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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ORGANIC ACIDS ON BARE SILICA

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SUMMARY

　　Unmodified silica gel in combination with mixtures of dichloromethane, methanol, buffer and water as the mobile phase was investigated as a phase system for the separation of aromatic acids by high-performance liquid chromatography. The influence of parameters such as the amounts of methanol and water and of the type, concentration and pH of the buffer in the mobile phase was systematically investigated. For many mobile phase compositions the composition of the adsorbed layer on the silica gel was also determined. The results show that the distribution process in these phase systems is very complex. However, these phase systems are efficient and applicable to the separation of carboxylic and even sulphonic acids. This is demonstrated with a number of separations of test mixtures and the analysis of additives in foods.

INTRODUCTION

　　Since the introduction of chemically modified silica gels in high-performance liquid chromatography (HPLC), interest in applying unmodified silica gel as an adsorbent for the separation of very polar substances, such as acids, has diminished considerably. It is doubtful whether this reduction in interest is justified nowadays, particularly if one considers the contribution that bare silica made to the popularity of thin-layer chromatography (TLC) for the separation of a great variety of substances, including acids, in recent decades[1–5].

　　In almost all TLC systems applied to the separation of organic acids the developing liquid consists of an apolar solvent acting as a diluent for a very polar solvent component, such as an alcohol, and further contains a considerable amount of an organic acid, such as acetic or formic acid, and in some instances a small amount of water. The results from these TLC experiments were adapted to compose mobile phases for the separation of carboxylic and sulphonic acids on bare silica by HPLC. Thus, diethyl ether–*n*-propanol–aqueous acetic acid was found useful for the separation of oxypurines[6] and dichloromethane–methanol–aqueous ammonium formate buffer was used for the rapid separation of nucleic acids[7]. Aromatic carboxylic and sulphonic acids could be well separated on bare silica by using *n*-butanol–etha-

nol–aqueous sodium acetate as the mobile phase[8]. In one study the possibility of chromatographing amino acids and sulphonic acids on bare silica with an aqueous mobile phase has been reported[9]. Recently, it was demonstrated that the pre-treatment of bare silica with a crystalline salt or acid allows tailing-free separations of a variety of aromatic carboxylic acids with mixtures of n-hexane and diethyl ether as the mobile phase[10].

All of these studies have clearly shown the potential of bare silica gel for the separation of organic acids. However, in most instances very little attention was given to the possibility of varying the retention via the mobile phase composition. In this paper we report the results of an extensive and systematic investigation on the retention behaviour of aromatic carboxylic acids on bare silica with dichloromethane–methanol–acetate buffer mixtures containing small amounts of water as the mobile phase.

It is shown that many parameters can be used to vary the retention and that the mobile phase composition has to fulfil certain requirements in order to be able to chromatograph carboxylic and sulphonic acids under optimal conditions. Various examples of the applicability of unmodified silica gel are demonstrated.

EXPERIMENTAL

Apparatus

The HPLC experiments were carried out with a Hupe-Bush 1010A liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a Rheodyne 7120 high-pressure sampling valve fitted with a 20-μl sample loop and a Pye-Unicam UV-LC variable-wavelength UV detector. The columns (250 × 4.6 mm I.D.) were made of 316 stainless steel. All measurements were performed at ambient temperature.

The gas chromatographic (GC) experiments were performed with a Packard-Becker 427 gas chromatograph equipped with a thermal conductivity detector (TCD). The column (1 m × 4 mm I.D.) was made of copper and was filled with Porapak Q (100–120 mesh) (Waters Assoc., Milford, MA, U.S.A.).

Materials

All chemicals and solvents were of analytical-reagent grade from commercial sources and were used as delivered. The sulphonic acids were kindly donated by Professor H. Cerfontain, University of Amsterdam. The silica gel used in HPLC was Hypersil, mean particle size 5 μm (Shandon, London, Great Britain).

Procedures

The HPLC columns were packed according to the procedure recommended by the supplier of the silica (Shandon). When changing the eluent the columns were washed successively with 25 ml of absolute ethanol, and 100 ml of dichloromethane and then equilibrated with the mobile phase until constant retention of the solutes was achieved. In all experiments toluene was used as the non-retarded solute.

In order to determine the composition of the adsorbed phase on the silica gel after each set of experiments, the column was washed with 25 ml of absolute ethanol. To the collected ethanol fraction 5 ml of ethyl acetate were added (as an internal

standard) and this mixture was then analysed by GC on a Porapak column using helium as the carrier gas at a flow-rate of 50 ml/min at 180°C. This gives the total amount of water, methanol and dichloromethane present in the column. The amount of mobile phase components adsorbed can then be calculated from the composition of this mixture, the mobile phase composition and from the mobile phase volume (V_m) which was determined from t_{R0} and the flow-rate. The volume of the adsorbed liquid phase (V_s) was determined from the void volume (V_t) of the column ($V_s = V_t - V_m$), which was determined by equilibrating the column with pure dichloromethane and determining the dichloromethane content after its elution with ethanol and GC analysis. The results agreed with those obtained according to ref. 11.
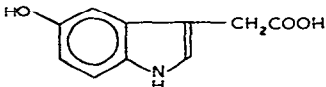
RESULTS AND DISCUSSION

Table I shows the structures of the carboxylic acids selected to study the retention behaviour on bare silica. The dependence of the capacity ratio (k') of these acids on the amounts of methanol and water and on the type, concentration and pH of the buffer present in the mobile phase was systematically investigated. The effect of the mobile phase composition on the peak shape and column efficiency was also studied.

In order to obtain some more insight into the distribution process, the composition of the adsorbed phase on the silica gel was determined as described under Experimental.

TABLE I

STRUCTURES OF THE ACIDS
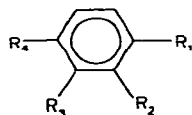
Name	Abbreviation	R_1	R_2	R_3	R_4	pK_a
4-Hydroxybenzoic acid	4HB	COOH	H	H	OH	4.67
3-Hydroxybenzoic acid	3HB	COOH	H	OH	H	4.30
4-Hydroxycinnamic acid	4HC	CH=CHCOOH	H	H	OH	4.64
4-Aminobenzoic acid	4AB	COOH	H	H	NH$_2$	4.68
Homovanillic acid	HVA	CH$_2$COOH	H	H	OH	4.41
Hippuric acid	HA	CONHCH$_2$COOH	H	H	H	–
2-Hydroxyphenylacetic acid	2HP	CH$_2$COOH	OH	H	H	–
5-Hydroxyindole-3-acetic acid	5HIA					–

Retention behaviour

Influence of water. The influence of water on k' is shown in Fig. 1; k' for each acid increases significantly with increasing water content and for some solutes it reaches a constant value in the region of 1–2.6% (v/v) of water. However, for hippuric acid (HA) and 5-hydroxyindoleacetic acid (5HIA) k' increases with increasing water content up to 1% and then gradually decreases again up to 2.6% of water.
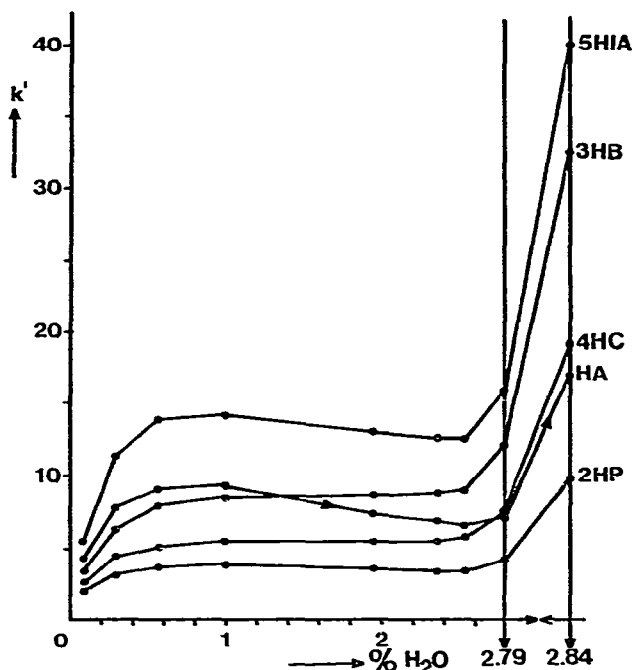
Fig. 1. Dependence of the capacity ratio (k') on the water content of the mobile phase. Mobile phase composition: dichloromethane–methanol (8:2) + 0.01 M acetic acid (HAc) + 0.01 M potassium acetate (KAc) + 0–2.84% (v/v) of water. Solutes: for abbreviations see Table I.

On increasing the water content from 2.6 to 2.79% (which is very close to the saturation point), a significant increase in k' for all solutes is observed. When the mobile phase is saturated with water (corresponding to 2.84% of water) k' for all solutes increases by a factor of about 2.5.

In order to obtain insight into the distribution process occurring in this phase system, the possible modification of the surface of the silica gel, by preferential adsorption of mobile phase components, was measured. Fig. 2 shows the amounts of methanol, water, dichloromethane and potassium acetate (obtained by evaporation of the collected ethanol fraction) adsorbed on the surface of the silica gel as a function of the water content of the mobile phase.

Comparison of Figs. 1 and 2 shows that there is no clear relationship between the variation of k' and the composition of the adsorbed layer up to 2.6% of water in the mobile phase. However, the significant increases in k' at 2.79 and 2.84% coincide very well with the steep increase in the adsorbed layer owing to partial (2.79%) and complete (2.84%) pore filling with a co-existing, relatively water-rich liquid phase[12]. The results in Figs. 1 and 2 suggest a complex mixed distribution process. The variation of the amount of adsorbed potassium acetate (KAc) as function of the water content indicates a similar effect. Also, the change in the selectivity factors with increasing water content might be an indication of the occurrence of a mixed distribution process.

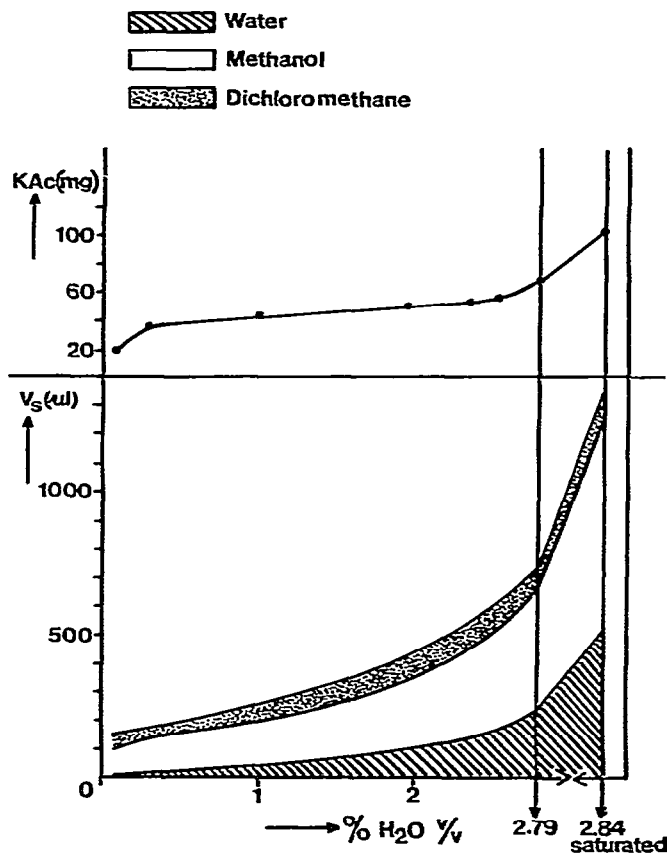*Influence of methanol.* The influence of methanol on k', other variables remain-

Fig. 2. Amount of adsorbed mobile phase components as a function of the water content of the mobile phase. Conditions as in Fig. 1. V_c = volume of the adsorbed liquid phase.

ing constant, is shown in Fig. 3. In contrast to water (see Fig. 1), methanol acts as a strong moderator and its concentration can be used to adjust the order and degree of retention over a wide range. As can be seen from Fig. 3, the graph of log k' versus log [methanol] is approximately linear for most acids in the region 15–40% of methanol, as is commonly found in adsorption systems[13]. For some solutes a deviation occurs at 10% of methanol, which might be attributed to significant pore filling as at lower methanol contents the actual amount of added water (0.6%) comes closer to the saturation point. For two acids (HA and HVA), significantly different behaviour of k' with respect to methanol content compared with the other acids is found. The graph of log k' versus log [methanol] is curved and shows a significantly different slope compared with the other acids.

Fig. 3 shows that the methanol content of the mobile phase is a valuable parameter for adjusting the degree and in some instances even the order of retention.

Influence of buffer concentration. The influence of the buffer concentration on k', with other parameters constant and keeping the ratio of HAc and KAc constant, is shown in Fig. 4. If no buffer is present in the mobile phase all acids are only slightly retained. However, after the addition of only $5 \cdot 10^{-4}$ M of buffer a considerable
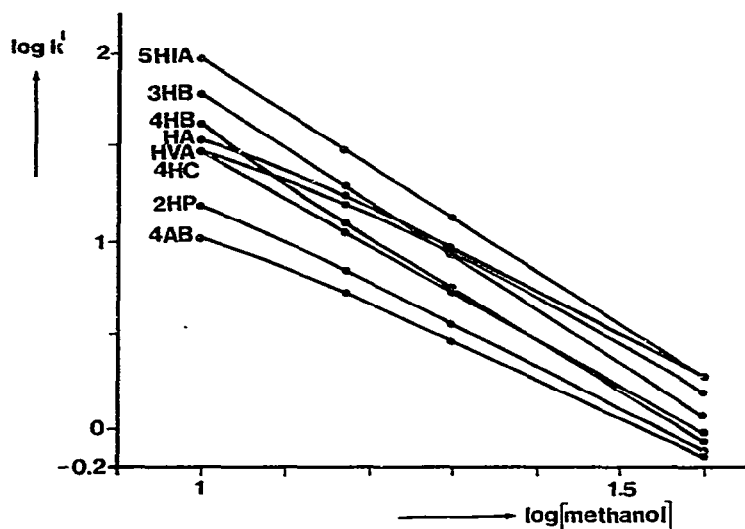
Fig. 3. Dependence of the capacity ratio on the methanol content of the mobile phase. Mobile phase composition: dichloromethane + 10–40 % (v/v) methanol + 0.01 M HAc + 0.01 M KAc + 0.6 % (v/v) H_2O.

increase in k' for all acids occurs. A further increase in the buffer concentration causes a small increase in k' up to 10^{-3} M, and from this point the k' values of the acids decrease again, with different slopes, up to about $2 \cdot 10^{-2}$ M of buffer. At very high buffer concentrations the k' values of all acids increase again. The last effect can be attributed to significant pore filling as the mobile phase comes closer to the water
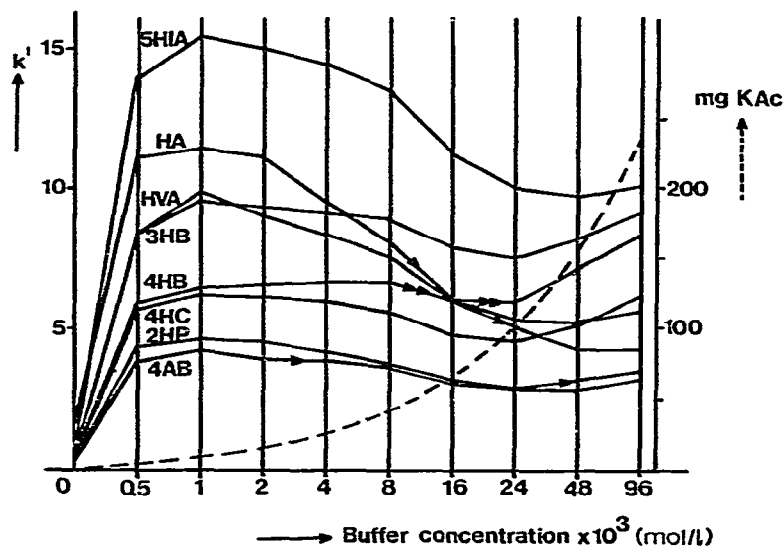

Fig. 4. Dependence of the capacity ratio on the buffer concentration in the mobile phase at a constant HAc/KAc ratio. Mobile phase composition: dichloromethane–methanol (8:2) + 2 % (v/v) water + 0–0.1 M HAc + 0–0.1 M KAc.

saturation point as a result of the high salt concentration (*i.e.*, the water saturation level is about 2.4% at 10^{-1} *M* of buffer). The significant increase in k' when only a small amount of buffer is added to the mobile phase leads to the conclusion that the solute has to be present in the mobile phase in an ionized form (possibly as an ion pair associated with potassium ions) in order to be transferred to the stationary phase. It can be seen from Fig. 4 that a buffer concentration of about 10^{-3} *M* is needed in order to create the adsorptive environment for the solutes. However, at a buffer concentration of 10^{-3} *M* k' decreases again, possibly owing to a competitive effect of KAc. The difference in the slope of this decrease for the acids might be attributed to the relative magnitude of the different distribution processes occurring.

Neglecting any speculation about the distribution process, Fig. 4 clearly shows that significant changes in the degree and order of retention can be introduced by variation of the buffer concentration.

Influence of pH of the buffer. The influence of the ratio of the amount of HAc and KAc added (for simplicity expressed in terms of the pH that an aqueous solution with the same concentrations would have) on k' was measured from 0.1 *M* HAc to 0.02 *M* KAc, and is shown in Fig. 5. It can be seen that the pH has a large influence on k'. At low pH (pure HAc) the solutes are almost unretained, indicating again that the acids in their undissociated form (and thus also HAc) show little affinity for the silica in combination with the selected mobile phase composition. Certainly the retention of the acids will increase with decreasing methanol content.

On changing the ratio of HAc and KAc in favour of the latter (increasing pH) k' starts to increase considerably and tends to level off at higher pH. The behaviour of k' as a function of pH coincides well with the degree of dissociation of weak acids in
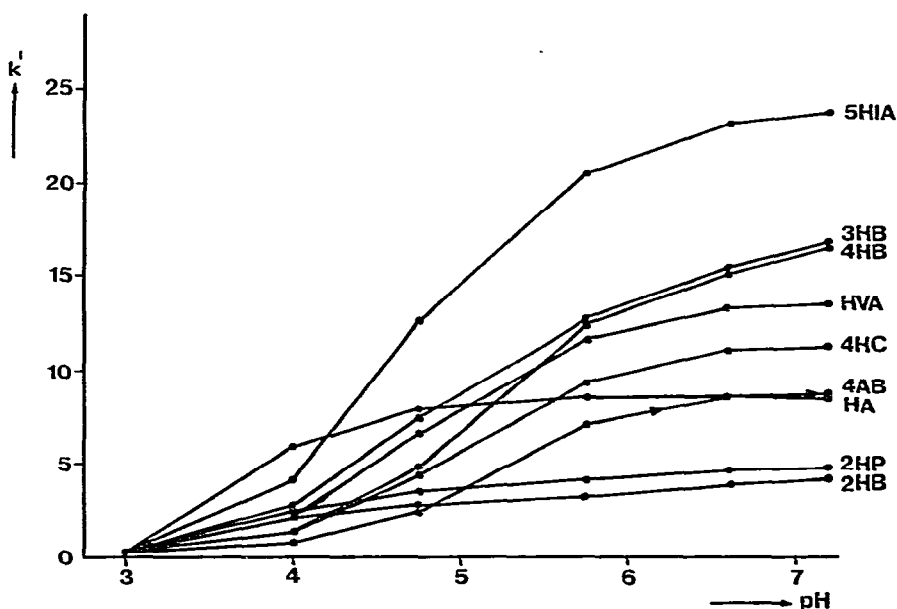

Fig. 5. Influence of the ratio of HAc and KAc (pH) in the mobile phase on the capacity ratio. Mobile phase composition: dichloromethane–methanol (8:2) + 1% (v/v) water + 0.02 *M* KAc and various amounts of HAc. Solutes: see Table I; 2HB = 2-hydroxybenzoic acid.

aqueous systems with pH and shows that the distribution coefficient, leaving the distribution process as unexplained, is roughly proportional to the term $(1 + [H^+]/K_a)^{-1}$. From Table I and Fig. 5 it can be seen that there is no clear correlation between the pK_a of the acids and their k' values.

Whether the pH effect occurs in the mobile and/or stationary phase is not yet clear. It seems that the effect is mainly due to the pH change of the mobile phase, as the amount of KAc adsorbed on the silica (and therefore probably the pH of the adsorbed layer) was found to be constant in the pH range 4–7. However, to understand the pH effect it is necessary to know precisely the pH of the adsorbed layer. So far attempts in our laboratory to measure the pH of the adsorbed layer have failed.

As a parameter for adjusting the retention, the ratio of HAc and KAc (the pH) seems to be one of the most effective.

Influence of the type of buffer. The effect of the cationic and anionic part of the buffer on k' was investigated with mobile phases containing a buffer composed of acetic acid (HAc) and potassium, sodium, ammonium and lithium acetate (KAc, NaAc, NH$_4$Ac, LiAc) and from potassium formate, acetate, propionate and chloroacetate and their corresponding acids. The results of these measurements are shown in Figs. 6 and 7.
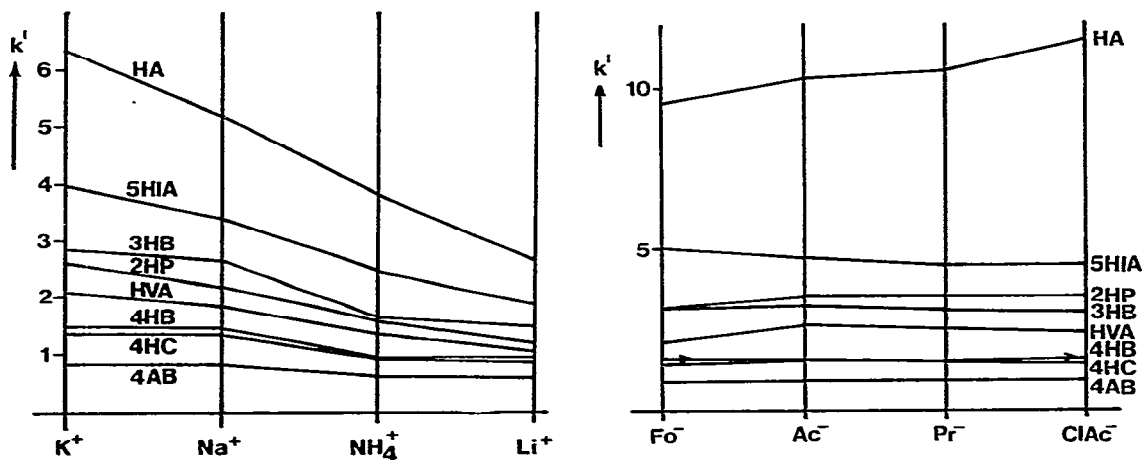

Fig. 6. Effect of the nature of the cationic part of the buffer on the capacity ratio. Mobile phase composition: dichloromethane–methanol (8:2) + 2% (v/v) water + 0.05 M HAc + 0.01 M KAc, NAc, NH$_4$Ac or LiAc. pH = 4.05.

Fig. 7. Effect of the nature of the anionic part of the buffer on the capacity ratio. Mobile phase composition as in Fig. 5; the buffer was composed of formic acid/potassium formate; HAc/KAc; propionic acid/potassium propionate (Pr$^-$); chloroacetic acid/potassium chloroacetate (ClAc$^-$).

As can be seen from Fig. 6, the type of the cationic part of the buffer has a significant effect on the retention. The degree of retention decreases in the order K$^+$ > Na$^+$ > NH$_4^+$ > Li$^+$ and the amount of adsorbed salts decreases in the order K$^+$ > Na$^+$ > Li$^+$ (NH$_4^+$ could not be determined). This result does not indicate a competition effect, but more to a pH effect on the surface of the silica due to the adsorption of different amounts of acetate with varying types of cation. The type of the anionic part of the buffer seems to have only a small effect on k', as can be seen

from Fig. 7. However, in some instances "fine tuning" of the selectivity can be introduced by varying the type of the anionic part of the buffer, as can be seen from Fig. 7.

Column performance

Apart from the influence of the various parameters on k', their effect on the peak shape and column efficiency was also studied. In order to determine whether the column efficiency decreases with the investigated phase systems, all freshly packed columns were first tested with pure dichloromethane as eluent and 4,4-dinitrodiphenyl ($k' \approx 0.3$) and acetophenone ($k' \approx 1.5$) as solutes. The plate numbers of the columns tested under these conditions ranged between 11,000 and 13,000 for a 25-cm column at a linear velocity of 2.5 mm/sec. This plate number decreased to 6000–7000 when using dichloromethane–methanol–buffer mixtures as the mobile phase and acids as the solutes.

Most of the variables seem to have only a small effect on the column efficiency. However, some restrictions apply with certain mobile phase compositions. Serious leading was noticed when mobile phases were used that contain less than $4 \cdot 10^{-3}$ M of KAc or of which the pH is lower than 3.0. Further, the water content must not be too low in order to obtain reproducible retention (*e.g.*, with 20 % of methanol the water content must be $>0.5\%$). On the other hand, maximal column efficiency (7000 plates) was found with eluents of higher pH (*ca.* 7) and of high KAc concentrations (*ca.* $2 \cdot 10^{-2}$ M) and with eluents with a water content near the saturation point or saturated with water.

The columns used in this study were used for several months with a great variety of eluents without any significant change in column characteristics. However, the performance of columns exposed to an eluent of high pH (8.8) for a longer period showed a large decrease in column efficiency (from 7000 to 3000) and a change in retention characteristics.
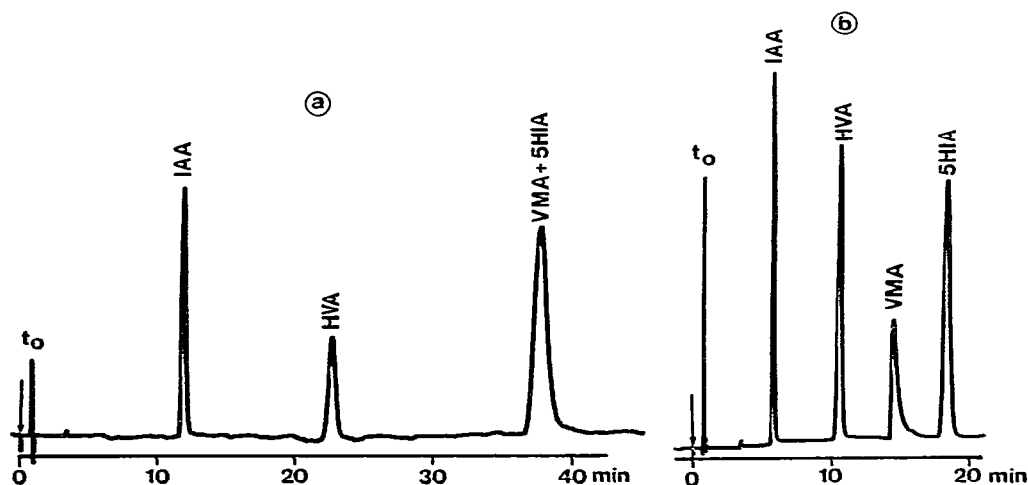

Fig. 8. Effect of the addition of 18-crown-6 to the mobile phase on the retention of some carboxylic acids. Stationary phase: silica. Mobile phase: (a) dichloromethane–methanol (8:2) + 0.01 M KAc, saturated with water; (b) dichloromethane–methanol (8:2) + 0.02 M KAc + 0.01 M 18-crown-6, saturated with water. Solutes: t_0 = toluene; indole-3-acetic acid (IAA); homovanillic acid (HVA); vanilmandelic acid (VMA); 5-hydroxyindole-3-acetic acid (5HIA).

Of about 40 carboxylic acids chromatographed so far with many phase systems, only a few acids showed bad peak shapes (broad and tailing) in all phase systems investigated. These are the acids that are able to form intramolecular hydrogen bonds such as the substituted mandelic acids and salicylic acid. However, promising results for these types of acids were obtained with water-saturated mobile phases and by the addition of a crown ether to the mobile phase[14]. The applicability of partition systems for carboxylic acids was demonstrated previously[15].

Fig. 8 shows the effect of the addition of 18-crown-6 to the mobile phase on the retention of some carboxylic acids of biomedical interest. Significant changes in the degree and order of retention can be introduced by adding 18-crown-6. Investigations of the favourable effects of crown ethers on the retention of acids are being continued.

Practical utility

The results obtained so far indicate that bare silica is applicable to the separation of carboxylic acids. With respect to efficiency, selectivity and stability bare silica can compete with alkyl-modified silica gels and is often a useful supplement. Moreover, bare silica is a relatively cheap and stable material compared with alkyl-modified silicas, which makes it attractive as a column support for preparative liquid chromatography, and also because of the ease of removing the organic solvent after collection of the sample.

The applicability of bare silica for the separation of a variety of acids is demonstrated in Figs. 9–12. Fig. 9 shows the rapid separation of a test mixture of
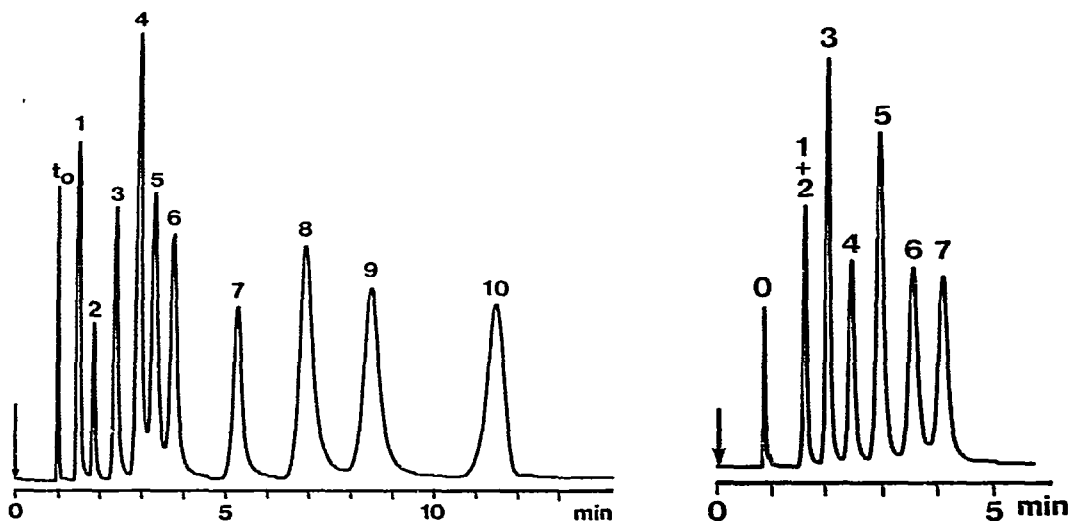

Fig. 9. Separation of a test mixture of aromatic carboxylic acids. Stationary phase: silica. Mobile phase: dichloromethane–methanol (8:2) + 2 % (v/v) water + 0.1 M HAc + 0.01 M KAc ("pH" = 3.75). Solutes: t_0 = toluene; 1 = 3,4-dimethoxybenzoic acid; 2 = benzoic acid; 3 = 4-hydroxybenzoic acid; 4 = 3-aminobenzoic acid; 5 = 4-hydroxyphenylacetic acid; 6 = 3-hydroxybenzoic acid; 7 = 3,4-furandicarboxylic acid; 8 = nicotinic acid; 9 = hippuric acid; 10 = 4-aminohippuric acid.

Fig. 10. Rapid separation of closely related cinnamic acids. Conditions as in Fig. 8, except 0.1 M HAc + 0.02 M KAc ("pH" = 4.0). Solutes: 1 = toluene; 2 = 2-methoxycinnamic acid; 3 = cinnamic acid; 4 = 4-hydroxy-3-methoxycinnamic acid; 5 = 4-hydroxycinnamic acid; 6 = 2-hydroxycinnamic acid; 7 = 3-hydroxycinnamic acid.
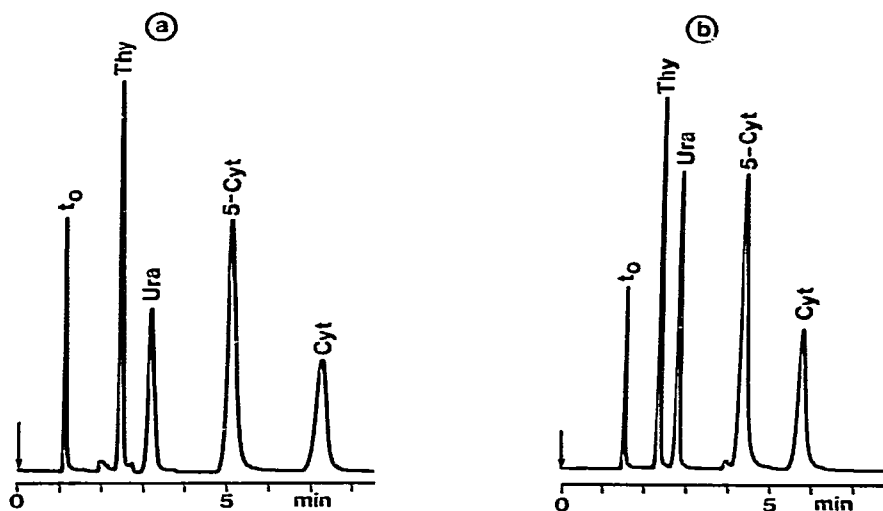
Fig. 11. Rapid separation of nucleobases. Mobile phase composition: (a) dichloromethane–methanol (8:2) + 1% (v/v) water + 0.2 M HAc + 0.02 M KAc (pH = 4.75); (b) dichloromethane–methanol (8:2) + 2% (v/v) water + 0.1 M HAc + 0.01 M KAc (pH = 3.75). Solutes: t_0 = toluene; Thy = thymine; Ura = uracil; 5-Cyt = 5-methylcytosine; Cyt = cytosine.

aromatic carboxylic acids of different classes and Fig. 10 shows the separation of closely related cinnamic acids. The developed phase system can also be applied to the efficient separation of a number of nucleobases, as can be seen from Fig. 11. Even aromatic sulphonic acids can be chromatographed on bare silica, as is demonstrated in Fig. 12.
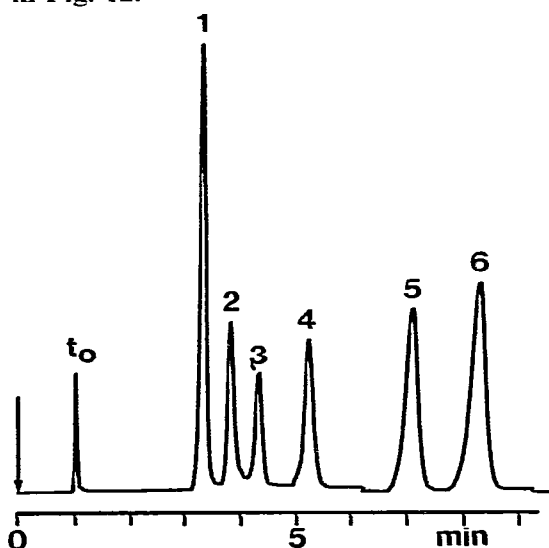

Fig. 12. Rapid separation of aromatic sulphonic acids. Mobile phase composition as in Fig. 11b. Solutes: t_0 = toluene; 1 = anthraquinone-2-sulphonic acid; 2 = benzosuberone-4-sulphonic acid; 3 = indane-4-sulphonic acid; 4 = benzenesulphonic acid; 5 = 1-naphthol-5-sulphonic acid; 6 = 6-hydroxynaphthalene-2-sulphonic acid.

Another advantage of bare silica is the possibility of injecting directly extracts of, for instance, foodstuffs, as is demonstrated in Fig. 13, which shows the analysis of benzoic and sorbic acids extracted with dichloromethane from rye bread and fruit juice.
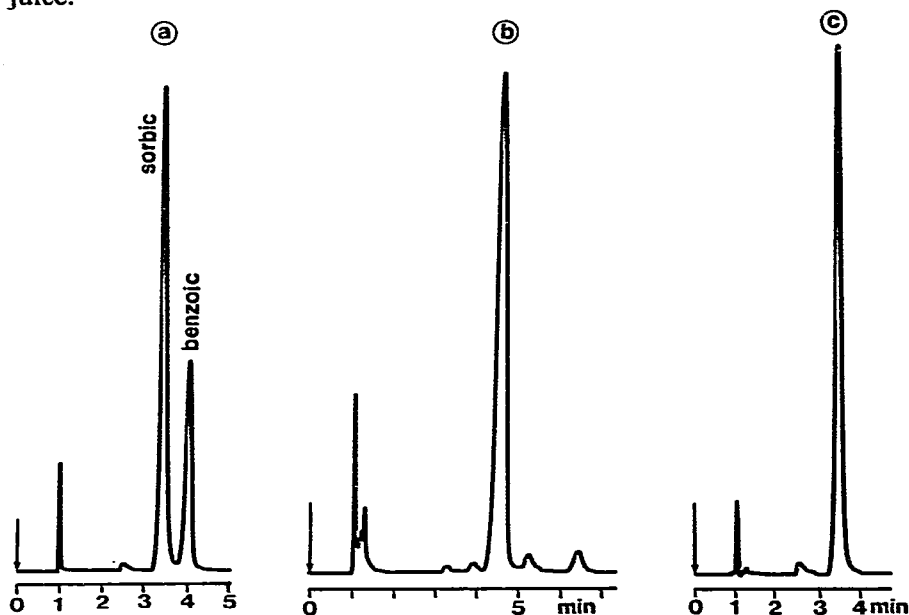

Fig. 13. Analysis of benzoic acid and sorbic acids extracted from foodstuffs and from fruit juice. Mobile phase composition: dichloromethane–methanol (8:2) + 2.3 % (v/v) water + 0.01 M HAc + 0.01 M KAc (pH = 4.75). (a) Separation of sorbic and benzoic acids; (b) benzoic acid in fruit juice; (c) sorbic acid in rye bread.

CONCLUSIONS

Bare silica in combination with mixtures of dichloromethane–methanol–acetate buffer as the mobile phase are very useful for the chromatography of carboxylic and even sulphonic acids. It seems to be necessary to add at least a small amount of water and acetate buffer in order to obtain symmetrical peaks and to retain the acids. Of the available parameters, the ratio of HAc and KAc (the pH) seems to be the most useful for adjusting the retention.

With the available data it is too speculative to postulate the distribution process that occurs. However, a complex mixed distribution process seems the most obvious process.

Future experiments in our laboratory will be devoted to the effect of the type of organic components of the eluent and of the effect of crown ethers on the retention of acids, and further attempts will be made to determine the magnitude of the various separate distribution processes.

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