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REVERSED-PHASE ION-PAIR CHROMATOGRAPHY WITH UV-ABSORBING IONS IN THE MOBILE PHASE

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SUMMARY

Reversed-phase systems have been developed that permit the detection and quantification of down to 0.1 nmole of non-UV-absorbing cations and anions using a UV detector. The samples give positive or negative peaks depending on their charge and retention relative to the UV-absorbing ionic component in the mobile phase. The relative detector response has a maximum, which can be considerably more than 100%, when the sample and the UV-absorbing mobile phase ion have about the same retention. Detection and separation studies on, *e.g.*, sulphonates, sulphates, carboxylates, amino acids, dipeptides and alkylammonium compounds of different degrees of substitution are described.

INTRODUCTION

In ion-pair chromatography it is usual to regulate the retention and separation selectivity for the ionic samples by changing of the nature and the concentration of the counter ion. The counter ion can, however, have a double function in these systems and it can also be used to improve the detection possibilities to a considerable extent. The technique has so far mainly been utilized on photometric detectors, where the ion-pair chromatographic principle offers excellent possibilities of a high detector response even for non-UV-absorbing compounds by using a counter ion of high molar absorptivity at the measuring wavelength.

The principle was applied initially to liquid–liquid straight-phase systems, where the connection to ion-pair extraction by the batch technique is striking. The UV-absorbing counter ion (X^-_{UV}) is applied in solution on a hydrophilic solid phase. A non-UV-absorbing sample with the opposite charge (Q^+) will migrate with the organic mobile phase as an ion pair which, owing to the high absorptivity of the counter ion, will have a high UV absorbance:

$$\underset{\text{aqueous stationary phase}}{Q^+ + X^-_{UV}} = \underset{\text{organic mobile phase}}{QX_{UV}}$$

The technique was first used in low-pressure systems[1–4] but its usefulness in

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high-performance systems has been demonstrated in several recent publications[5–10].

The ion-pair principle can, however, also be applied in reversed-phase systems with a hydrophobic adsorbent as the stationary phase. The sample is distributed to the adsorbent which, owing to the prerequisite for electroneutrality in the phases, might give rise to changes in the concentration of the UV-absorbing counter ion in the mobile phase. The basic principle can be illustrated by

$$\underset{\text{aqueous mobile phase}}{Q^+ + X^-_{UV}} = \underset{\text{stationary phase}}{QX_{UV}}$$

Parris[11,12] used diisobutylethoxyethyldimethylbenzylammonium and aromatic sulphonates as UV-absorbing counter ions in the aqueous mobile phase in chromatographic separations of bile acids and ionic surfactants. Improvements in detection by using UV-absorbing counter ions in reversed-phase systems have also been obtained by Bidlingmeyer and co-workers[13,14], with phenethylammonium and cetylpyridinium as counter ions and alkylsulphonates as samples.

Our studies on reversed-phase ion-pair chromatographic systems with a UV-absorbing counter ion in the mobile phase have shown that they can be highly suitable for the detection of non-UV-absorbing anions and cations of widely different kinds. The samples give rise to positive or negative peaks depending on their charge and retention and both positive and negative peaks are well suited for quantification. The response depends basically on the molar absorptivity of the counter ion, but it can be changed by varying the composition of the chromatographic systems and it is even possible to limit the response to certain groups of compounds.

This paper describes studies of the influence of the mobile phase composition on the response for cationic and anionic compounds of different kinds using one cation, 1-phenethyl-2-picolinium, and one anion, naphthalene-2-sulphonate, as UV-absorbing components and a moderately hydrophobic adsorbent, μBondapak Phenyl, as stationary phase.

EXPERIMENTAL

Apparatus

The detectors were an LDC UV-III-Monitor and an LDC Spectromonitor III. The pumps were a Gynkotek 600/200 and an Altex 100 A. Rheodyne 70-10 and 71-25 injectors with loop volumes of 25.2 and 15.5 μl, respectively, were used.

The columns (100 × 3.2 mm I.D.) were made of stainless steel with a polished inner surface, equipped with modified Swagelok connectors and Altex 250-21 filters.

The pH measurements were made with an Orion Research Model 801 instrument with Ingold Type 401 combined electrode. The spectrophotometric measurements were made with a Zeiss PMQ II Spektralphotometer.

Chemicals and reagents

Sodium naphthalene-2-sulphonate was obtained from Eastman-Kodak (Rochester, NY, U.S.A.) and was recrystallized from water before use. 1-Phenethyl-2-picolinium bromide (Eastman-Kodak) was converted into the hydroxide by use of an

ion exchanger in the hydroxide form and freed from UV-absorbing impurities by repeated extractions with methylene chloride.

All other chemicals were of analytical-reagent grade.

Chromatographic system

μBondapak Phenyl (10 μm) (Waters Assoc., Milford, MA, U.S.A.) was used as packing. The mobile phases were aqueous solutions of naphthalene-2-sulphonate or 1-phenethyl-2-picolinium, which usually also contained buffering compounds or other salts.

Column preparation

The columns were packed by a slurry technique using water–ethanol (58:42) as the suspending medium. They were washed with 200 ml of water–methanol (1:4) before use. Column testing was carried out with water–methanol (2:3) as mobile phase and toluene and mesitylene as retained samples, giving capacity ratios (k') of about 1 and 4 respectively. Columns that gave reduced plate heights of less than 10 were accepted.

Chromatographic technique

The eluent reservoir, injector, column and connecting tubes were thermostated at 25.0 ± 0.1°C in a water-bath. Detection was effected at 254 nm.

The mobile phase flow-rate was 0.50 ml/min. Equilibrium was obtained after passage of 30–50 ml of mobile phase. No recirculation of mobile phase was used. The samples were injected dissolved in the mobile phase, if not stated otherwise. The volume of mobile phase in the column, V_m, was obtained from the front peak of the chromatogram. Peak areas were determined by planimetry.

RESULTS AND DISCUSSION

The basic reversed-phase chromatographic studies were performed with alkylsulphates, alkylsulphonates, alkylcarboxylates and alkylammonium ions of different degrees of substitution. The only UV-absorbing components were the counter ions, naphthalene-2-sulphonate (NS) or 1-phenethyl-2-picolinium (PEP), and the observed chromatographic peaks were due to changes in the concentration of the counter ions.

Injections of ionized samples in systems of this kind gives rise to two kinds of migrating zones: one for each of the ionized components in the sample and one zone that is typical of the chromatographic system (the system zone). If the sample ion and the UV-absorbing ion have opposite charges, the first peak in the chromatogram is negative, and it can be given by the sample or by the system zone, whichever comes first. The sums of the areas of the positive and the negative peaks are equal. The system peak always has the same capacity ratio, whether it is negative or positive. It is also easy to recognize: injection of mobile phase containing an excess or a deficiency of the counter ion gives a positive or negative peak at the k' of the system peak.

Injection of a sample with the same charge as the UV-absorbing ion in the mobile phase also gives a chromatogram with a sample peak and a system peak, but the direction of the peaks is reversed. The first peak is positive and the second is negative.

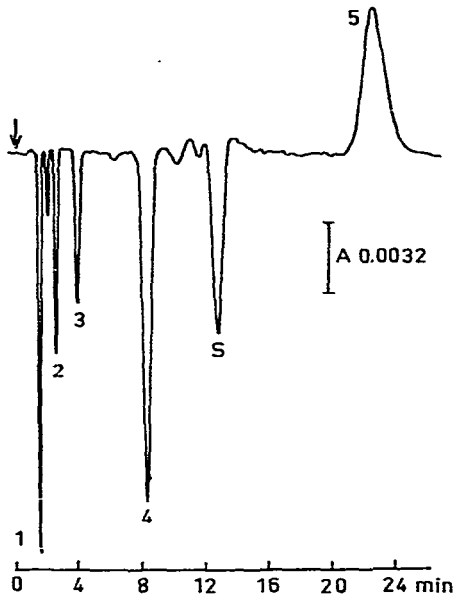

Fig. 1. Carboxylic acids in a system with PEP as UV-absorbing ion. Mobile phase: 1-phenethyl-2-picolinium (PEP), $3 \cdot 10^{-4}$ *M* in acetate buffer (pH 4.6). Solid phase: μBondapak Phenyl. Sample: 1 = acetic acid; 2 = propionic acid; 3 = butyric acid; 4 = valeric acid; 5 = caproic acid (12 nmole of each). S = system peak.

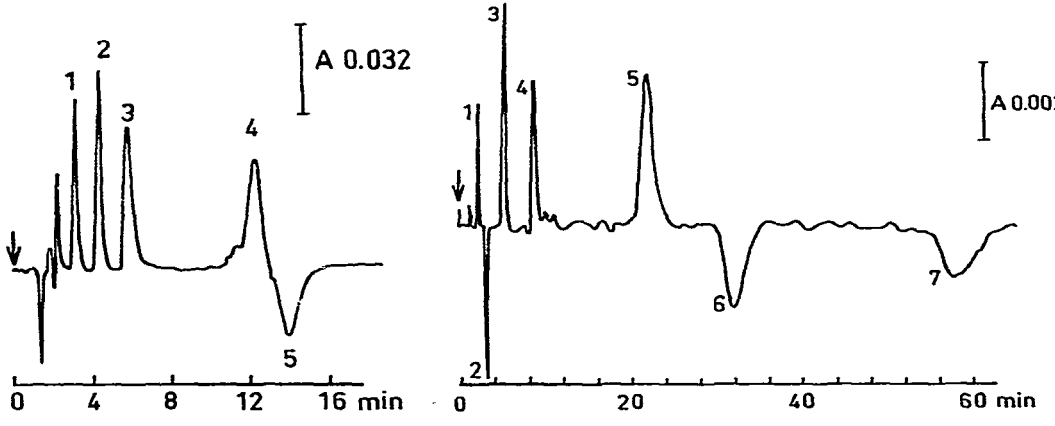

Fig. 2. Quaternary ammonium ions in a system with PEP as UV-absorbing ion. System as in Fig. 1. Sample: 1 = trimethylphenylammonium; 2 = trimethylbenzylammonium; 3 = methyltripropylammonium; 5 = tetrapropylammonium (1.5 nmole of each); 4 = system peak.

Fig. 3. Anionic and cationic compounds with NS as UV-absorbing ion. Mobile phase: naphthalene-2-sulphonate (NS), $4 \cdot 10^{-4}$ *M* in 0.05 *M* phosphoric acid. Solid phase: μBondapak Phenyl. Sample: 1 = butyl sulphate; 2 = pentylamine; 3 = hexanesulphonate; 4 = system peak; 5 = heptylamine; 6 = octanesulphonate; 7 = octyl sulphate.

It has been verified that the total amount of an injected compound is eluted in the sample peak. The tests were made with compounds with inherent UV absorbance in other wavelength regions than that of the counter ion and measurement of peak areas at the wavelength specific for the sample.

A typical chromatogram is given in Fig. 1, which shows the separation of carboxylates with 1–5 alkyl carbon atoms in a system with the cationic PEP as counter ion. The separation factor is about 3 per alkyl carbon atom. The same system can also be used for quaternary ammonium ions but the number of carbon atoms in this case must be higher, 9–12 (Fig. 2).

Fig. 3 shows the separation of a mixture of anionic and cationic compounds, two alkylamines, two alkylsulphates and two alkylsulphonates, with the anionic NS as the UV-absorbing component. The separation factors in this case are also 2.5–3 per alkyl carbon atom. The direction of the peaks follows the general principle, without exception. The anionic samples give positive peaks when they appear before the sample peak (butyi sulphate, hexanesulphonate) and negative after (octanesulphonate, octyl sulphate). The cations are negative before (pentylamine) and positive after (heptylamine) the system peak.

This NS system has pH 2 and some amino acids and dipeptides have acidities such that they are ionized and can be detected at that pH. Some examples are shown in Figs. 4 and 5.

Substances with inherent UV absorbance should, if possible, be chromatographed in systems where they give positive peaks, as inherent UV absorbance decreases the height of a negative peak. An example is given in Fig. 6. Tyrosine and phenylalanine, with low molar absorptivities, give negative peaks, whereas DOPA, with a considerably higher molar absorptivity, gives a positive peak.

In the above cases, the sample was dissolved in the mobile phase. Dissolution of the sample in water does not seem to affect the sample peak, as demonstrated in Fig. 7. The main difference from the previous systems is that the system peak is strongly

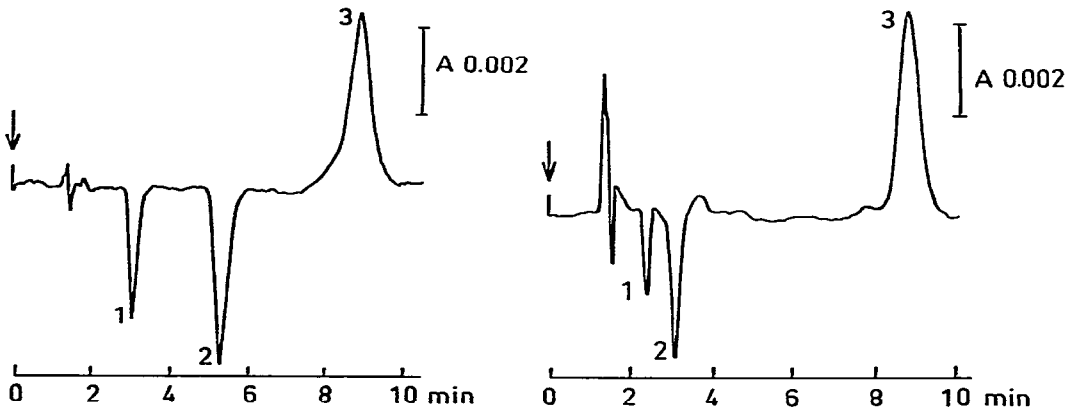

Fig. 4. Amino acids with NS as UV-absorbing ion. System as in Fig. 3. Sample: 1 = norleucine (2 nmole); 2 = phenylalanine (0.8 nmole); 3 = system peak.

Fig. 5. Dipeptides with NS as UV-absorbing ion. System as in Fig. 3. Sample: 1 = leucylserine; 2 = leucylalanine; 3 = system peak.

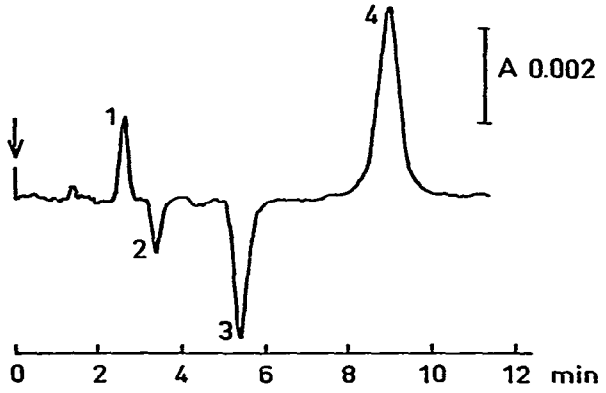

Fig. 6. Amino acids with NS as UV-absorbing ion. System as in Fig. 3. Sample: 1 = DOPA (1.3 nmole); 2 = tyrosine (0.7 nmole); 3 = phenylalanine (0.6 nmole); 4 = system peak.

negative, exactly as when pure water is injected. Some extra front peaks are also obtained.

Retention model

Negative peaks are used for detection purposes in "vacancy chromatography"[15], in which the mobile phase contains a series of detectable compounds at constant concentration. On injection of a sample, negative or positive peaks can be obtained depending on whether the sample contains these components at lower and higher concentration than the mobile phase. Scott *et al.*[16] used this principle for detection of nucleic acid bases separated by cation exchangers.

Šlais and Krejčí[17] used a different technique for the detection of organic sol-

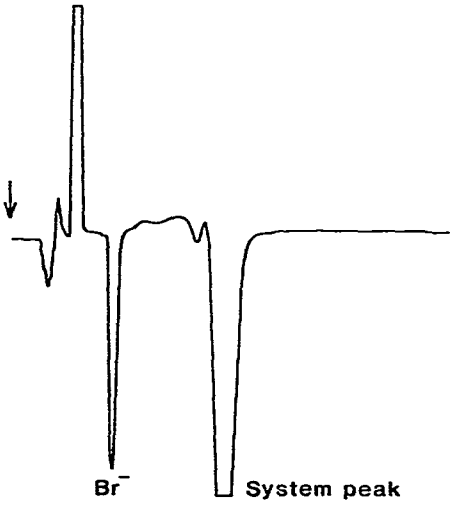

Fig. 7. Sample dissolved in water. Mobile phase: $3 \cdot 10^{-4}$ *M* 1-phenethyl-2-picolinium in 0.1 *M* acetic acid. Solid phase: μBondapak Phenyl. Sample: bromide.

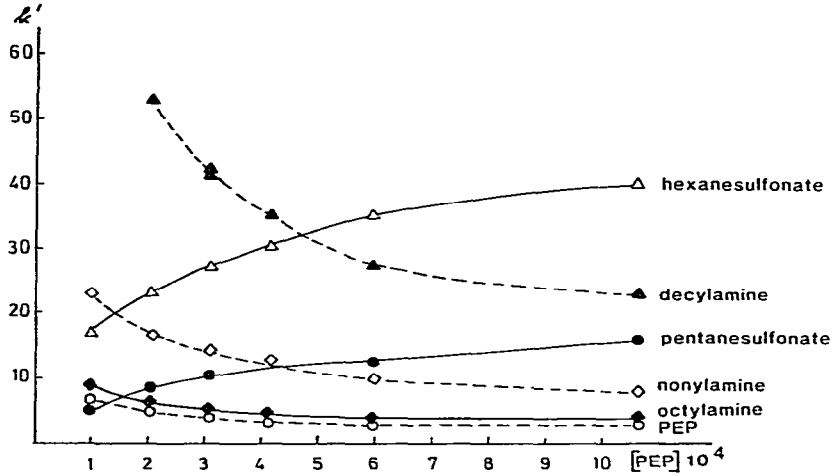

Fig. 8. Retention of anionic and cationic compounds with PEP in mobile phase. Mobile phase: 1-phenethyl-2-picolinium (PEP) in 0.1 *M* acetic acid. Solid phase: μBondapak Phenyl.

vents with a refractive index detector after separation on charcoal. Cyclohexane with a low content of diethyl ether was used as the mobile phase and chromatograms were obtained that contained a sample peak and a "system peak" with constant retention. The two peaks had opposite directions. No mechanism for the observed effect was suggested.

Chromatograms with positive and negative peaks are also obtained by gel permeation chromatography of proteins on columns equilibrated with UV-absorbing, low-molecular-weight cofactors. The phenomenon is due to binding between protein and cofactor and binding constants have been calculated from the peak areas[18–20].

In the present case, the column is equilibrated with a mobile phase containing a UV-absorbing ion and it is likely that the observed peaks are due to changes in its distribution to the solid phase as an ion pair. The distribution changes can be elucidated to a certain extent by studies of the influence of the composition of the mobile phase on the capacity ratio of samples of different charge.

The relationship between the capacity ratio and the concentration of the UV-absorbing mobile phase component (the counter ion) is demonstrated in Fig. 8. The k' of the counter ion (PEP) and k' of all samples of the same charge decrease with increasing PEP concentration, whereas the retention of samples of opposite charge increases.

Addition of other ions to the mobile phase will also affect the retention. In a system with an anionic UV-absorbing mobile phase component, NS, an increase in the concentration of an ion of opposite charge, tetramethylammonium (TMA^+), increases the retention of the UV-absorbing component, whereas samples of the same charge as the added ion show decreased retention (Fig. 9).

Further illustrations are given in Fig. 10, which shows the influence of the hydrophobicity of the ions that are added to the mobile phase. A change in the cationic component from Na^+ to tetraethylammonium increases the retention of the

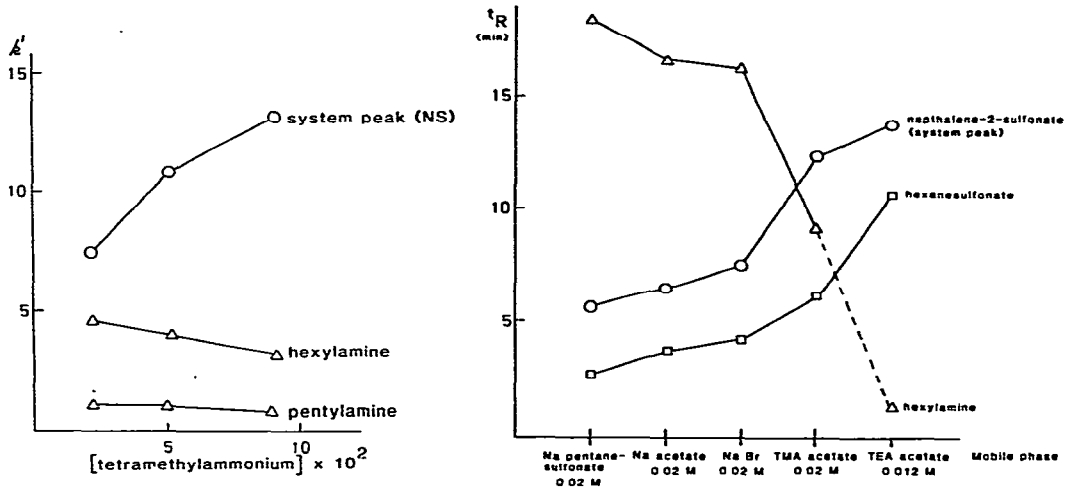

Fig. 9. Retention of cationic compounds with NS and tetramethylammonium in mobile phase. Mobile phase: $4 \cdot 10^{-4}$ *M* naphthalene-2-sulphonate (NS) and tetramethylammonium in phosphate buffer (pH 2.0). Solid phase: μBondapak Phenyl.

Fig. 10. Retention of cationic and anionic compounds with NS and different cations and anions in mobile phase. Mobile phase: $4 \cdot 10^{-4}$ *M* naphthalene-2-sulphonate in aqueous salt solutions (pH 5). Solid phase: μBondapak Phenyl.

sulphonates considerably whereas the retention of the ammonium ion decreases to such an extent that a sample peak can no longer be observed. A change in the anionic component from acetate to pentanesulphonate has the opposite effect.

The response pattern seem to depend on the presence of ions in the mobile phase besides the UV-absorbing component. A system with $4 \cdot 10^{-4}$ *M* sodium naphthalene-2-sulphonate in water as the mobile phase only gives a normal response for strongly hydrophobic ions.

These results indicate that the retention of the ionic samples and the UV-absorbing mobile phase ion follows the general rules for reversed-phase ion-pair chromatography with a hydrophobic adsorbent as the stationary phase[21–23]. The UV-absorbing mobile phase component is distributed to the hydrophobic adsorbent as an ion pair with other mobile phase components (cations for NS; anions for PEP). The sample is distributed to the stationary phase as an ion pair with the UV-absorbing ion or with other mobile components of the opposite charge. There is a competition between the ion pairs for the limited capacity of the adsorbent. Measurements of the adsorption of NS from a mobile phase with 0.05 *M* phosphoric acid as solvent have shown that it follows a Langmuir expression, as indicated by a reciprocal plot of amount adsorbed *versus* mobile phase concentration[21].

These facts can be summarized in the following expressions for the capacity ratio of a cationic sample, HA^+, and an anionic sample, Z^-, when the mobile phase contains NS^- (UV-absorbing component), Y^- and Q^+:

$$\log k'_{HA} = \log K_0 \cdot q + \log (K_{HANS}[NS] + K_{HAY}[Y]) - \log \{1 + [HA](K_{HANS}[NS] + K_{HAY}[Y]) + [Q](K_{QNS}[NS] + K_{QY}[Y])\} \quad (1)$$

$$\log k'_Z = \log K_0 \cdot q + \log K_{QZ}[Q] - \log \{1 + [Q](K_{QZ}[Z] + K_{QNS}[NS] + K_{QY}[Y])\} \quad (2)$$

[NS], [Z], [HA], [Q] and [Y] represent concentrations of the ionic components in the mobile phase in the migrating zone, K_{HANS}, K_{HAY}, K_{QNS}, K_{QZ} and K_{QY} are constants for ion-pair distribution between mobile and stationary phase, K_0 is the capacity of the adsorbent and q the phase ratio in the column[21-23]. It is assumed that the adsorbent has one kind of adsorption site only.

Response

The injection of an ionic sample can be assumed to give rise to changes in the concentrations of all ionic mobile phase components in the injection zone. All of these ions (UV-absorbing ion, other anions and cations) will migrate in separate zones with the mobile phase, which is supplied to the system at constant composition.

The sample usually has a low concentration (about 10^{-4} *M*) and it will give rise to small changes in the concentrations of the other mobile phase ions. The effect on the capacity ratio, which is controlled by the concentration of these ions, is therefore very limited: an increase in the concentration of a cationic sample ($k' = 16$) from $2 \cdot 10^{-5}$ to $2 \cdot 10^{-4}$ *M* decreases its retention by about 2% in an NS system similar to that used in Fig. 3. A small change in the concentration of a highly UV-absorbing ion can, however, be measured with high precision with a UV detector, which registers the difference in concentration.

The response pattern in the chromatograms is due to competing distribution processes comprising of binding and displacement of the UV-absorbing ion. A qualitative interpretation can be based on assumptions regarding the distribution changes on application of the sample. An example is given in Table I. The concentration of the UV-absorbing ion in the mobile phase, [NS], changes in the injection zone. This gives rise to a migrating NS-zone beside the sample zone, and the change of [NS] is transferred to the faster of the migrating zones. The change of NS-concentration in the system zone compensates that in the sample zone.

The UV-absorbing ion is usually the only mobile phase component that gives

TABLE I

RESPONSE WITH ANIONIC UV-ABSORBING ION IN MOBILE PHASE

Ions in mobile phase: NS^- (UV-absorbing), Y^- and Q^+.

Sample	*Capacity ratio*	*Distribution processes*	*NS^- peak (system peak)*	*Sample peak*
HA^+	$k'_{HA} < k'_{NS}$	Binding of HANS dominates over displacement of QNS	Positive	Negative
	$k'_{HA} > k'_{NS}$		Negative	Positive
Z^-	$k'_Z < k'_{NS}$	Displacement of QNS dominates over binding of QZ	Negative	Positive
	$k'_Z > k'_{NS}$		Positive	Negative

TABLE II

OBSERVED MOLAR ABSORPTIVITY (ε') OF NAPHTHALENE-2-SULPHONATE

Solvent: water.

Maximum concentration in the range ($mol/l \cdot 10^4$)	$\Delta C \cdot 10^5$ (mol/l)	ΔA	ε'
2.41	5.98	0.155	2595
3.70	4.24	0.100	2374
3.91	2.11	0.0494	2341
4.23	3.18	0.0740	2327
5.43	6.04	0.135	2243

an easily observed chromatographic peak. Q^+ and Y^- are often highly hydrophilic with short retentions and give peaks that appear close to the front.

The amount of counter ion in the sample peak can be calculated from the peak area and the molar absorptivity of the ion. The absorbance of the mobile phase at 254 nm ranges between 0.3 and 1.2, depending on the concentration of the UV-absorbing counter ion. The observed molar absorptivity of the counter ions decreases gradually with increasing absorbance in the range $A = 0.1$–1.0, probably owing to stray-light disturbances in the UV detector. The changes obtained with naphthalene-2-sulphonate are demonstrated in Table II, which gives the observed molar absorptivity, $\varepsilon' = \Delta A/\Delta C$, in different concentration ranges, where ΔA is the change in absorbance given by a certain change in concentration, ΔC.

The peak heights (positive and negative) correspond under normal chromatographic conditions to an absorbance change of less than 0.05 units. The observed molar absorptivity under these conditions can be considered as almost constant, as seen from Table II, and it can be used in a calculation of the amount of counter ion in a chromatographic peak from the measured peak area.

The response factor, *i.e.*, the amount of counter ion in the peak divided by the

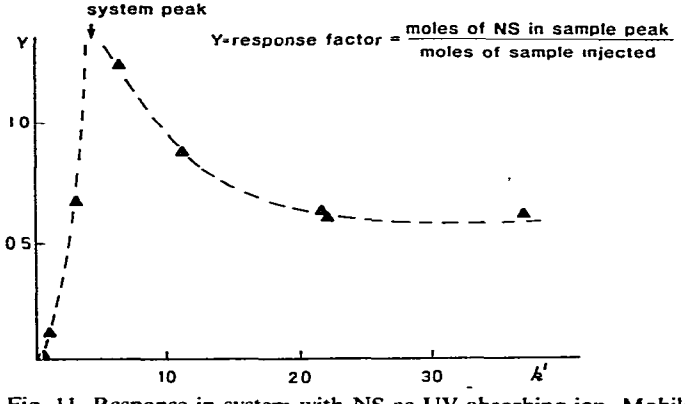

Fig. 11. Response in system with NS as UV-absorbing ion. Mobile phase: $3.7 \cdot 10^{-4}$ *M* naphthalene-2-sulphonate in 0.01 *M* phosphoric acid. Solid phase: μBondapak Phenyl.

amount of sample injected, is strongly dependent on the capacity ratio of the sample, as illustrated in Fig. 11. The response factor increases with increasing k', goes through a maximum when the sample and the system peak have about the same retention and levels out at higher k'. The influence of the composition of the system on the response factor at high k' has so far not been elucidated.

Remarkably high response factors are often obtained when the sample and the counter ion have about the same capacity ratio and even response factors higher than 4 have been obtained. The effect seems to be due to interaction between the ions in the two zones.

Optimization of the response factor of a sample can be achieved by changing the mobile phase composition in such a way that the k' values of the sample and system peak coincide. If the sample and the UV-absorbing ion have different charges, an increase in the response factor might be obtained by changing the concentration of the UV-absorbing component, as demonstrated by Fig. 8 and Table III.

TABLE III

RESPONSE FACTORS WITH 1-PHENETHYL-2-PICOLINIUM AS UV-ABSORBING ION

Conditions as in Fig. 8.

$[PEP] \cdot 10^4$ (mol/l)	*Response factor*		
	Pentanesulphonate	*Octylamine*	*Nonylamine*
1.01	1.14	2.16	0.63
2.07	0.95	2.16	0.58
3.08	0.60	1.97	0.60
4.13	0.60	1.75	0.53

Pentanesulphonate and PEP have about the same retention when the PEP concentration is about $1 \cdot 10^{-4}$ M (Fig. 8). The response factor is about 1.1 under these conditions (Table III), but it decreases with increasing PEP concentration and increasing deviation in retention. Octylamine, which has the same charge and also about the same retention as PEP, retains a very high response in the whole concentration range. The more strongly retained nonylamine gives a considerably lower response.

The retention and the response factors can also be regulated by other mobile phase components, as has been demonstrated for hexylamine and hexanesulphonate in Fig. 10 and Table IV. An increase in the size of the cation in the mobile phase has a drastic effect on the response of hexylamine. In the presence of 0.02 M tetramethylammonium the sample peak is close to the system peak (NS) and a response of more than 1.5 is obtained. Addition of tetraethylammonium, on the other hand, will decrease the retention to such an extent that no response at all is obtained.

Quantitation

The relationship between amount injected and peak height has shown good linearity up to 10 nmoles for both positive and negative peaks. The counter ions used

TABLE IV

RESPONSE FACTORS WITH NAPHTHALENE-2-SULPHONATE AS UV-ABSORBING ION

Conditions as in Fig. 10.

Mobile phase	*Response factor*	
	Hexylamine	*Hexanesulphonate*
Sodium pentanesulphonate (0.02 *M*)	0.24	0.05
Sodium acetate (0.02 *M*)	0.62	0.30
Sodium bromide (0.02 *M*)	0.60	0.67
Tetramethylammonium acetate (0.02 *M*)	1.58	0.18
Tetraethylammonium acetate (0.012 *M*)	—	0.12

in these studies have molar absorptivities between 2300 and 3100 and they permit the quantitation of about 0.1 nmole of a sample with acceptable precision if the conditions are such that the response factor is about 0.5.

The background absorbance of the mobile phase is high, as mentioned above, and the observed changes in absorbance are often only a few parts per thousand. It is obvious that the precision is highly dependent on the quality of the equipment and the stability of the experimental conditions. High-quality pumps with pulseless flow and careful thermostating of the whole system are prerequisites for good precision in quantitation at the lowest levels.

The systems are usually very easy to prepare: a stable baseline is normally obtained within 1 h after the introduction of a new mobile phase. The stability is extremely good: with mobile phases of pH 2–6 it is possible to run the systems for several months without a significant change in their properties.

Studies have so far been performed only with UV-absorbing counter ions and UV detectors. It might also be possible, however, to use fluorescent or electroactive counter ions to obtain higher sensitivity.

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