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

III. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF QUATERNARY ALKYLAMMONIUM IONS AS ION PAIRS WITH NAPHTHALENE-2-SULPHONATE, USING A SILICA SUPPORT OF LOW SURFACE AREA

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SUMMARY

Ion-pair chromatographic systems suitable for the separation and determination of non-absorbing quaternary ammonium ions in the nanogram range have been devised. An aqueous stationary phase containing the highly absorbing counter ion naphthalene-2-sulphonate has been applied on a support of microparticulate silica with a low surface area.

The separation efficiency and selectivity of these systems are demonstrated with some examples of the separation of closely related quaternary ammonium ions.

The possibilities of the direct injection of aqueous samples and subsequent quantitation by peak-area measurement have been studied.

Investigations on the retention behaviour of quaternary ammonium ions in these systems have shown that there is still an important influence of adsorption effects, especially at low loadings. A particularly strong influence of adsorption on the retention of long-chain ammonium ions has been observed.

INTRODUCTION

In straight-phase systems for ion-pair chromatography, the detection sensitivity can be considerably improved by a proper choice of the counter ion, as in this instance the samples are eluted as ion pairs in the organic mobile phase. This principle for the enhancement of the detector response has been applied in high-performance liquid chromatographic (HPLC) systems for determining nanogram amounts of alkylamines, amino acids and dipeptides with UV detection at 254 nm[1,2]. An aqueous solution of the highly absorbing counter ion naphthalene-2-sulphonate (NS) was used as the stationary phase. A maximum amount of stationary phase was applied on the silica support (LiChrospher SI 100) to minimize the influence of adsorption effects on the migration of the sample.

Quaternary alkylammonium ions are often used in ion-pair systems and there is a need for rapid and sensitive methods for the determination of those non-absorbing compounds. The ion-pair chromatographic method with NS seems to be suitable but the behaviour of quaternary ammonium ions in such liquid–liquid systems is greatly influenced by adsorption on the silica support, even at the maximum loading, resulting in highly deformed and retarded peaks. The strong tendency of quaternary ammoniums to be adsorbed on silica from aqueous solutions, even at low pH has been demonstrated by static experiments[1,3] and by chromatographic means[4]. Adsorption decreases with decreasing surface area of silica and on the addition of different kinds of competitors, such as other quaternary ammonium ions or lipophilic alcohols such as 1-pentanol[4].

This paper reports on studies of the chromatographic properties of quaternary alkylammonium ions as NS ion pairs, using silica with a low surface area (LiChrospher SI 500) as the chromatographic support. Investigations on retention effects have been made under different chromatographic conditions. The possibilities of the quantitative determination by direct injection of aqueous samples have been examined.

EXPERIMENTAL

Apparatus

The pump was a Model 6000 A solvent delivery system and the detector was Model 440 absorbance detector measuring at a wavelength of 254 nm, both from Waters Assoc. (Milford, Mass., U.S.A.). The injector was a high-pressure valve from Valco Instruments, with 10–25-μl loops.

The columns were made of 316 stainless steel and had a length of 250 mm and an I.D. of 4.0 mm.

A Heto type 02 PT 923 water-bath (Birkeröd, Denmark) was used to thermostat the chromatographic equipment.

In the batch experiments, the photometric measurements were made with a Perkin-Elmer Model 200 spectrophotometer. A Corning-EEL Model 109 digital pH meter was used for pH measurements.

Chemicals and reagents

The chromatographic support was LiChrospher SI-500 (10 μm) from E. Merck (Darmstadt, G.F.R.) with a specific surface area of 50 m^2/g and a pore volume of 0.8 ml/g.

Chloroform and 1-pentanol were of pro analysi quality from Merck.

Naphthalene-2-sulphonic acid, sodium salt, was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.).

The aqueous stationary phases were purified by repeated extraction with chloroform–1-pentanol (9:1) until the organic phase had a constant absorbance.

The quaternary alkylammonium ions used in the chromatographic experiments were mainly obtained from Eastman-Kodak and Fluka (Buchs, Switzerland), as bromides or iodides. Tetrabutylammonium, tetrapropylammonium and tetraethylammonium hydrogen sulphates (puriss) from Labkemi (Göteborg, Sweden) were used for the quantitative determinations and in the partition studies, after neutralization

with sodium hydroxide. The alkylamines, obtained from Fluka, were distilled and converted into chlorides before use.

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

Aqueous solutions of sodium or choline dihydrogen citrate (0.1 *M*) were used as buffers.

Column preparation and chromatographic technique

The columns were packed by a balanced-density slurry technique and coated according to the pumping or injection techniques described earlier[1].

The mobile phase reservoir, the injector and the column were thermostated in a water-bath at 25.0 ± < 0.1 °C. A small amount of aqueous stationary phase was present as an upper layer in the reservoir to ensure complete saturation of the recycled mobile phase.

The samples were dissolved in the mobile phase as halide salts, unless otherwise stated.

All chromatographic experiments were carried out at a flow-rate of 1.0 ml/min, which corresponds to a mobile phase speed of 2.2–2.9 mm/sec, depending on the volume of mobile phase in the column. This volume of mobile phase, V_m, was determined by injection of toluene, which is unretained in these systems.

RESULTS AND DISCUSSION

The following abbreviations and symbols are used: TBA = tetrabutylammonium; TPrA = tetrapropylammonium; TEA = tetraethylammonium; TMNA = trimethylnonylammonium; Q^+ = ammonium ion; X^- = NS; $K_{ex(QX)} = [QX]_{org}/[Q^+][X^-]$ = extraction constant of the ion pair QX; $K_{a(QX)} = [QX]/[Q^+][X^-]$ = constant for ion-pair formation between Q^+ and X^- in the aqueous phase. Concentrations without an index refer to the aqueous phase. All concentrations are given in moles per litre.

The principles of ion-pair extraction and its application in liquid–liquid chromatography have been described in previous papers[1,5,6]. In straight-phase ion-pair chromatography, the capacity ratio of the cation Q^+ can be expressed by

$$k'_Q = \frac{V_s}{V_m} \cdot \frac{1 + K_{a(QX)} \cdot [X^-]}{K_{ex(QX)} \cdot [X^-]} \quad (1)$$

where V_s/V_m is the phase-volume ratio and $[X^-]$ represents the concentration of the counter ion NS in monomeric form[2]. Eqn. 1 is valid if the retention of Q^+ is due entirely to liquid–liquid distribution and if ion-pair formation in the aqueous phase is the only side-reaction which influences significantly the ion-pair distribution equilibrium.

Stability of stationary phase loadings

The maximum volume of stationary phase (V_s) that could be applied on LiChrospher SI 500 was 1.43 ml for the type of column used. This corresponds to a phase-volume ratio (V_s/V_m) of 1.04. The fraction of the column volume occupied by

the mobile phase (ε_m) was 0.44, which means that the pores were almost completely filled with stationary phase in this instance[1,2]. This maximum loading was not stable and a slow stripping of stationary phase was observed ($\pm$ 0.1 ml bleeding in 24 h).

A better stability was obtained when V_s was about 1.20–1.25 ml (V_s/V_m = 0.75–0.80 and $\varepsilon_m \approx 0.5$). Most chromatographic experiments were carried out with such stationary phase loadings. Good peak symmetry and separating efficiency were obtained for quaternary ammonium ions in that V_s range, together with the interesting properties of heavily loaded columns, such as high sample capacity and selectivity.

The long-term stability of V_s in columns with LiChrospher SI 500 as the support is not exactly as good as that obtained with LiChrospher SI 100, however. This is due to the lower adsorptive properties of LiChrospher SI 500, and also to the presence of larger pores, as the contribution of capillary forces to the stability of the stationary phase volume held in the pores is far from negligible[7]. If necessary, V_s can easily be readjusted by injection of stationary phase via the sample loop.

Ion-pair distribution of quaternary ammonium ions

Constants for the ion-pair extraction [$K_{ex(QX)}$] of TPrA and TEA with NS as counter ion and chloroform containing 5–20% (v/v) of 1-pentanol as the organic phase are listed in Table I. Constants for ion-pair formation in the aqueous phase [$K_{a(QX)}$] are also given. The batch experiments were carried out under conditions of concentration such that both kinds of constants could be obtained simultaneously by slope analysis[1]. Table I also contains a few constants from previous publications. Some of them were determined with pure chloroform as the organic phase.

TABLE I

EXTRACTION CONSTANTS OF ION PAIRS BETWEEN QUATERNARY AMMONIUM IONS AND NAPHTHALENE-2-SULPHONATE

Organic phase: chloroform or chloroform–1-pentanol. Aqueous phase: citrate buffer (pH 3.8).

Cation (Q^+)	*1-Pentanol (%,v/v)*	$C_X \cdot 10^4$	$C_Q \cdot 10^2$	$C_{QX_{org}} \cdot 10^4$	*log* $K_{ex(QX)}$	*log* $K_{a(QX)}$
TPrA	—				1.24*	0.9*
	5	2.1–2.6	1.0–1.8	1.4–1.9	1.77	1.0
	10	0.7–2.0	0.8–1.5	0.8–2.4	2.08	1.1
	20	1.2–1.7	0.7–1.3	2.3–2.8	2.33	1.1
TEA	5				0.10*	—
	10	3.3–5.3	9.7–14	0.6–0.7	0.46	0.9
	20	2.9–3.2	7.3–13	0.8–0.9	0.76	1.0
TMNA	—				2.27**	—

* Constants from ref. 6.

** Constant from ref. 8.

The constants given in Table I indicate that the extraction of quaternary ammonium ions as NS ion pairs is much lower than that of primary or secondary ammonium ions with the same number of alkyl carbons (*cf.*, constants in ref. 1). On the other hand, the tendency of quaternary ammonium ions to form ion pairs in the aqueous phase seems to be higher than that observed with other ammonium ions [log $K_{a(QX)} \approx 0.6$ (*cf.*, ref. 2)].

There is a linear relationship between the logarithms of the extraction constants of TPrA and TEA and the concentration of 1-pentanol in the organic phase, as already observed with other ammonium ions. The slope of the lines is around unity, *i.e.*, about the same as that given by secondary amines[2].

Influence of the adsorption properties of the support

An estimation of the importance of adsorption effects on retention can be obtained by comparing the capacity ratios found (k'_f) and calculated according to eqn. 1 (k'_c).

A decrease in k'_f/k'_c from 14 to 2.9 was obtained for TPrA when LiChrospher SI 500 was used as the support instead of SI 100, the columns being loaded with the maximum amount of stationary phase in both instances. This indicates that the influence of adsorption on the retention of quaternary ammonium ions in liquid–liquid systems can be reduced considerably by using a silica support with a low surface area.

The values of k'_f/k'_c obtained for alkylamines with LiChrospher SI 500 as the support were in the range 0.7–1.0 at the maximum loading, which means that the influence of adsorption on the migration of these samples is negligible. The fact that in some instances the found capacity ratios were even lower than the calculated values is probably due to ion-pair dissociation in the organic phase[5,6]. A certain degree of dissociation of the sample ion pairs might occur, owing to the very low distribution of the counter ion NS to the mobile phase when sodium is the only cation present in the stationary phase, as shown in Table II.

TABLE II

ABSORBANCE AND CONCENTRATION OF NAPHTHALENE-2-SULPHONATE IN THE MOBILE PHASE

Mobile phase: chloroform–1-pentanol. Stationary phase: naphthalene-2-sulphonate (NS), 0.1 *M* in citrate buffer (pH 3.8). Support: LiChrospher SI-500. Measuring wavelength: 254 nm. The ε_{254} values given in the text were used to estimate the NS concentrations.

Cationic component of the buffer (C = 0.1)	*1-Pentanol (%, v/v)*	*Absorbance of the mobile phase*	*NS concentration (mol/l · 10⁴)*
Sodium	10	0.160	0.5
Choline	5	0.218	0.7
	10	0.673	2.1
	20	2.05	10

Influence of the volume of stationary phase

Fig. 1 shows the variation of the observed k' values with the phase-volume ratio. The change in V_s/V_m was obtained by spontaneous stripping of aqueous stationary phase from a column loaded with the maximum V_s. The bleeding process was very slow, especially at low V_s/V_m.

The drastic increase of the influence of adsorption effects on the retention behaviour of quaternary ammonium ions at lower loadings is demonstrated in Fig. 1: an increase in k' with decreasing V_s/V_m is obtained for all quaternary ammonium ions, but this increase is particularly strong for the long-chain ammonium ion TMNA. Other indications of a stronger adsorption of ammonium samples with long alkyl chains

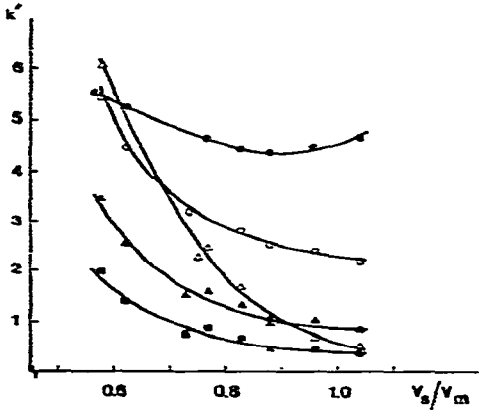


Fig. 1. Variation of retention with the phase-volume ratio. Mobile phase: chloroform–1-pentanol (9:1). Stationary phase: naphthalene-2-sulphonate, 0.1 *M* in sodium citrate buffer (pH 3.8). Support: LiChrospher SI 500. Samples: ●, *n*-butylamine; ○, tripropylmethylammonium; ▲, tetrapropylammonium; △, trimethylnonylammonium; ■, tripropylbutylammonium.

(six carbon atoms or more) have been reported earlier[1]. The order of elution of the two isomers TMNA and TPrA at the highest loading corresponds to that which could be expected from batch distribution data (*cf.*, Table I), but this order is reversed at lower V_s/V_m values.

Fig. 1 also shows that the capacity ratio of a primary amine, such as *n*-butylamine, decreases with decreasing V_s/V_m in the high loading range, which is in accordance with a retention behaviour entirely governed by liquid–liquid distribution (*cf.*, eqn. 1). An increase in k' is observed, however, at lower loadings but it is much less pronounced than that observed with quaternary ammonium ions.

Influence of the addition of choline

The possible effects on retention of the addition to the stationary phase of a cation which could compete for adsorption with the samples were studied. The quaternary ammonium ion choline was chosen because of its low distribution to the mobile phase as an NS ion pair. As can be seen from Table II, the absorbance of the mobile phase in the presence of choline is still acceptable.

In the usual range of V_s/V_m values (0.75–0.80), a decrease in retention on addition of choline was obtained only for long-chain ammonium ions, such as TMNA and *n*-hexylamine. The values of k'_s/k'_c for the symmetrically substituted quaternary ammonium ions TPrA and TEA, which are about 8 and 4.5, respectively, in that V_s/V_m range, were not significantly reduced in the presence of choline. The influence of the addition of choline seems, however, to be more important at lower loadings, resulting in a less drastic increase in the capacity ratios of quaternary ammonium ions.

Influence of 1-pentanol concentration

Fig. 2 shows the relationship between the log k' values of quaternary ammonium ions and $\log[\text{1-pentanol}]_{org}$. Fairly straight lines were obtained in all instances,

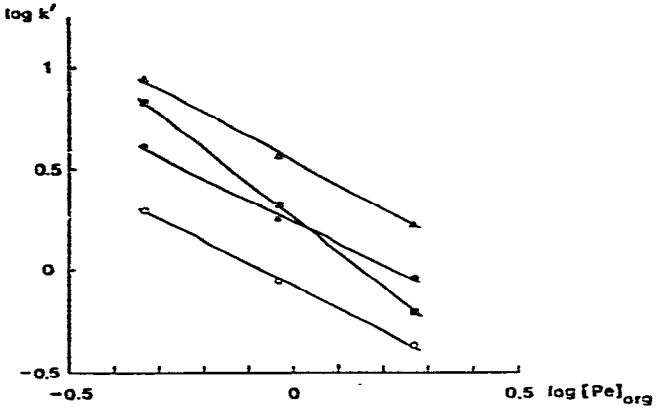


Fig. 2. Influence of 1-pentanol concentration on the retention of quaternary ammonium ions. Mobile phase: chloroform–1-pentanol. Stationary phase: naphthalene-2-sulphonate, 0.1 *M* in choline citrate buffer (pH 3.8). Support: LiChrospher SI-500. V_s/V_m: 0.75. Samples: ▲,triethylbenzylammonium; ●, tetrapropylammonium, ■, trimethylnonylammonium; ○, tripropylbutylammonium.

and the slope of the lines was usually close to the value expected from batch distribution data. This allows one to regulate systematically the retention of quaternary ammonium ions by changing the concentration of 1-pentanol in the mobile phase.

TMNA again shows a particular behaviour, its slope being much higher than that of other quaternary ammonium ions. It has been reported that 1-pentanol can compete with ammonium ions for adsorption to silica[4] and it is probable that the contribution of adsorption effects to the retention of TMNA decreases significantly with increasing concentration of 1-pentanol. Fig. 2 shows that, at a V_s/V_m of 0.75, the two isomers TPrA and TMNA are eluted in the order expected from batch distribution only when chloroform + 20% (v/v) of 1-pentanol is used as the mobile phase.

Al already observed with other ammonium ions[2], an increase in the slope was obtained when the quaternary ammonium ions had additional hydrophilic groups.

Separation efficiency

The chromatographic systems with LiChrospher SI 500 as the support had a good efficiency at high loadings ($V_s/V_m > 0.7$) but the height of a theoretical plate, H, obtained with quaternary ammonium ions as samples ($H = 0.1$–0.2 mm) was often slightly higher than that observed with primary, secondary and tertiary amines ($H < 0.1$ mm) under the same conditions of retention. This might be related to the differences in retention behaviour between these two kinds of samples[9].

Selectivity

The selectivity that can be obtained for quaternary ammonium ions in systems such as those described above is illustrated by some examples of separations. Fig. 3 shows the separation of tripropylalkylammonium ions. The change in log k' between two samples with a difference of one methylene group in the alkyl chain, such as compounds 1 and 2 in Fig. 3, is usually around 0.3. This value is lower than expected but it is still sufficient for the complete resolution of homologous am-

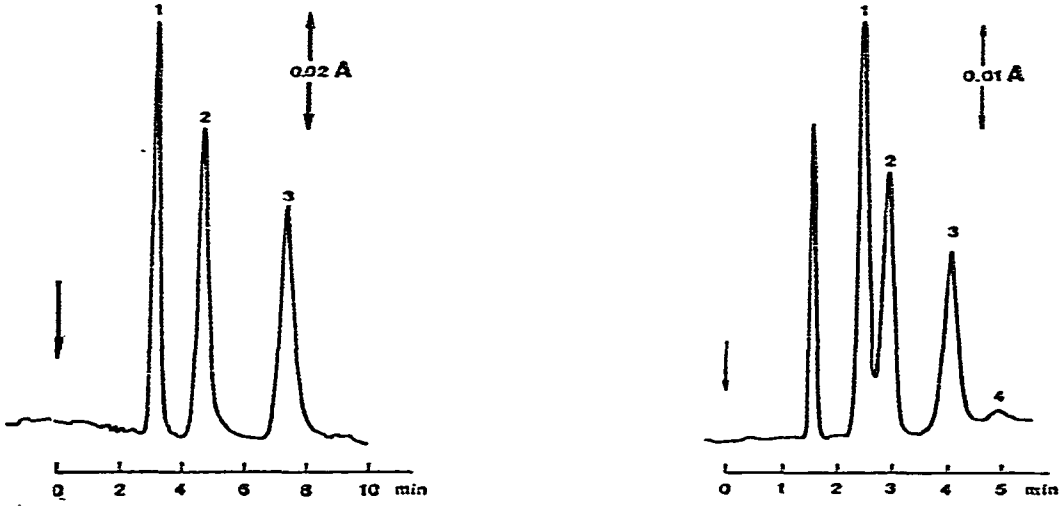


Fig. 3. Separation of tripropylalkylammonium ions. Mobile phase: chloroform–1-pentanol (9:1); 2.5 mm/sec; 4.4 MPa. Stationary phase: naphthalene-2-sulphonate, 0.1 *M* in choline citrate buffer (pH 3.8). Support: LiChrospher SI 500 (10 μm). V_s/V_m: 0.75. Samples: 1, tripropylbutylammonium bromide; 2, tetrapropylammonium bromide; 3, tripropylmethylammonium iodide.

Fig. 4. Separation of quaternary ammonium ions. Mobile phase: chloroform–1-pentanol (9:1); 2.8 mm/sec; 4.9 MPa. Stationary phase: naphthalene-2-sulphonate, 0.1 *M* in sodium citrate buffer (pH 3.8). Support: LiChrospher SI 500 (10 μm). V_s/V_m: 0.96. Samples: 1, trimethylnonylammonium bromide; 2, tetrapropylammonium bromide; 3, triethylbenzylammonium chloride; 4, triethylamine hydrochloride.

monium ions. The decrease in selectivity is probably related to the dominating influence of adsorption on the retention of these compounds[2].

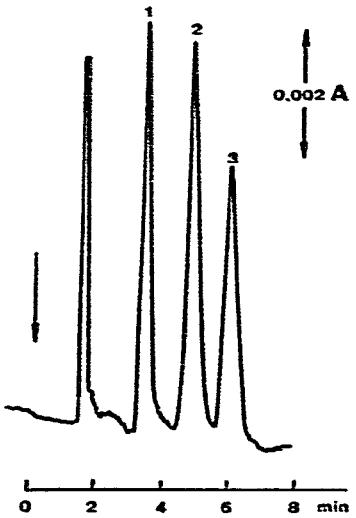
As shown in Fig. 1, the best separation between the two isomers TPrA and TMNA in the high V_s/V_m range is obtained at the maximum loading. A chromatogram obtained under these conditions is shown in Fig. 4. Triethylbenzylammonium ion, which contains six aromatic carbon atoms, is less hydrophobic and therefore more retained than samples which have only alkyl carbons.

A small secondary peak (peak 4 in Fig. 4) was always observed on injection of triethylbenzylammonium ion. This peak is probably due to the presence of a small amount of triethylamine in the triethylbenzylammonium sample tested, as its k' values, obtained under various chromatographic conditions, were always identical with those of triethylamine.

The NS method has also been applied to the determination of quaternary ammonium ions used as drugs, which have a low UV absorbance. Fig. 5 shows the separation of three closely related anticholinergics. The only difference between compounds 1 and 3 is that the former has a cyclohexane instead of a benzene ring in its molecule. Compound 2 contains one alkyl carbon atom more than compound 3.

Injection and quantitative analysis of aqueous samples

In chromatographic systems with an organic mobile phase, an extraction step is usually needed before injection if the samples are initially present in aqueous



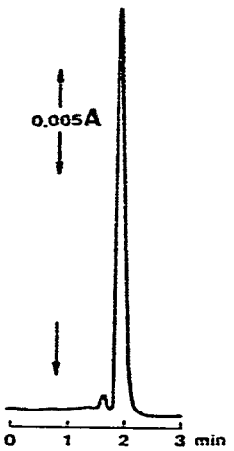


Fig. 5. Separation of anticholinergics. Conditions as in Fig. 3. Samples: 1, oxypyrronium bromide; 2, benzilonium bromide; 3, poldine methylsulphate.

Fig. 6. Chromatogram of tetrabutylammonium. Mobile phase: chloroform–1-pentanol (9:1); 2.6 mm/sec; 6.7 MPa. Stationary phase: naphthalene-2-sulphonate, 0.1 *M* in sodium citrate buffer (pH 3.8). Support: LiChrospher SI 500 (10 μm). V_s/V_m: 0.82. Sample: tetrabutylammonium in citrate buffer (388 ng per 10-μl injection).

solutions. It has been found, however, that aqueous samples can be injected directly in straight-phase systems under certain conditions.

In order to avoid the presence of water droplets in the eluate, the total amount of aqueous solution injected should be taken up by the support. This is not possible, of course, in columns loaded with the maximum amount of stationary phase but, in general, lower loadings are used with supports of low surface area such as LiChrospher SI 500, so that a certain volume of aqueous phase can still be taken up. This volume will depend on the size of the column and on the initial V_s/V_m: with the type of column used (250 × 4 mm I.D.) and an initial V_s/V_m of 0.8, up to 15 successive 10-μl injections of aqueous solution could be made without disturbance to the detector baseline. The number of injections can no doubt be increased if smaller sample volumes are used.

An example of a chromatogram obtained by injection of an aqueous sample is shown in Fig. 6. A small peak was often observed at the front but it had no influence on the sample peak. Only aqueous samples with approximately the same pH and buffer composition as the stationary phase were injected in these systems. Owing to the relatively low stability of the maximum loading on LiChrospher SI 500, the usual V_s/V_m (around 0.8) was restored spontaneously after 1–2 days. A more rapid stripping might be obtained by use of non-equilibrated mobile phase.

Quantitative experiments were made with TBA as the sample, dissolved in a citrate buffer. A slight decrease in the capacity ratio was observed during the injections, which corresponds to the usual behaviour of quaternary ammonium ions when V_s/V_m increases from 0.8 to 1.0 (*cf.*, Fig. 1). The separating efficiency and peak

symmetry were not affected and a good estimation of the peak areas could be obtained by triangulation. Calibration graphs with high correlation coefficients ($r \geqslant 0.998$) were obtained in the concentration range studied (0.1–1 μg per injection). Some examples of chromatographic quantitation of TBA in aqueous solutions by the NS method are given in Table III.

TABLE III

QUANTITATIVE DETERMINATION OF TETRABUTYLAMMONIUM

Mobile phase: chloroform–1-pentanol (9:1); 2.6–2.8 mm/sec; 6.7–7.0 MPa. V_s/V_m: 0.8–1.0. Other conditions as in Fig. 6. Sample: TBA in citrate buffer (pH 3.8). Volume injected: 10 μl. Relative standard deviation: <2%.

Sample amount (ng)	*Recovery (%)*
194	101.7
388	98.7
776	100.2

Molar absorptivity of naphthalene-2-sulphonate ion pairs

A decrease in the molar absorptivity (ε) of NS ion pairs with increasing 1-pentanol content of the mobile phase has been reported[2]. In the systems described here, stationary phases with a higher pH value were used and a slight decrease in the molar absorptivity was observed only at a 1-pentanol content of 20% (log ε_{254} = 3.29). The expected molar absorptivity for ion pairs between NS and alkylammonium ions (log ε_{254} = 3.50; *cf.*, ref. 6) was obtained at lower 1-pentanol concentrations, which seems to confirm that the ε-value is related to the co-extraction of NS in acidic form to the mobile phase. This extraction can be considered to be negligible at pH 3.8 when the 1-pentanol content of the mobile phase is 5–10%[2].

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