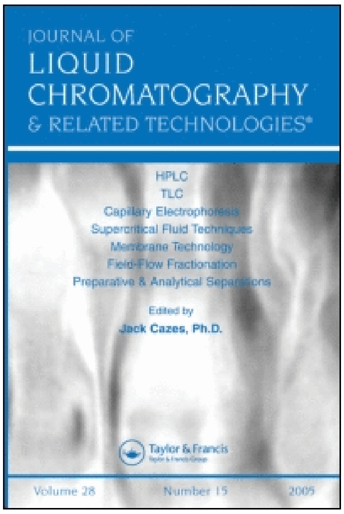

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Sorel, R. H. A. , Hulshoff, A. and Wiersema, S.(1981) 'Reversed Phase High Performance Liquid Chromatography with Cetrimide Containing Eluents', Journal of Liquid Chromatography & Related Technologies, 4: 11, 1961 — 1985

To link to this Article: DOI: 10.1080/01483918108067555

URL: http://dx.doi.org/10.1080/01483918108067555

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(11), 1961-1985 (1981)

REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH CETRIMIDE CONTAINING ELUENTS

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ABSTRACT

A radially compressed column, packed with micro particle, reversed phase (C 18) material, was used to study HPLC with cetrimide containing eluents. The amount of cetrimide adsorbed onto the stationary phase was measured; not the number of available adsorption sites, but rather the presence of micelles in the eluent appears to be the limiting factor for the uptake of cetrimide from the eluent.

The capacity factors, k', of several - mainly acidic - compounds were determined in this system, with varying pH and cetrimide concentration of the eluent (methanol-water, 50% w/w). The results obtained upon changing the pH of the eluent can not all be explained with an ion-exchange (or ion-pair) model.

Upon increasing the cetrimide concentration, maximum values in k' are reached at about the critical micelle concentration (cmc) of cetrimide in the eluent. The results of conductimetric experiments suggested, that the decrease of k' at cetrimide concentrations above this CMC, observed for most of the compounds, can be explained by partitioning of the solutes between the micelles and the bulk of the mobile phase. From these experiments it was also clear that ion-pair formation between cetrimide and solutes is possible.

INTRODUCTION

Recently, chromatography on non-polar phases with ion-pair reagents, with or without marked surface activity, added to the eluent has been the subject of many studies. Different theories have been developed to explain the retention behaviour of solutes in these systems. Most of these theories have been discussed recently by Bidlingmeyer (1) and by Knox and Hartwick (2). One of the points which have not been settled yet is, how to explain a maximum in the capacity factor, k', when k' is plotted against the pairing-ion concentration in the eluent.

Three theories have been proposed to explain this phenomenon which was observed by many authors (2-10);

a) "The decrease in k' at higher counter ion concentrations may be due to the combined effect of a decrease in the adsorption capacity of the retaining phase and an ion-pair formation ...." (7);

b) Above a critical concentration a number of surface active ions are able to form micelles; solutes are supposed to partition between the micelles and the bulk of the mobile phase (2-4, 10);

c) Hung and Taylor (9) explain the decrease in k' by assuming a decrease of the surface which is available for hydrophobic interaction, caused by the larger amount of pairing-ion adsorbed onto the surface.

Secondly, according to these authors, the increased concentration of the counter ions accompanying the pairing ions results in less possibilities for ion-exchange of the solute ions with the adsorbed pairing-ions.

In this investigation the retention behaviour of various compounds, with different acidic and basic properties, were studied at different cetrimide (the "pairing-ion") concentrations. Possible interactions between solutes and cetrimide above as well as below the critical micelle concentration (CMC) were studied by conductimetry.

The factors which influence the adsorption of cetrimide onto the stationary phase are also of interest. Different authors studied the adsorption of cetrimide as a function of the cetrimide concentration in the eluent (3, 4, 9, 10); little is known about the influence of the pH on the adsorption phenomena (4).

In the present study the influence of pH on the chromatographic behaviour of mainly acidic compounds is described. However, the chosen pH range is higher than usual, so the effect of pH on k' could be studied under conditions where for instance carboxylic acids are completely deprotonated.

MATERIALS

Apparatus

The chromatographic system consisted of a 6000 A solvent delivery system, a U6K injection system and a Radial-Pak A cartridge, 10 cm x 8 mm I.D., in combination with an RCM-100 Radial Compression Module (all from Waters Assoc., Milford, Mass., USA). The column was installed between the U6K injection system and two on-line detectors, a model 440 differential UV absorbance detector, operated at 254 nm and an R401 refractive index detector (both from Waters Assoc.). By means of a Valco rotary six port 7000 psig valve (Chrompack, Middelburg, The Netherlands), connected between the column and the detectors, a bypass could be used to flush the entire system except the column.

Retention times were obtained by means of an SP4000 (Spectra-Physics, Santa Clara, CA, USA) data system.

For measuring pH values a Radiometer (Copenhagen, Denmark) PHM 64 pH meter was used.

The conductance was measured with a Consort (Turnhout, Belgium) K620 conductometer in combination with a type EA 645 titration cell (Metrohm, Herisau, Switzerland).

Conductance and chromatographic experiments were carried out at 25.0 ±0.1° C and 25.0 ±0.2°C, respectively.

Chemicals

Methanol, potassium bromide and sodium hydroxyde were of analytical reagent grade and were obtained from Merck (Darmstadt, G.F.R.). Cetyl trimethylammonium bromide (cetrimide) was purchased from B.D.H. (Poole, England). Boric acid, "analyzed" reagent, was obtained from Baker (Deventer, The Netherlands). 2-Methoxy methyl benzoate was prepared by methylation of methyl salicylate with methyl iodide, in an analogous manner as was reported for the propylation of nalidixic acid with propyl iodide (11). The other compounds under investigation were commercially available products and were used without further purification.

Cetyl trimethyl ammonium (CTA) containing eluents were prepared in water-methanol mixtures (final ratio in the eluent, 1:1 w/w) with 0.1% w/w up to 1.6% w/w (= 2.74 x 10^{-3} - 4.39 x 10^{-2} mol•kg^{-1}) cetrimide; potassium bromide was added to a final bromide concentration of 0.1 mol•kg^{-1}; the eluents were buffered with boric acid buffers, 0.025 mol•kg^{-1} eluent and adjusted to the desired pH with sodium hydroxide, measured against water-methanol buffers (12). The pH values in the water-methanol system are indicated by pH*.

METHODS

The amount of cetrimide adsorbed onto the column was studied as a function of pH and of the cetrimide concentration in the eluent.

First the chromatographic system was equilibrated with the eluent of the desired pH without the cetrimide; after that the cetrimide containing eluent with the same pH was pumped through the column. The amount of cetrimide adsorbed would then be (V_r-V_o) . C, where V_r= breakthrough volume of cetrimide as indicated by the refractive index detector, V_o= void volume and C= concentration of cetrimide in the eluent.

After the breakthrough volume of cetrimide was registered more solvent was pumped through the column, until the pH of the eluate had become exactly the same as the pH of the eluent (12). Cetrimide was removed from the column with the same eluent without cetrimide. This stripping process was followed with the refractive index detector. In order to check whether all of the adsorbed cetrimide had been removed, the retention volume of salicylic acid was determined, because this volume was found to be strongly influenced by the presence of even small amounts of cetrimide.

The pKa values of some of the compounds under investigation in water-methanol, 1:1 w/w, were determined according to the method described by Hulshoff and Perrin (14); these pKa values are indicated by pKa*.

Critical micelle concentrations of cetrimide in the different eluents were measured conductimetrically as described before (10). Other conductimetric measurements were carried out to study possible interactions between some of the compounds under investigations and cetrimide. In these measurements 6.25 x 10^{-6} mol of these compounds were added to 40 g water-methanol mixtures, containing varying concentrations of cetrimide (below and above the CMC).

RESULTS AND DISCUSSION

Adsorption of cetrimide onto the column packing material

We reported earlier on the breakthrough patterns of cetrimide containing eluents and on the pH-shifts which can be observed during these processes (13). The amount of cetrimide adsorbed onto the column is shown as a funciton of pH* in Fig. 1a and in Fig. 1b as a function of the cetrimide concentration in the eluent. The phenomenon of increasing amounts of cetrimide adsorbed onto the column at increasing pH we have discussed before (13). Knox and Laird (3) found, that the adsorption isotherm of cetrimide on SAS silica obeyed a Freundlich type equation.

Hung and Taylor (9) state that neither a Freundlich nor a Langmuir type of equation can be used to describe the adsorption of quaternary nitrogen compounds. Assuming that the carbon chains lie flat on the surface, they made the contradictory observation that the larger the alkyl groups of the quaternary nitrogen compounds, the more surface area seemed to be available. They therefore introduced the wellknown Hansch theory to describe the adsorption as a function of the hydrophobicity of the longest alkyl group of the pairing ion. However, we suppose that the carbon chains of the pairing ions are situated between the C_{18} brushes of the stationary phase. In principle for each type of reagent molecule an equal number of sites of adsorption will then be

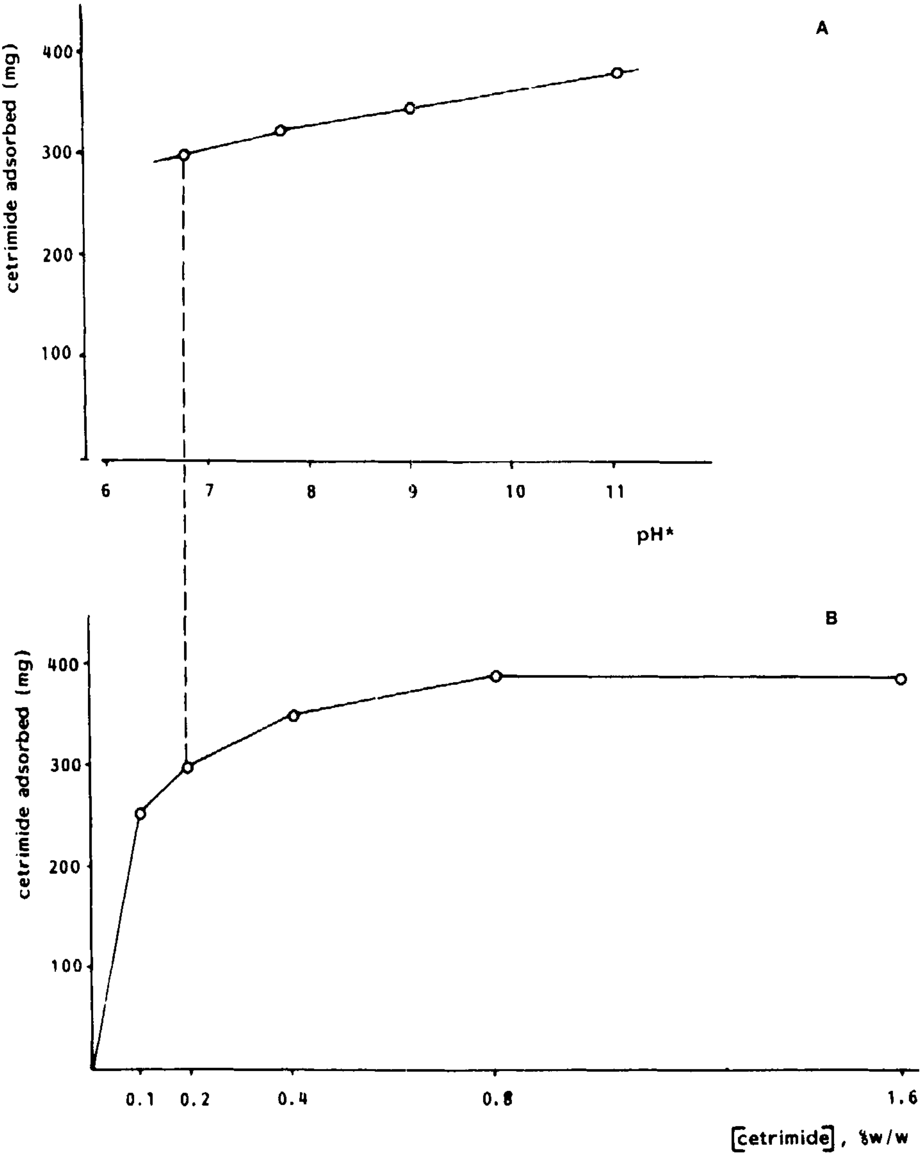


FIGURE 1 Adsorption of cetrimide onto the column, (A) as a function of the pH* of the eluent; cetrimide concentration, 0.2% w/w, and (B) as a function of the cetrimide concentration in the eluent at pH* 6.8. For other conditions, see Materials.

available and for each of these reagents an adsorption isotherm can be used. The hydrophobic interaction increases with the length of the carbon chain, resulting in a higher level of maximal adsorption for the reagents with longer alkyl groups. The adsorption isotherms found in our experiments, particularly with respect to the data at the higher cetrimide concentrations, did not fit quite well to the empirical Freundlich type equation. The reason for this is simply the presence of cetrimide micelles above 0.7% w/w cetrimide in the eluent. The CMC values, as determined in the eluents and in different methanol-water mixtures, are summarized in Table I.

At increasing cetrimide concentration above the CMC the cetrimide monomer concentration will only slightly increase (dependent on the hydrophobicity of the eluent). So the limiting factor for the uptake of cetrimide out of the mobile phase is not necessarily the surface coverage of the stationary phase, but more likely the cetrimide monomer concentration in the eluent. As is evident from Figure 1b, the maximal adsorption is indeed reached near the CMC of cetrimide.

Although our results fit quite well with a Langmuir type of equation, in fact neither a Freundlich nor a Langmuir isotherm can therefore be used when concentrations above the CMC are included in the data.

TABLE I

Critical Micelle Concentrations of Cetrimide

Solvent		CMC ± SD (% w/w)
eluent[1]	pH* 6.8	0.70 ± 0.03
	pH* 9.0	0.70 ± 0.03
	pH* 11.0	0.85 ± 0.04
water		0.036 ± 0.001[2]
methanol/water	10% w/w	0.045 ± 0.001
	20% w/w	0.090 ± 0.004
	30% w/w	0.21 ± 0.01
	40% w/w	0.47 ± 0.04
	50% w/w	0.95 ± 0.04

1) see MATERIALS, *Chemicals*

2) the same value has been reported in REFERENCE (25)

Recently Knox and Hartwick (2) published adsorption isotherms of some anionic surfactants onto Hypersil ODS. They estimated the CMC values of these surfactants in the eluents from data reported in the literature. Their results show that the adsorption of these reagents increases even above these CMC values, so we suppose that their estimations are not accurate enough. The adsorption curve for cetrimide, as reported by Hung and Taylor (9) reaches a maximum close to the CMC of cetrimide in water (which is the eluent they used). The consequences of this for their hypothesis concerning the retention mechanism will be considered further below.

Influence of pH on the retention

In Figure 2, k' values for the different compounds are plotted versus the pH* of the eluent. Some unexpected results were obtained. 2-Methoxy-methyl benzoate, a neutral compound, shows a marked decrease in k' with increasing pH*. The conclusion has to be that at higher

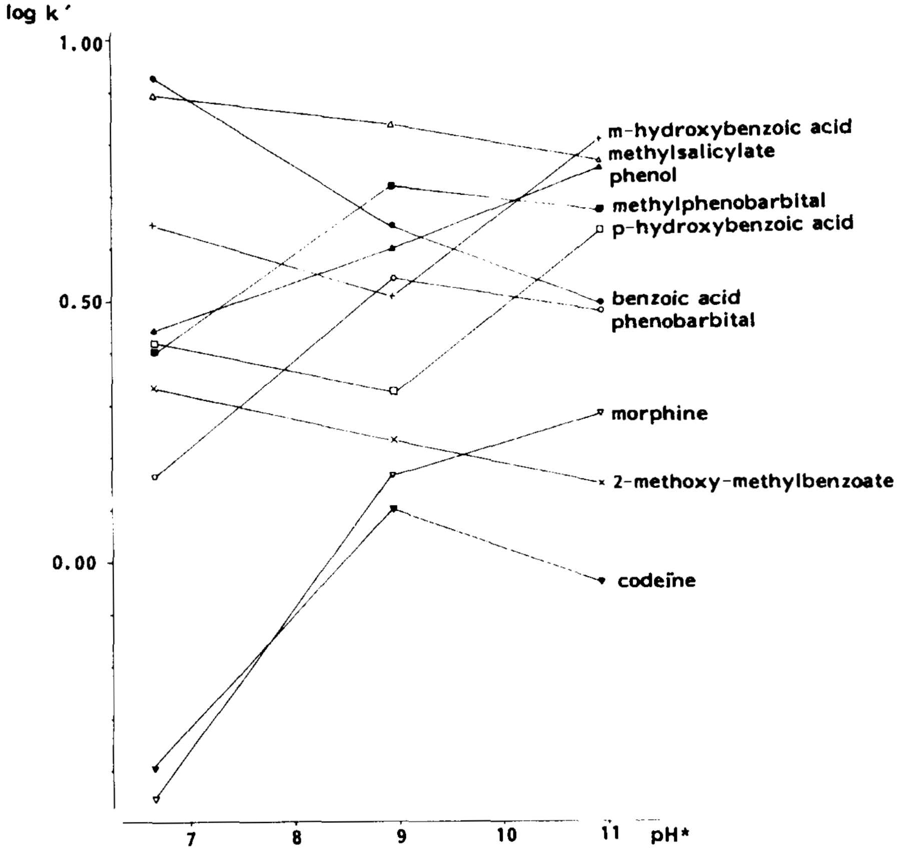


FIGURE 2 Influence of the pH* of the eluent on log k'; cetrimide concentration, 0.2% w/w; for other conditions, see Materials.

pH values the available area for hydrophobic interactions is decreased. A possible explanation can be found in the work of Hung and Taylor (9), who stated that the more ion-pairing reagent is adsorbed onto the surface, the less sites are available for desolvation of solutes during chromatography.

We observed that more cetrimide was bound onto the column at higher pH (Fig. 1a); therefore their hypothesis might help to explain this phenomenon of decreasing k' with increasing pH. With benzoic acid (pKa* = 5.2), present as anion in the entire pH range, the same behaviour was observed as the neutral compound.

Although it was expected that k' would increase, because the increased amount of adsorbed cetrimide should give more possibilities for ion-exchange chromatography, we have to conclude that another (unknown) mechanism is working in the opposite direction.

On introducing a phenolic function into the benzoic acid molecule, k' increases between pH* 9 and 11 (See data for m-hydroxybenzoic acid and p-hydroxybenzoic acid). This is in agreement with an increase in retention due to anion-exchange, for in this pH range the phenolic functions are getting deprotonated, thus doubling the negative charge of the ions.

Phenol itself shows the same behaviour in the pH range (9-11). Of the other phenolic compounds only

methyl salicylate is an exception; it shows a decrease in k'.
The intramolecular hydrogen bonding, which is known to exist with this type of compound, is probably responsible for this different behaviour.

For phenobarbital (pKa* = 8.2) and mephobarbital (pKa* = 8.4) k' increases between pH* 7 and 9, this is also in good agreement with the ion-exchange theory. However, at pH* 11, k' for both compounds is decreased. Possibly the same mechanism is responsible as the mechanism causing the decrease in k' for e.g. benzoic acid and 2-methoxy-methyl benzoate.

For codeine (pKa* = 7.6) and morphine (pKa* = 7.7) k' increases from pH* 7 to 9; in this range the nitrogen groups are getting deprotonated, so the possibilities for hydrophobic interaction are increased. Above pH* = 9, the phenolic function of morphine causes an increase in k'.

Similar patterns of log k' versus pH* were obtained at 1.6% w/w cetrimide (above the CMC) in the eluent (Results not shown).

Influence of the cetrimide concentration on k'

In figure 3, k' is plotted versus the cetrimide concentration in the eluent at pH* 9. At this pH the CMC is 0.70% w/w (see Table I). For all weak and strong acids there is a sharp increase in k' when cetrimide is added to the eluent, till a maximum value is reached.

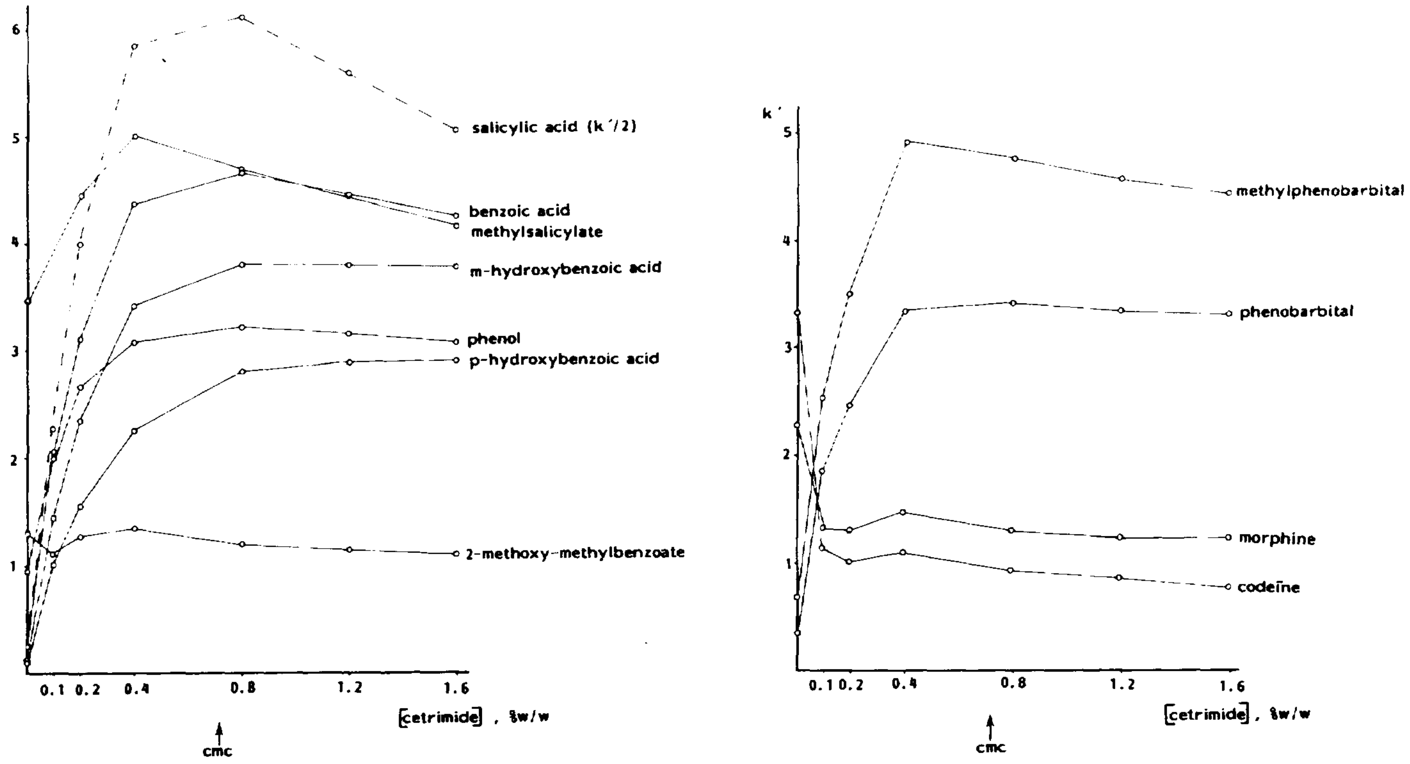


FIGURE 3 Influence of the cetrimide concentration in the eluent on k' at pH* 9. For other conditions, see Materials.

These first parts of the different plots correspond very well with the adsorption isotherm of cetrimide (Fig. 1b). The ion-exchange mechanism, therefore, seems to be an important retention mechanism for these compounds. This is in agreement with the results found by several investigators (2,4, 9, 15-19). The maximum in k' for the acidic compounds coincides rather well with the CMC of cetrimide in the eluent.

At cetrimide concentrations above the CMC, for some compounds, k' decreases (e.g. salicylic acid) or k' does not change anymore (e.g. m-hydroxybenzoic acid).
For the two basic compounds, codeine and morphine, the addition of cetrimide to the eluent results in a decrease of k', caused by the loss of adsorption sites onto the silica matrix, as was apparent from the improved peak symmetry. Furthermore, k' of morphine is larger than k' of codeine in the presence of cetrimide; this is caused by the anion-exchange possibilities for morphine due to its phenolic function.

Hung and Taylor (9) claim that the decrease in k' at higher pairing-ion concentrations has nothing to do with the occurrence of micelles, because they also found a decrease in k' with non-surfactants as ion-pair reagents (tetramethyl ammonium, tetraethyl ammonium and tetrabutyl ammonium). This general decrease in k' is ascribed by them to two factors: a) a decrease in the C_{18} surface available for desolvation of the solutes, and

b) the increasing counterion concentration at higher concentrations of the ion-pair reagents, which will lead to a decrease in retention due to ion-exchange processes.

However, in the present investigation, the surface area available for desolvation will not change anymore above the CMC (as discussed before). Secondly, the counterion concentration was kept constant, so we have to conclude that another mechanism is responsible for the decrease in k'.

In fact the same conclusions can be drawn for their (9) own results with cetrimide. Because the eluent used by Hung and Taylor was water, they also have to deal with micelles above 0.036% cetrimide.

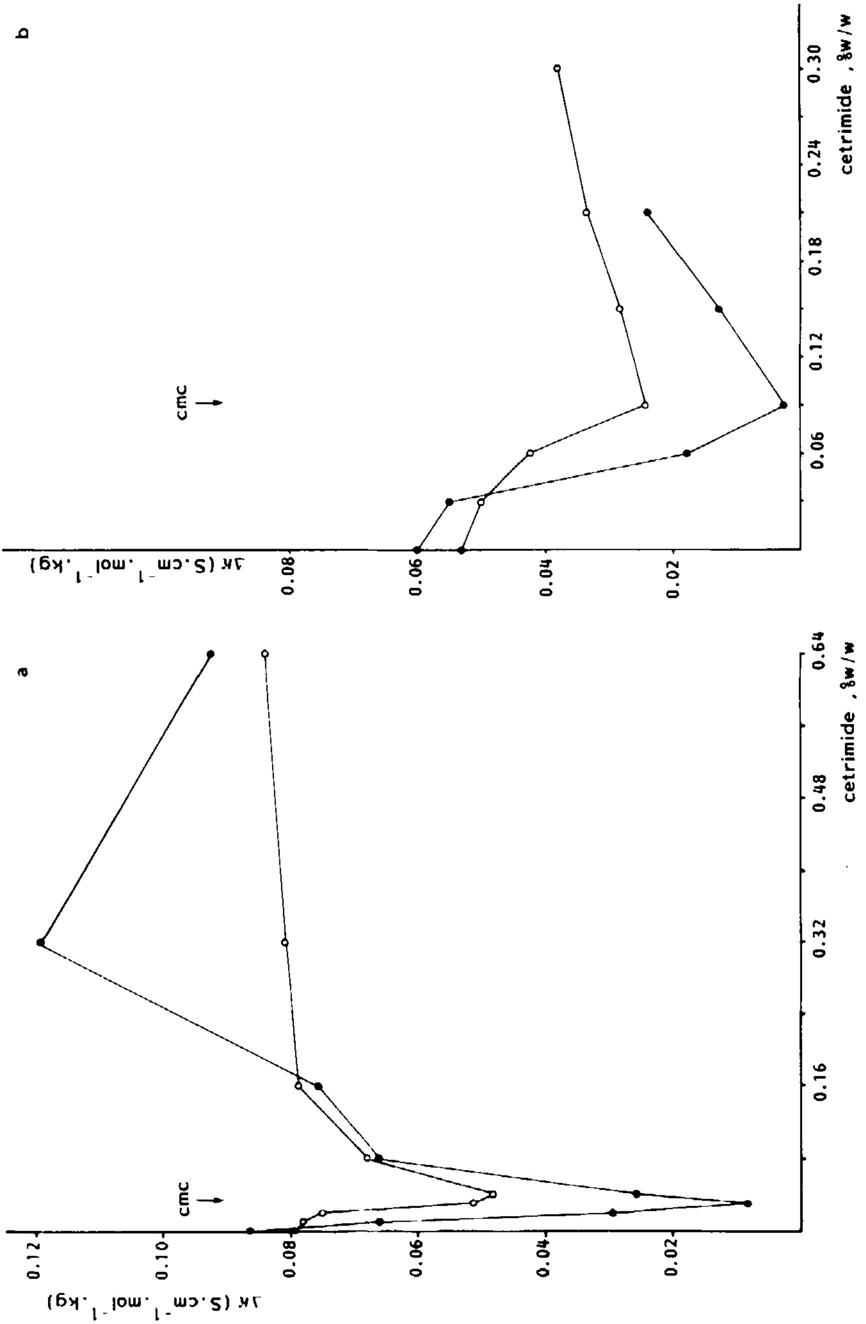
Once the CMC has been reached, the addition of more cetrimide to the eluent will only result in an increase of the number and size of the micelles. The monomer concentration, and therefore also the amount of adsorbed cetrimide and the free counterion concentration will remain practically constant above the CMC. The sharp decrease in k' values observed by Hung and Taylor in cetrimide containing eluents must therefore be attributed to another mechanism than those proposed by them. However, for the other pairing ions in their investigation, their theory is possibly correct, because indeed the counterion concentration increases at increasing pairing ion concentration.

Interaction between cetrimide and solute anions

Possible interactions between some of the anions under investigation and cetrimide were studied by conductimetric experiments. Salicylicate and m-hydroxy benzoate were chosen; the first anion, because it shows a strong decrease in k' above the CMC of cetrimide, while for the second anion k' seems to be independent of the cetrimide concentration above the CMC (Fig. 3). Both compounds were added as their sodium salts to solutions of different concentrations of cetrimide (below and above the CMC). With the offset of the conductometer the contribution of the cetrimide itself to the conductance was corrected for.

The eluents used in this study, contain comparatively high concentrations of ionic substances; this would prevent the measurement of small changes in conductivity, so pure water-methanol mixtures were used.

Figure 4 shows the *increase* in specific conductivity, Δk expressed in $S \cdot cm^{-1} \cdot mol^{-1} \cdot kg$, of different cetrimide containing solutions after the addition of sodium salicylate or sodium m-hydroxy benzoate. It is apparent from these figures that in solvents containing up to 30% methanol a minimum value for Δk exists, which coincides with the CMC of cetrimide, determined at each of these methanol concentrations. There is obviously an interaction between salicylate (and m-hydro-


b
cmc
Jκ (S.cm^{-1}.mol^{-1}.kg)
0.08
0.06
0.04
0.02
0.06
0.12
0.18
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cetrimide , %w/w
a
cmc
Jκ (S.cm^{-1}.mol^{-1}.kg)
0.12
0.10
0.08
0.06
0.04
0.02
0.16
0.32
0.48
0.64
cetrimide , %w/w

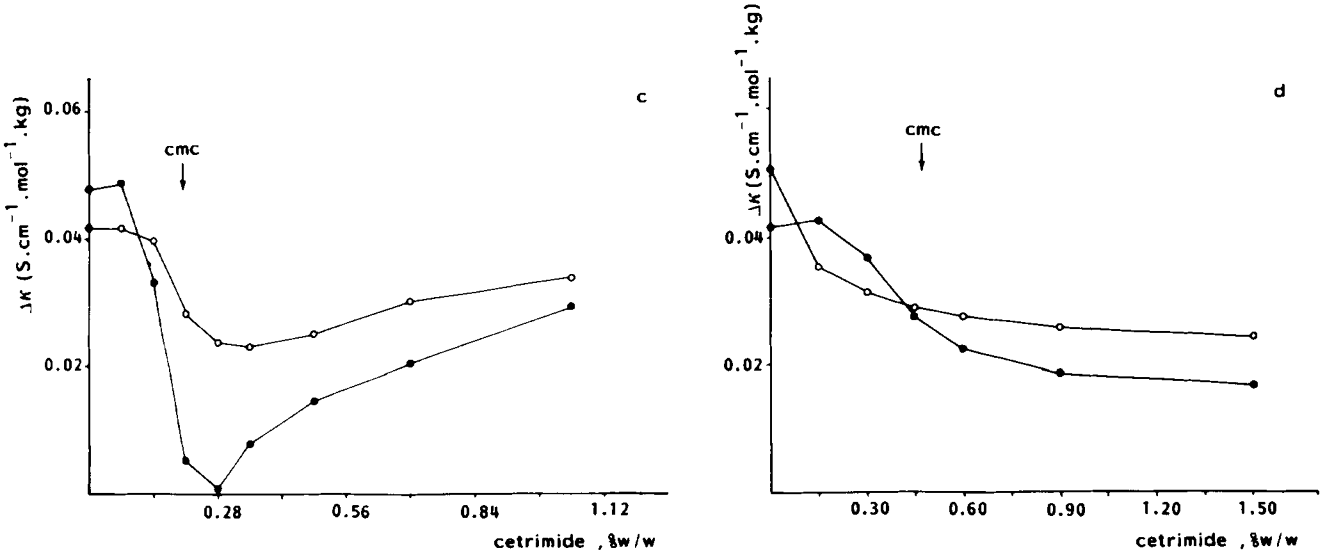

FIGURE 4 The increase in the specific conductivity, ΔK, of cetrimide containing solutions after the addition of 6.25 x 10^{-6} mol sodium salicylate (●—●) or sodium m-hydroxy benzoate (○—○); a, water; b, 20% w/w methanol; c, 30% w/w methanol; d, 40% w/w methanol.

xy benzoate) and cetrimide below the CMC, for otherwise Δk should be the same value at each cetrimide concentration, resulting in a horizontal straight line.

Bidlingmeyer et al. (20) also using conductimetric experiments, found no interactions between octane sulphonate and octyl amine (and some drugs) in water-methanol mixtures. However, Melander et al. (21), using the data of Bidlingmeyer et al. (20), concluded that ion-pair formation can not be excluded.

In our study the values for Δk were found to increase above the CMC, probably by partitioning of the solutes between the micelles and the bulk solution, resulting in a bromide (micelle counter-ion) release. From the literature (22) it is known that anions are able to release bromide from cetrimide micelles. The observed increase in Δk is more pronounced for salicylate than for m-hydroxy benzoate, so this phenomenon offers a possible explanation for the decrease of k' of salicylate as compared with m-hydroxy benzoate.

Assuming that below the CMC of cetrimide ion-pair formation occurs, theoretically the ion-association constant, K_{ip}, can be calculated from the measurements in the following way:

For ion-pair formation the following equation can be written,

$$CTA^{+} + S^{-} \rightleftarrows CTAS$$

and $$K_{ip} = \frac{[CTAS]}{[CTA^+][S^-]} = \frac{C_{ip}}{C \cdot C^1} \qquad (1)$$

where $[CTA^+] = C$ is the cetrimide cation concentration, $[S^-] = C^1$ is the solute anion concentration and $[CTAS] = C_{ip}$ is the concentration of the ion-pair (all in $mol \cdot kg^{-1}$).

For the specific conductivity, k, the following equation can be written, when no ion association occurs,

$$k \times 1000 = \Lambda_{CTABr} \cdot C + \Lambda_{NaS} \cdot C^1 \qquad (2)$$

where Λ_{CTABr} and Λ_{NaS} are the equivalent conductivities of CTABr and NaS respectively.

The following equation for K_{ip} can be derived, in an analogous way as was done by Tomlinson and Davis (23), using Kohlrausch's law of the independent migration of ions and the Law of Mass Action.

$$K_{ip} = \frac{\dfrac{1000 \cdot \Delta k}{\lambda_{CTA^+} + \lambda_{S^-}}}{\left(C - \dfrac{1000 \cdot \Delta k}{\lambda_{CTA^+} + \lambda_{S^-}}\right)\left(C^1 - \dfrac{1000 \cdot \Delta k}{\lambda_{CTA^+} + \lambda_{S^-}}\right)} \qquad (3)$$

where λ is the equivalent conductivity for each ion.

The value Δk can be calculated from Λ_{CTABr} and Λ_{NaS} which are determined separately; from the sum of these two values the measured value of k, obtained after addition of NaS to the cetrimide solution, is subtracted. The values λ_{CTA^+} and λ_{S^-} can be obtained by subtracting the equivalent conductivities of bromide and sodium ions (24) from Λ_{CTABr} and Λ_{NaS}, respectively.

However, with the available equipment the standard deviations obtained for measured values were such, that the comparatively small Δk values lacked sufficient precision to allow the determination of reliable K_{ip} values. This was also concluded by Melander et al. (21) for the octyl amine - octyl sulfonate system. For instance in water the K_{ip} for cetrimide salicylate was found to be 2×10^3 kg•mol^{-1} at 0.01% cetrimide and 2×10^4 kg•mol^{-1} at 0.02% cetrimide.

The conclusions from these conductimetric experiments are therefore qualitative in nature. Below the CMC interaction between cetrimide and the solute anions in the mobile phase seems likely, particularly at lower methanol concentrations.

Above the CMC of cetrimide the results suggest the partitioning of solutes between micelles and bulk phase; for salicylate the interaction with the micelles is more pronounced than for m-hydroxy benzoate, the decrease in k' of salicylate relative to m-hydroxy benzoate could be explained in this way.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. O.A.G.J. van der Houwen and Prof. Dr. A.W.M. Indemans for helpful discussions and Mrs. H. Verleun and Mr. B. Loriaux for their technical assistance.

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