitor (Waters Assoc.) with a 12.5-μl cell, or a Model 1203 UV III monitor (Laboratory Data Control) with an 8-μl cell. The measuring wavelength was 254 nm in both instances. The pump was a Laboratory Data Control Model 711-26 solvent delivery system comprising a Milton-Roy Minipump and a pulse dampener. The injector was a high-pressure valve from Valco Instruments.

The columns were made of 316 stainless steel with a polished surface, length 150 mm, I.D. 4.5 mm, equipped with modified Swagelok connectors and Varian stainless-steel frits (2 μm). Punched PTFE filters, Mitex Type LS (5 μm) from Millipore, were fitted on both sides of the frits.

The photometric measurements in the partition studies were performed with a Zeiss PMQ II Spektralphotometer. The pH measurements were made with an Orion Research Model 801 digital pH meter equipped with an Ingold Type 401 combined electrode.

Chemicals and reagents

LiChrospher SI-100 (10 μm), obtained from E. Merck (Darmstadt, G.F.R.), was used as the chromatographic support.

1-Pentanol was of Fischer Scientific (Pittsburgh, Pa., U.S.A.) A.C.S. quality. Only batches with a residue after evaporation of 0.001% and Color 10 were used. Chloroform was of pro analysi quality from Merck.

Naphthalene-2-sulphonic acid, sodium salt, was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). Aqueous solutions, used as stationary phases,

were purified by repeated extraction with chloroform–1-pentanol (9:1) until the extract had a constant absorbance.

N,N-Dimethylprotriptyline bromide was synthesized from protriptyline according to Borg and transferred to phosphate and purified according to Lagerström[6].

The amines and the amino acids were mainly obtained from Fluka (Buchs, Switzerland). The amines were distilled and converted into chlorides before use. The dipeptides were obtained from Sigma (St. Louis, Mo., U.S.A.) and Serva (Heidelberg, G.F.R.).

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

The buffers that were prepared from phosphoric acid and sodium phosphates had an ionic strength of 0.1.

Column packing

The columns were packed by a balanced-density slurry technique with tetrachloroethane as the suspending medium. After packing, *n*-hexane was passed through the column to remove the suspending liquid.

The columns were first tested in the adsorption mode with *n*-hexane–1-butanol (199:1) as the mobile phase. The interstitial volume was determined with toluene, which is unretained in this system. Determinations made on several column gave a porosity of 0.77 ± 0.01. The efficiency was tested with some retained samples (1,4-dinitrobenzene and 2,4-dinitrotoluene).

Column coating

Two different coating techniques were used. In both instances the columns were eluted with acetone before applying the aqueous phase on the support. The whole procedure was usually carried out at a flow-rate of 1.0 ml/min, *i.e.*, the same rate as that used in the analytical experiments. The flow-rate was reduced when the pressure tended to increase above 10 MPa.

(1) Injection technique. Mobile phase (chloroform–1-pentanol, 5–15%, not equilibrated with the stationary phase) was pumped through the column. The stationary phase was introduced in 10–30-μl portions with intervals of 1–2 min between the injections. The application of stationary phase was stopped as soon as water droplets appeared in the detector. A change to mobile phase, equilibrated with stationary phase, was then made and recycling of the mobile phase started.

By change of the coating, the column was eluted with ethanol followed by phosphate buffer (pH 2), water and finally acetone. The column was then re-equilibrated with mobile phase and coated with the new stationary phase as given above.

(2) Pumping technique. The detector was disconnected and 50 ml of stationary phase were pumped through the column, followed directly by mobile phase, saturated with stationary phase. When no droplets of water could be observed in the eluate, the detector was connected and recycling of the mobile phase started.

By change of coating, the column was eluted with ethanol followed by phosphate buffer (pH 2). The application of the new stationary phase was then started.

The columns were ready for use when retained samples showed stable capacity ratios and a high separating efficiency ($H < 0.1$ mm at a mobile phase flow-rate of 2.5 mm/sec). In order to ensure stable conditions during the recycling, the reservoir

should contain about 500 ml of mobile phase and a layer of stationary phase should always be present.

Chromatographic technique

The samples were injected in uncharged form or as ion pairs, dissolved in the mobile phase. The sample volume was 10–30 μl.

The mobile and stationary phase were equilibrated with each other at the experimental temperature (25.0°). No pre-column was used.

The mobile phase, the reservoir, the injector and the column were thermostated in a water-bath at 25.0 $\pm <$ 0.1°. To ensure the thermostating of the mobile phase, the injector was preceded by a tube of length 1 m and volume 3 ml placed in the bath. All tubing outside the bath was well insulated. The detector cell was kept at a temperature of about 35°. Cooling of the pump-head was also necessary. The best results were obtained by using a modified pump-head with channels fed with water at 22°.

Determination of constants

Constants for ion-pair extraction, including side-reactions, were determined by the partition technique. The partition experiments were performed in centrifuge tubes with equal phase volumes using mechanical shaking in a thermostated water-bath at 25.0 $\pm$ 0.1°. The equilibration time was 30 min (*cf.*, ref. 3).

One of the ion-pair components was usually present in large excess, and the concentration of the other was measured by photometry in the aqueous phase. The concentration of the ion pair in the organic phase was obtained as the difference between the total and the aqueous concentration of the ion measured.

The calculation of the constants was made by slope analysis[7]. Constants for ion-pair formation in the aqueous phase and ion-pair dimerization in the organic phase were calculated according to the principles given by Modin and Schill[8].

RESULTS AND DISCUSSION

The ion-pair extraction principle can be used in partition chromatography both in reversed- and straight-phase systems, but an amplification of the detector response can be obtained only when the substrate migrates as ion pair with an organic mobile phase.

A quantitative expression for the distribution of an ion pair QX is given by the extraction constant, E_{QX}, which is defined as

$$E_{QX} = [QX]_{org} \cdot [Q^+]_{aq}^{-1} \cdot [X^-]_{aq}^{-1} \quad (1)$$

The distribution of Q^+ between the two phases is given by the expression for the distribution ratio, D_Q:

$$D_Q = [QX]_{org} \cdot [Q^+]_{aq}^{-1} = E_{QX} \cdot [X^-]_{aq} \quad (2)$$

Control of the retention

In a system where X^- is present in the aqueous stationary phase, the capacity ratio of the sample Q^+, k'_Q, is given by

$$k'_Q = (E_{QX} \cdot [X^-]_{aq})^{-1} \cdot V_s \cdot V_m^{-1} \quad (3)$$

where $V_s \cdot V_m^{-1}$ is the phase volume ratio.

The magnitude of k'_Q can be regulated to some extent by the concentration of X^-, but the range is limited by the fact that a very low concentration will give an unstable chromatographic system while a high concentration might give rise to disturbing side-reactions (*cf.*, ref. 9).

The capacity ratio can, of course, be varied by changing the nature of the counter ion, which can change the extraction constant within wide limits[10]. However, when the counter ion is also used to increase the detector response, the choice is limited and the regulation of E_{QX} is mainly effected by altering the composition of the organic phase. It is then often suitable to use an organic phase that consists of one weakly and one strongly solvating agent (*e.g.*, a strongly hydrogen-bonding solvent), as this will give simple means for the systematic regulation of E_{QX} and k' (ref. 11).

Extraction constants

Batch extraction studies were made on naphthalene-2-sulphonate (NS) ion pairs of primary, secondary and tertiary amines of different hydrophobic character. Constants for extraction, ion-pair formation in the aqueous phase and ion-pair dimerization in the organic phase are given in Table I.

With the organic phase used (chloroform plus 5 or 10% of 1-pentanol) there are small differences in the extraction constants between tertiary, secondary and primary amines with straight alkyl chains (*cf.*, *n*-hexylamine, di-*n*-propylamine and triethylamine as well as *n*-octylamine and di-*n*-butylamine). With chloroform as the organic phase, alkylamines with the same number of carbon atoms but different degrees of substitution at the nitrogen atom usually have considerably different extraction constants, with a decrease in the order tertiary > secondary > primary[12]. Addition of 5–10% of pentanol has a drastic effect and the order of the constants is reversed.

Branching of the alkyl chain also gives a significant decrease in the extraction constant, as illustrated by the series di-*n*-propylamine–diisopropylamine and *n*-octylamine–1-methylheptylamine–*tert.*-octylamine.

The constants for ion-pair formation in the aqueous phase as well as the constants for ion-pair dimerization in the organic phase are rather low.

Columns with naphthalene-2-sulphonate as counter ion

The chromatographic studies on amino acids, peptides and alkylamines were performed with naphthalene-2-sulphonate (NS) as counter ion in the stationary phase. Ion pairs between NS and alkylamines have a high molar absorptivity (about 1400 at 254 nm[13]). NS is almost aprotic and can be used as a counter ion at a low pH ($\pm$ 2) where the distribution of the amines and the amino acids in non-charged form is negligible in most instances. NS has been the subject of thorough liquid–

TABLE I

EXTRACTION CONSTANTS OF ION PAIRS BETWEEN ALKYLAMINES AND NAPHTHALENE-SULPHONATE

Organic phase: chloroform–1-pentanol. NS (X^-) measured in the aqueous phase at 275 mn[14]. Aqueous phas phosphate buffer (pH 2.2).

Cationic component (HA^+)	*Pentanol (% v/v)*	$C_X \cdot 10^5$	$C_{HA} \cdot 10^2$	$C_{HAXorg} \cdot 10^5$	*Log* E_{HAX}	*Log* k_a*	*Log* $k_{2(HAX)}$**
n-Butylamine***	10				0.47	—	§§
n-Pentylamine***	5				0.47	—	2.6
n-Hexylamine***	5				0.92	0.5	2.9
Di-*n*-propylamine	5	2.70–11.8	8.10–29.4	2.30–13.2	0.92	0.4	§§§
Diisopropylamine***	5				0.66	0.5	§§
Diisopropylamine***	10				1.21	—	§§
Triethylamine	5	6.14–11.8	4.32–13.0	3.05–4.83	0.85	0.6	§§§
n-Octylamine (OA)	5	2.20–10.0	0.33– 3.35	5.30–15.3	2.36	§	§§§
1-Methylheptylamine	5	2.39–30.8	0.22– 0.68	0.94– 3.59	2.24	§	§§§
tert.-Octylamine (*tert.*-OA)	5	7.07-32.1	0.41– 1.21	1.79– 5.70	1.71	§	§§§
Di-*n*-butylamine (DBA)	5	2.41–12.0	0.25– 0.75	1.59– 8.00	2.12	§	§§§
N-Methyloctylamine (MOA)	5	2.20– 8.80	0.17– 0.36	7.10–41.1	3.15	§	§§§
N,N-Dimethyloctylamine (DMOA)	5	2.01–28.8	0.01– 0.06	2.21–15.0	3.72	§	§§§

* $k_a = [HAX]_{aq} \cdot [HA^+]^{-1} \cdot [X^-]^{-1}$.
** $k_{2(HAX)} = [H_2A_2X_2]_{org} \cdot [HAX]_{org}^{-2}$.
*** Constants from ref. 5.
§ Log $k_a < 2$.
§§ Log $k_{2(HAX)} < 2$.
§§§ Log $k_{2(HAX)} < 3.7$.

liquid distribution studies and its association equilibria in both aqueous and organic phase are well known[14]. Its solubility in water is good.

Conditioning and stability. The columns with NS as the stationary phase showed some changes in properties during the first few hours of recycling that followed the coating. Fig. 1 gives the capacity ratios of five alkylamines during the passage of about 4000 ml of mobile phase (corresponding to a running time of 67 h).

The extent and the rapidity of the change of the capacity ratios were not the same for all of the amines, the more hydrophobic OA, MOA and DMOA giving a larger and more rapid change than the less hydrophobic *tert.*-OA and DBA.

For the first group of amines (OA, MOA and DMOA) there was also a decrease in the plate height, H, during the conditioning (Fig. 2). In the latter group (*tert.*-OA and DBA), H was low at the beginning of the equilibration and only small changes were observed.

A change to a new batch of mobile phase was usually followed by a certain increase in both k' and H for a short period of time. No change in the interstitial volume was observed during the conditioning period.

Columns prepared by the pumping and by the injection techniques showed the same behaviour. Stable capacity ratios and high efficiency were usually obtained after the passage of about 500 ml of mobile phase. No further changes were observed for a long period of time (more than a month).

The content of stationary phase was always 1.0 ml per gram of support,

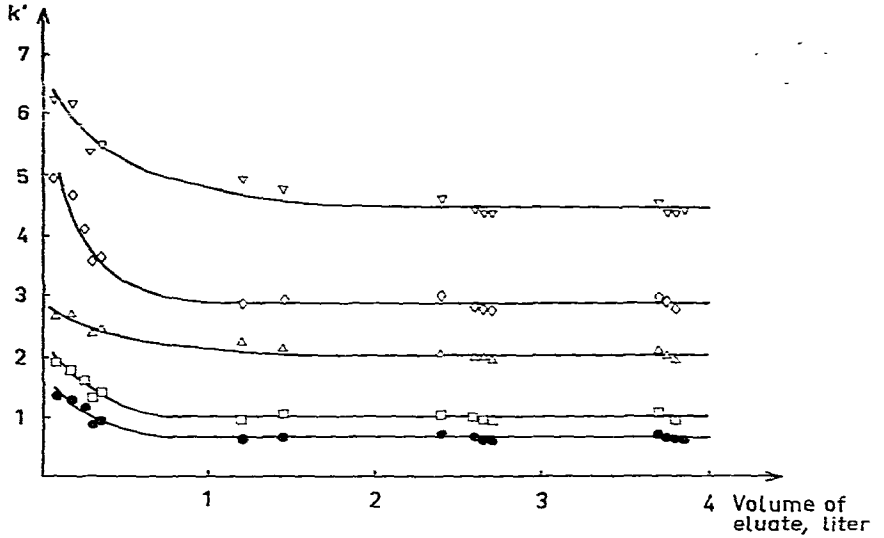


Fig. 1. Capacity ratio during the conditioning of naphthalene-2-sulphonate columns. Mobile phase: chloroform–1-pentanol (19:1); 2.5 mm/sec; 4.5 MPa. Stationary phase: naphthalene-2-sulphonate 0.01 *M*, pH 2.1. Support: LiChrospher SI-100 (10 μm). Samples: ● = N,N-dimethyloctylamine; □ = N-methyloctylamine; △ = di-*n*-butylamine; ◇ = *n*-octylamine; ▽ = *tert.*-octylamine.

calculated from the difference in interstitial volume before and after the coating. This corresponds to a porosity ($\varepsilon_{mt} = V_{mi}/V_0$, where V_0 is the volume of the empty column) of 0.38. This is close to the calculated interparticle porosity of 0.4 (ref. 15) which is considered to be equal to ε_m when the pores are completely filled with stationary phase. Further injections of stationary phase can give a higher loading, but such columns are not stable, and stationary phase is stripped off until the stable level is reached.

Retention. The amino acids, the peptides and the more hydrophilic amines

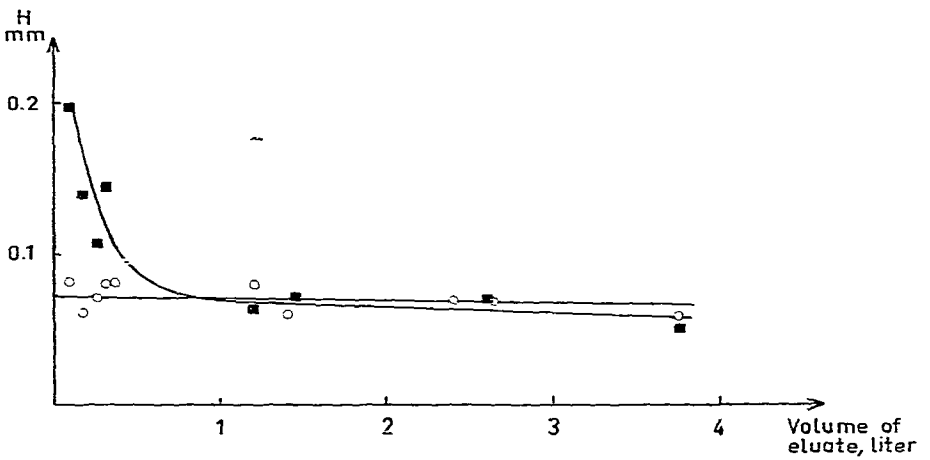


Fig. 2. Separating efficiency during the conditioning of naphthalene-2-sulphonate columns. Conditions as in Fig. 1. Samples: ■ = N-methyloctylamine; ○ = *tert.*-octylamine.

needed an NS concentration of 0.1 *M* for a suitable retention to be obtained. The found capacity ratios, k'_f, are given in Tables II and III.

A comparison of k'_f with capacity ratios calculated from the known equilibrium constants, k'_c, is made for the amines. It should be noted that NS at this concentration is tetramerized to some extent in the aqueous phase[14]. Ion-pair formation in the aqueous phase must also be taken into account. The agreement between the found and the calculated capacity ratios, as reflected by the ratio k'_f/k'_c, is very good in most instances.

The more hydrophobic amines were run with a stationary phase of 0.01 *M* NS and the results are given in Table II. In this instance, no side-reactions have any significant influence on k'_c. The agreement between the found and the calculated capacity ratios is fairly good, when the alkyl chain contains six carbon atoms or less, k'_f/k'_c being of the order of 1–2. Above this level, the deviation between the found and the calculated capacity ratios increases drastically. The deviation is correlated with the extraction constant, but the strong difference in chromatographic behaviour between DBA and OA seems more dependent on the increase in the chain length from four to eight carbon atoms than on the rather small difference in extraction constants (about 0.2 log units).

It is highly likely that ion-pair distribution is not the predominant factor for the retention of the most hydrophobic amines.

TABLE II

CHROMATOGRAPHIC BEHAVIOUR OF ALKYLAMINES WITH NAPHTHALENE-2-SULPHONATE (NS) AS COUNTER ION IN THE STATIONARY PHASE

Mobile phase: chloroform–1-pentanol; 2.5–2.8 mm/sec; 3.8–4.6 MPa. Stationary phase: NS in phosphate buffer (pH 2.2). Support: LiChrospher SI-100 (10 μm).
Sample: amount, 10–80 ng; volume, 10–30 μl; V_s/V_m, 1.01. H = 0.06–0.10 mm. Asymmetry factor*: 0.8–1.8.

Cationic component	*NS concentration (M)*	*Pentanol (%)*	k'_f	k'_f/k'_c
n-Hexylamine	0.1	5	2.4	0.9
Di-*n*-propylamine	0.1	5	2.9	1.1
Diisopropylamine	0.1	5	5.5	1.1
n-Pentylamine	0.1	5	7.4	0.9
Triethylamine	0.1	5	7.5	2.2
n-Hexylamine	0.1	10	0.6	
n-Pentylamine	0.1	10	2.5	
Diisopropylamine	0.1	10	2.8	2.0
Triethylamine	0.1	10	5.3	
n-Butylamine	0.1	10	6.5	1.0
Isobutylamine	0.1	10	7.9	
tert-Butylamine	0.1	10	10.6	
N,N-Dimethyloctylamine (DMOA)	0.01	5	0.6	30.0
N-Methyloctylamine (MOA)	0.01	5	1.0	14.3
Di-*n*-butylamine (DBA)	0.01	5	2.0	2.6
1-Methylheptylamine	0.01	5	2.1	3.5
n-Octylamine (OA)	0.01	5	2.8	6.2
tert.-Octylamine (*tert*.-OA)	0.01	5	4.3	2.2
n-Hexylamine	0.01	5	12.1	1.0

* Back part of peak base/front part of peak base.

TABLE III

CHROMATOGRAPHIC BEHAVIOUR (k'_f VALUES) OF AMINO ACIDS AND DIPEPTIDES WITH NAPHTHALENE-2-SULPHONATE (NS) AS COUNTER ION IN THE STATIONARY PHASE

Stationary phase: NS (0.1 *M*) in phosphate buffer (pH 2.2). Sample: amount, 50–100 ng; volume, 10–30 µl. Other conditions as in Table II. H = 0.05–0.10 mm. Asymmetry factor: 1.2–1.5.

Cationic component	*Pentanol content of mobile phase (%)*		
	5	*10*	*15*
8-Aminocaprylic acid	—	8.1	3.3
Phenylalanine	—	13.5	5.7
Leucine	—	—	8.6
Tranexamic acid	—	35.7	13.2
6-Aminocaproic acid	—	—	32.7
Phenylalanylphenylalanine	4.5	0.6	
Phenylalanylleucine	5.6	0.8	
Leucylphenylalanine	7.1	1.0	
Leucylleucine	—	1.2	0.5
Methionylleucine	—	1.8	0.7
Phenylalanylvaline	—	1.9	0.8
Leucylmethionine	—	2.2	0.9
Valylphenylalanine	—	2.5	1.1
Leucylvaline	—	3.4	1.4
Valylleucine	—	3.9	1.5
Phenylalanylproline	—	4.6	1.9
Methionylvaline	—	5.3	2.2
Leucylproline	—	9.6	4.0
Phenylalanylalanine	—	—	4.4
Leucylalanine	—	—	8.9

TABLE IV

EFFECT OF SAMPLE CONCENTRATION ON CHROMATOGRAPHIC PROPERTIES

Mobile phase: chloroform–1-pentanol (19:1), 2.5 mm/sec, 4.5 MPa. Stationary phase: naphthalene-2-sulphonate (0.01 *M*) in phosphate buffer (pH 2.1). Support: LiChrospher SI-100 (10 µm). Sample volume: 10 µl.

Sample	*Sample concentration* $\times 10^6$ *(mole/l)*	k'_f	H *(mm)*	*Asymmetry factor*
N,N-Dimethyloctylamine	6.5	0.6	0.06	1.6
	13	0.6	0.09	1.7
	26	0.6	0.09	2.0
	65	0.6	0.09	2.5
	130	0.7	0.08	2.4
Di-*n*-butylamine	12.5	2.0	0.11	1.3
	25	2.0	0.12	1.1
	50	2.0	0.11	1.5
	125	1.9	0.12	2.0
	250	2.1	0.13	2.1
tert.-Octylamine	22.5	4.4	0.06	1.2
	45	4.4	0.09	1.6
	90	4.3	0.12	2.3
	225	4.2	0.17	3.3
	450	4.4	0.23	3.6

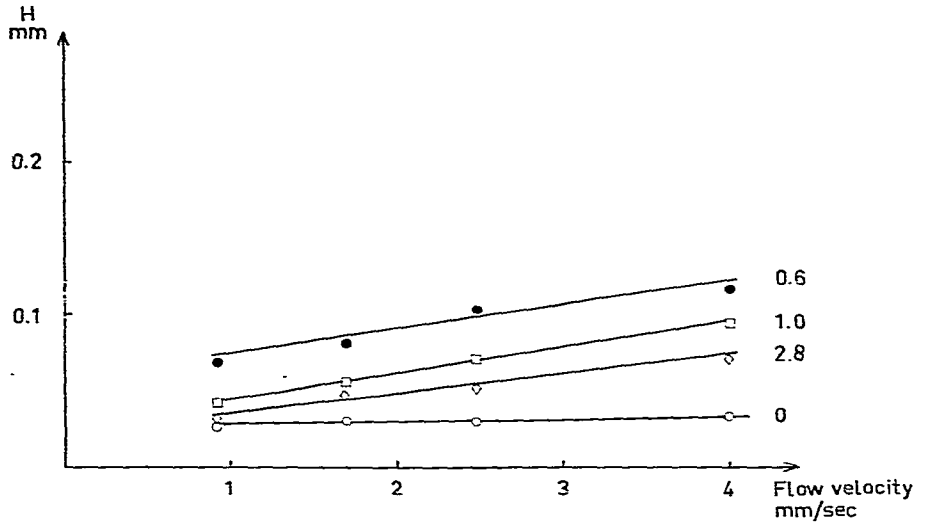


Fig. 3. Influence of mobile phase flow-rate and capacity ratio on the column efficiency. Conditions as in Fig. 1. Samples: ○ = toluene; ◇ = *n*-octylamine; □ = N-methyloctylamine; ● = N,N-dimethyloctylamine.

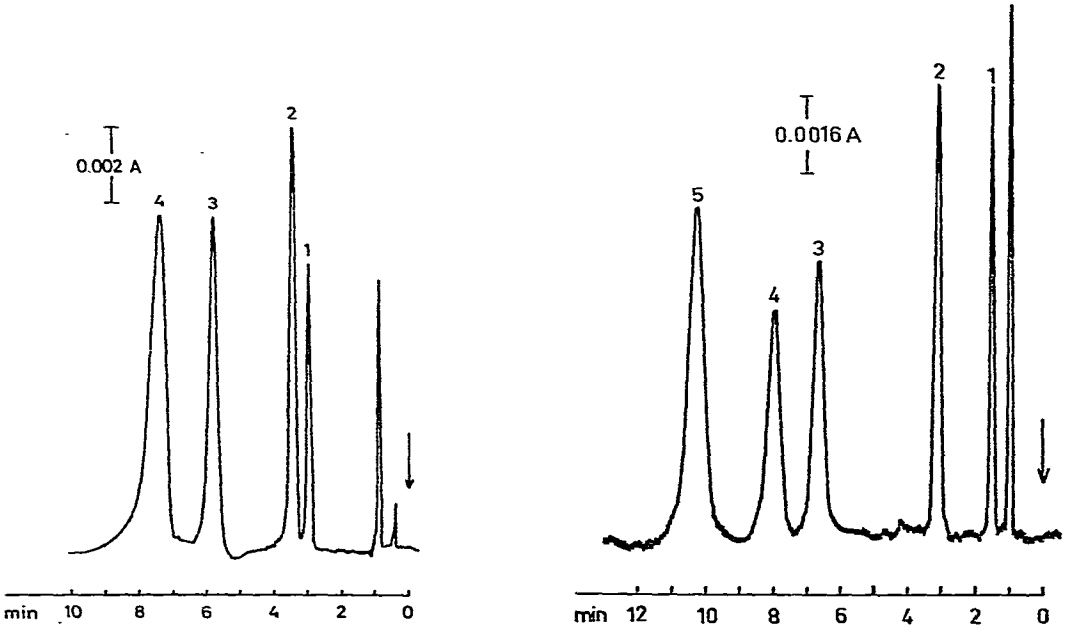


Fig. 4. Separation of isomeric alkylamines. Mobile phase: chloroform–1-pentanol (19:1); 2.8 mm/sec; 3.8 MPa. Stationary phase: naphthalene-2-sulphonate, 0.1 *M*, pH 2.3. Support: LiChrospher SI-100 (10 μm). Samples: 1 = *n*-hexylamine (31 ng); 2 = di-*n*-propylamine (63 ng); 3 = diisopropylamine (80 ng); 4 = triethylamine (107 ng).

Fig. 5. Separation of primary alkylamines. Mobile phase: chloroform–1-pentanol (9:1); 2.8 mm/sec; 4.6 MPa. Other conditions as in Fig. 4. Samples: 1 = *n*-hexylamine (35 ng); 2 = *n*-pentylamine (51 ng); 3 = *n*-butylamine (45 ng); 4 = isobutylamine (44 ng); 5 = *tert.*-butylamine (92 ng).

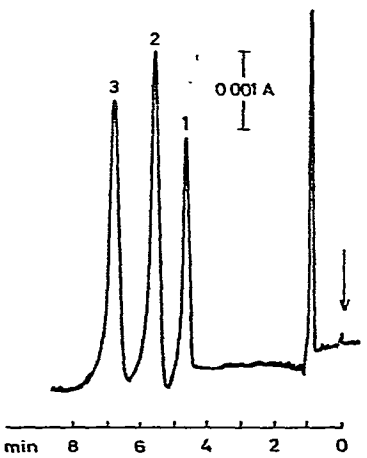
Peak symmetry. The more hydrophilic amines, the amino acids and the peptides gave good peak symmetry at sample concentrations of 10^{-6}–10^{-4} *M* (corresponding to 0.01–1 μg). The more hydrophobic amines also gave symmetrical peaks at concentrations below $2 \cdot 10^{-5}$ *M*, but a tendency to tailing was observed at higher concentrations, as shown in Table IV. The tailing seems to increase with increasing hydrophobic character, DMOA giving a significant asymmetry (asymmetry factor $\geqslant 2$) at a much lower cencentration than DBA and *tert.*-OA.

As no difference in the capacity ratio was observed, it is improbable that the asymmetry is due to dimerization of the ion pair in the organic phase[5]. It must be emphasized that the tailing has a rather limited effect on the separating efficiency at sample concentrations below 10^{-4} *M*.

Column efficiency. The separating efficiency of the systems was good for all of the compounds studied, $H \leqslant 0.1$ mm being obtained in most instances at a mobile phase flow-rate of 4 mm/sec.

The relationship between *H* and the mobile phase flow-rate is demonstrated in Fig. 3. There is a slight increase in plate height with increasing flow-rate, *H* having a maximum at low *k'*. The decrease in *H* with increasing capacity ratio indicates a predominating effect of stationary phase mass transfer.

Selectivity. Some examples of separations of nanograms amounts of samples are presented in Figs. 4–7. The separation of four amines containing six alkyl carbon atoms but with different degrees of substitution at the nitrogen atom is demonstrated in Fig. 4. The separation factors in relation to the primary amine are 1.2 for the



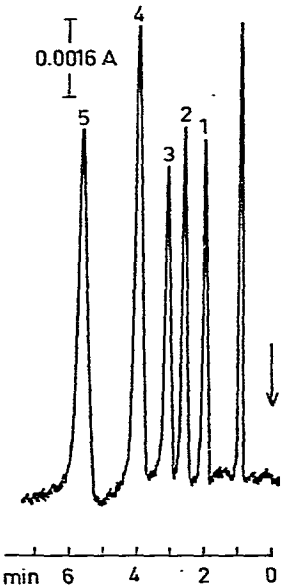


Fig. 6. Separation of dipeptides. Conditions as in Fig. 4. Samples: 1 = phenylalanylphenylalanine (56 ng); 2 = phenylalanylleucine (90 ng); 3 = leucylphenylalanine (90 ng).

Fig. 7. Separation of dipeptides. Conditions as in Fig. 5. Samples: 1 = leucylleucine (74 ng); 2 = phenylalanylvaline (104 ng); 3 = valylphenylalanine (106 ng); 4 = leucylvaline (170 ng); 5 = methionylvaline (214 ng).

secondary amine and 3.1 for the tertiary amine, in spite of the fact that the extraction constants are about equal.

Fig. 5 shows the separation of homologous primary alkylamines as well as the influence of chain branching. The separation factors between homologous, straight-chain amines are 3–4, *i.e.*, of the expected magnitude. Branching in this system gives an increase in log k' of 0.1 unit, which is about half the value given by Leo *et al.*[16] in their prediction system.

Figs. 6 and 7 demonstrate that dipeptides containing the same amino acids in reverse order can also be separated in this system.

Minimum detectable amount. The minimum concentration that is photometrically detectable depends on the molar absorptivity of the migrating sample and the noise of the detector. In the present system, the highly absorbing counter ion, NS, is to a certain extent distributed to the mobile phase as an acid and an ion pair with sodium[5,13], and this can give rise to considerable noise if the system is not in complete balance. It has been observed that one of the most important factors for the balance of the system is the thermostating, and by careful temperature control it has been possible to reduce the noise to $6 \cdot 10^{-5}$ absorbance units.

The minimum detectable concentration (MDC) by photometry can be defined by (2 × noise)/molar absorptivity. At the given noise level, an NS ion pair with a molar absorptivity of $1.4 \cdot 10^3$ will have an MDC of $8 \cdot 10^{-8}$ *M*.

In liquid chromatography, where samples of rather large volume can be used, it is often of greater interest to give the minimum detectable amount (MDQ) that can be calculated by the expression

$$\mathrm{MDQ} = \mathrm{MDC} \cdot \sqrt{2\pi} \cdot V_m(1+k')/\sqrt{N} \qquad (4)$$

The application of this equation to the chromatogram given in Fig. 8 (V_m = 0.9 ml) shows that DMOA with $k' = 0.6$ and a number of theoretical plates $N = 1600$ should have an MDQ of $7 \cdot 10^{-12}$ moles, *i.e.* 1 ng. It is easy to confirm this calculation from Fig. 8.

Columns with cationic counter ions

Many cationic compounds with highly conjugated structures that will give ion pairs with high molar absorptivity are commercially available as drugs. For use as an amplifier in the detection of anionic samples in ion-pair chromatographic systems, quaternary ammonium ions are preferable as they can be used at any pH. Dimethylprotriptyline (MPT) and higher homologues have been used in ion-pair chromatography on cellulose by Borg and Schill[17] for barbiturates and by Eksborg *et al.*[18] for carboxylic acids. Lagerström[6] has studied the extraction and association equilibria of MPT and found that it is highly associated in aqueous solution at a concentration above 0.03 *M*. The molar absorptivity of the ion pairs is $3 \cdot 10^3$ at 254 nm. Methylimipramine, with similar association properties, has a molar absorptivity of $8 \cdot 10^3$ (ref. 6) and should give an even higher amplification.

Stability of MPT columns. A stationary phase of MPT in phosphate buffer (pH 6.5) was applied by the pumping technique. Immediately after the coating, the MPT columns showed the same stationary phase content as the NS columns, *i.e.*, 1.0 ml per gram of support. During the recycling period, a slow stripping off of the

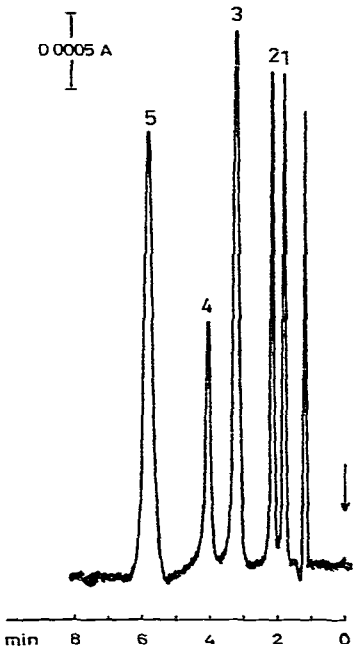


Fig. 8. Separation of hydrophobic alkylamines. Mobile phase: chloroform–1-pentanol (19:1); 2.5 mm/sec; 4.5 MPa. Stationary phase: naphthalene-2-sulphonate, 0.01 *M*, pH 2.1. Support: LiChrospher SI-100 (10 μm). Samples: 1 = N,N-dimethyloctylamine (26 ng); 2 = N-methyloctylamine (24 ng); 3 = Di-*n*-butylamine (46 ng); 4 = *n*-octylamine (16 ng); 5 = *tert.*-octylamine (54 ng).

stationary phase was observed, V_s/V_m decreasing from 1.0 to 0.4 during the passage of 15 l of mobile phase.

Tests with samples of sulphonates and sulphates showed that the decrease in V_s was accompanied by a significant increase in k' (Fig. 9). Good column efficiency could not be obtained ($H \geqslant 0.15$ mm) and a considerable increase in H was observed during the recycling.

It has been reported[9] that silica supports have a strong tendency to adsorb quaternary alkylammonium ions from the stationary phase and acquire a hydrophobic character. Elution of the stationary phase after passage of about 15 l of mobile phase showed that the amount of MPT in the stationary phase had increased about 20-fold. The capacity ratios found were much higher than those calculated from extraction constants and the nominal counter ion concentration, and the separation factors were considerably lower than expected (*cf.*, ref. 9).

Adsorption on silica. The adsorption of different quaternary ammonium ions on silica was studied on suspensions of the micro-particles in aqueous solutions, using a shaking time of 30 min, which was sufficient for equilibrium to be reached.

The influence of pH on the adsorption of MPT is demonstrated in Fig. 10. A drastic increase in the amount adsorbed occurs at about pH 7, which is close to the assumed pK_a value of silica[19].

The addition of competing quaternary alkylammonium ions (tetramethyl-, tetraethyl-, tetrapropyl- and trimethylnonylammonium, choline and carbacholine)

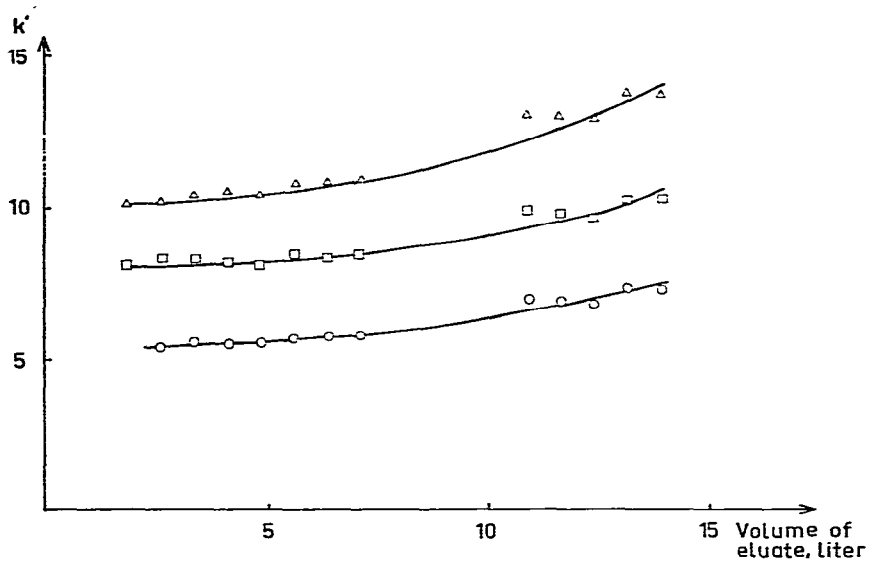


Fig. 9. Variation of the capacity ratio on dimethylprotriptyline columns. Mobile phase: chloroform–1-pentanol (19:1). Stationary phase: dimethylprotriptyline, 0.025 *M*, pH 6.5. Support: LiChrospher SI-100 (10 μm). Samples: ○ = *n*-butyl sulphate; □ = *n*-butane sulphonate; △ = toluene 4-sulphonate.

decreased the adsorption of MPT in batch experiments but tests on-column did not indicate a significant improvement of the stability.

It seems that the adsorption increases with increasing hydrophobic character of the quaternary ammonium ion, but other structural features appear to be even more important, as columns with a high and stable V_s as well as stable k' and $H < 0.1$ mm were obtained with tetrabutylammonium (0.025 *M*, pH 6.5) as stationary phase.

Tests with other quaternary ammonium ions with high UV absorbance are in progress.

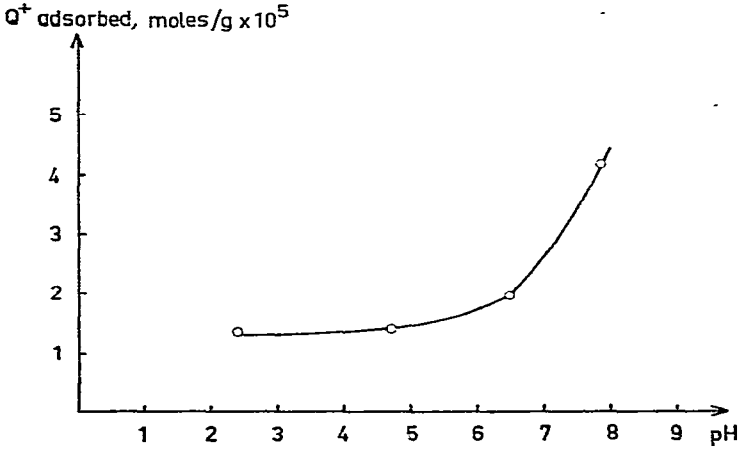


Fig. 10. Adsorption of dimethylprotriptyline (Q^+) on silica. Aqueous phase: dimethylprotriptyline (1.1×10^{-3} *M*) in phosphate buffer (10 ml). Solid phase: LiChrospher SI-100 (10 μm) (0.12 g).

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REFERENCES

1 G. Schill, K. O. Borg, R. Modin and B. A. Persson, in L. F. Chasseaud and J. Bridges (Editors), *Progress in Drug Metabolism*Vol. 2, Wiley, New York, 1977, ch. 5.
2 G. Schill, K. O. Borg, R. Modin and B. A. Persson, in E. R. Garrett and J. Hirtz (Editors), *Drug Fate and Metabolism: Methods and Techniques*, Vol. 1, Marcel Dekker, New York, 1977, Ch. 4.
3 S. Eksborg and G. Schill, *Anal. Chem.*, 45 (1973) 2092.
4 S. Eksborg and B. A. Persson, *Acta Pharm. Suecica*, 8 (1971) 205.
5 S. Eksborg, *Acta Pharm. Suecica*, 12 (1975) 19.
6 P. O. Lagerström, *Acta Pharm. Suecica*, 12 (1975) 215 and 429.
7 R. Modin and G. Schill, *Acta Pharm. Suecica*, 7 (1970) 585.
8 R. Modin and G. Schill, *Acta Pharm. Suecica*, 4 (1967) 301.
9 B. Fransson, K.-G. Wahlund, I. M. Johansson and G. Schill, *J. Chromatogr.*, 125 (1976) 327.
10 G. Schill, in J. A. Marinsky and Y. Marcus (Editors), *Ion Exchange and Solvent Extraction*, Vol. 6, Marcel Dekker, New York, 1974, ch. 1.
11 R. Modin and G. Schill, *Talanta*, 22 (1975) 1017.
12 K. Gustavii, *Acta Pharm. Suecica*, 4 (1967) 233.
13 J. Crommen, in preparation.
14 M. Schröder-Nielsen, *Acta Pharm. Suecica*, 11 (1974) 541.
15 J. C. Giddings, *Dynamics of Chromatography, Part 1*, Ma;cel Dekker, New York, 1965.
16 A. Leo, C. Hansch and D. Elkins, *Chem. Rev.*, 71 (1971) 525.
17 K. O. Borg and G. Schill, *Acta Pharm. Suecica*, 5 (1968) 323.
18 S. Eksborg, P.-O. Lagerström, R. Modin and G. Schill, *J. Chromatogr.*, 83 (1973) 99.
19 D. N. Strazhesko, V. B. Strelko, V. N. Belyakov and S. C. Rubanik, *J. Chromatogr.*, 102 (1974) 191.