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## Retention behaviour of mono- and dicarboxylic acids, carbohydrates and alcohols in ion-exclusion chromatography

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### SUMMARY

The retention behaviour of mono- ( $C_1$ – $C_5$ ) and dicarboxylic acids ( $C_2$ – $C_8$ ), alcohols ( $C_1$ – $C_4$ ) and carbohydrates (sucrose,  $\alpha$ -D- and  $\beta$ -D-glucose, L-sorbose, D-xylose, D-mannose, D-galactose, D-fucose, D-lyxose, L- and D-arabinose, D-fucose and D-ribose) was studied using a poly(styrene–divinylbenzene) sulphonate ( $H^+$ ) cation-exchange resin (Catex-H). The effects of the concentration of sulphuric acid and acetonitrile in the eluent and the column temperature (20–35°C) on the retention of compounds and on the number of the theoretical plates were investigated. For detection, UV and refractive index detectors connected in series were used.

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### INTRODUCTION

In an other paper<sup>1</sup>, the separation of different short alkyl chain mono- and dicarboxylic acids was studied by reversed-phase high-performance liquid chromatography (RP-HPLC) and reversed-phase ion-pair HPLC (RP-IP-HPLC). A wide scope of these methods was achieved by the application of two stationary phases with different polarities (octylsilica and PRP-1), by adding methanol to the mobile phase, by changing the concentrations of the salt and the phosphoric acid and by the application of an ion-pair forming reagent (tetrabutylammonium hydrogensulphate, TBAHSO<sub>4</sub>). Despite the advantage of the RP-HPLC and RP-IP-HPLC methods, the separation of hydrophilic carboxylic acids of low molar mass is difficult.

A versatile approach is ion-exclusion chromatography (IEC), in which hydrophilic weak acids, alcohols and carbohydrates<sup>2–16</sup> are separated on a cation-exchange resin in the  $H^+$  form, using an acidic eluent. In IEC strong acids, being highly ionized, pass quickly through the column. They are excluded from the negatively charged resin phase according to the Donnan membrane equilibrium principle and elute at the dead volume of the column. Non-ionic compounds (undissociated acids, alcohols and carbohydrates) can enter the resin network. Acids of intermediate strength such as

most carboxylic acids are partially ionized, depending on the pH of the medium. They generally eluted in order of their  $pK_a$  values<sup>17</sup>. In the separation of weak acids ( $2.5 < pK_a < 6.0$ ) by IEC, other mechanisms also play a role, such as adsorption (partition) on the surface of the matrix of the cation-exchange resin.

Long alkyl chain carboxylic acids in the molecular form are retained on the stationary phase usually by a combination of ion exclusion, size exclusion and hydrophobic interaction.

In this work, a poly(styrene-divinylbenzene) sulphonate ( $H^+$ ) cation-exchange resin (Catex-H) was applied to elucidate the retention mechanism by studying the effect of the experimental conditions on the retention of various solutes.

## EXPERIMENTAL

The laboratory-made instrument used consisted of a pump (Model 6000 A, Waters Assoc.), variable-wavelength UV detector (Model OE-308, Labor MIM), an injector valve (Model 7125, Rheodyne) with a 10- $\mu$ l loop, a differential refractometer detector (LC 4010, Varian) and two recorders [Model 4225 (1 V), Knauer, and Model 9176 (1 mV), Varian].

The analytical column was Catex-H, 8% cross-linked (exchange capacity 4.0 mequiv./g,  $250 \times 4.0$  mm I.D., 6  $\mu$ m) (Bio-Separation-Technologies, Budapest, Hungary). The column temperature was maintained by means of a water-bath and a water-jacket.

The eluents were dilute sulphuric acid of different concentrations at a flow-rate of 0.2 ml/min. Standard solutions of compounds were prepared from analytical-reagent grade chemicals without further purification.

## RESULTS AND DISCUSSION

Changes in the retention of the compounds were studied as a function of the concentration of the sulphuric acid eluent between  $5 \cdot 10^{-4}$  and  $3 \cdot 10^{-3}$  M. The retention of the carbohydrates was found to be constant. The retention of the mono- and dicarboxylic acids was determined by their ionization ability, while the retention of the alcohols decreased as the concentration of sulphuric acid in the eluent increased. Fig. 1 shows the  $\log k'$  (capacity factor) vs.  $\log c_{H_2SO_4}$  (sulphuric acid concentration) functions obtained for the alcohols. The dead volume ( $v_0$ ) data required for the calculation of the capacity factor,  $k'$ , were obtained by injection of strong acid samples (nitric, hydrochloric and sulphuric) ( $v_0 = 1.15$  ml).

As can be seen, the decrease in retention is accompanied by a change in selectivity. With  $10^{-3}$  M sulphuric acid eluent, *n*- and isopropanol, *n*- and isobutanol (Fig. 2) and *tert.*-, *sec.*- and *n*-butanol ( $k' = 2.13, 2.94$  and  $4.03$ ) can be well separated. In  $2 \cdot 10^{-3}$  M eluent, however, the *n*- and iso-alcohols cannot be separated.

The retention behaviour of the carboxylic acids is related to the difference in the pH of the eluent and the  $pK_a$  value of the acids. Retention of the acids, occurring in fully or partially ionized form with variation in the pH of the eluent, decreases as the

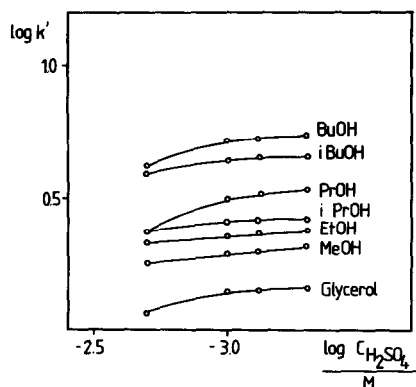


Fig. 1. Retention of alcohols as a function of sulphuric acid concentration in the eluent at 25°C. Me = Methyl; Et = ethyl; iPr = isopropyl; Pr = propyl; iBu = isobutyl; Bu = butyl.

hydrogen-ion concentration decreases. The capacity factor and the hydrogen-ion concentration of the eluent are interrelated by the following equation<sup>18</sup>:

$$k' = \frac{a}{1 + \frac{K_a}{[H^+]}} \quad (1)$$

where  $a$  is a constant in the partition coefficient. This equation was applied to some partially ionized dicarboxylic acids in the  $1 \cdot 10^{-3}$ – $6 \cdot 10^{-3}$  M hydrogen ion concentration range. Fig. 3 shows the results for maleic ( $pK_{a1} = 1.8$ ), citraconic ( $pK_{a1} = 2.42$ ) and fumaric ( $pK_{a1} = 3.0$ ) acids. The slopes of the straight lines are 0.732, 0.766 and 0.949, respectively. Similar results were obtained by Walser<sup>10</sup> for maleic, fumaric and pyruvic acids on a PRP-X300 medium-capacity cation-exchange resin column.

Comparing the straight lines obtained with the PRP-X300 (0.17 mequiv./g)<sup>10</sup> and Catex-H (4.0 mequiv./g) resins, it can be seen that the retention values are signif-

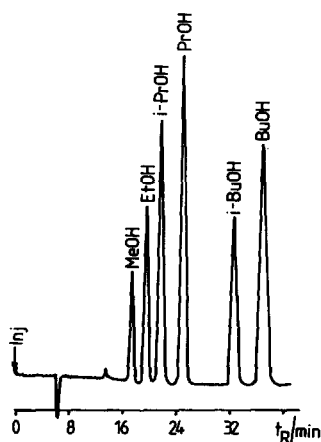


Fig. 2. Chromatogram of alcohols using  $10^{-3}$  M sulphuric acid eluent at 35°C. Refractive index detector.  $t_R$  = Retention time.

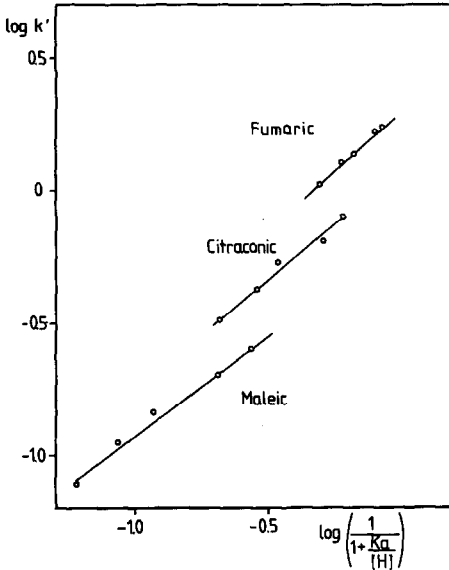


Fig. 3. Variation of retention of relatively strong dicarboxylic acids as a function of  $H^+$  concentration in the eluent.

icantly higher for maleic and fumaric acids on the lower capacity PRP-X300 cation exchanger.

The retention of the weaker ( $pK_a > 3.5$ ) mono- and dicarboxylic acids did not change appreciably in the pH range 2.2–3.0. The relationship between the logarithm of the capacity factor of the various carboxylic acids and their  $pK_a$  values ( $pK_{a1}$  for dicarboxylic acids) was also studied. The  $\log k'$  values obtained with  $10^{-3} M$  sulphuric acid eluent are plotted as a function of the  $pK_a$  of the carboxylic acid in Fig. 4.

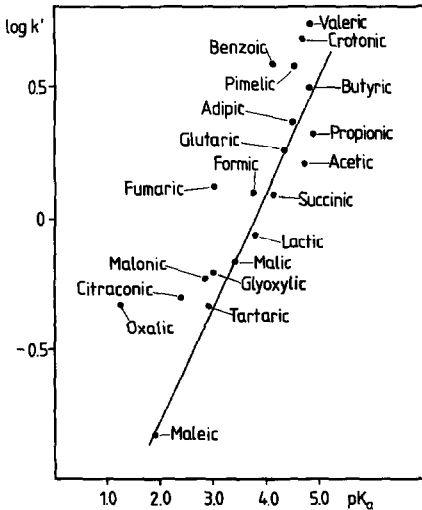


Fig. 4. Relationship between the retention of acids and their  $pK_a$  values. Eluent,  $10^{-3} M$  sulphuric acid; 25°C.

Most of the acids are eluted in order of their  $pK_a$  values. A straight line can be fitted to the points obtained. For some acids the points do not be on the straight line. The deviation is most significant for oxalic acid ( $pK_a = 1.23, 3.80$ ), whose degree of ionization is the highest at the pH of the eluent. The deviation is also significant for fumaric and citraconic acids. Both acids have a higher retention than could be expected on the basis of their  $pK_{a1}$  values.

The retention of the mono- ( $n_C > 3$ ) and dicarboxylic acids with longer carbon chains ( $n_C > 5$ ) and that of the unsaturated crotonic acid is again higher than could be predicted by considering the  $pK_a$  values. This indicates that in these instances the retention deviation can be interpreted on the basis of a hydrophobic-type reversed-phase mechanism on the surface of the (apolar) cation-exchange stationary phase. This is corroborated by the results shown in Fig. 5, where the  $\log k'$  values of the mono- and dicarboxylic acids are plotted against their carbon atom number. Similarly to the linear  $\log k' - n_C$  relationships known in reversed-phase liquid chromatography, a straight line is obtained but only for higher carbon atom number carboxylic acids on the strong acid cation-exchange resin. The deviating retention values obtained for the  $C_4$  and  $C_5$  dicarboxylic acids can presumably be understood by assuming a mixed retention mechanism (ion exclusion + hydrophobic interaction).

The effect of the concentration of sulphuric acid on the theoretical plate number ( $N$ ) was also studied. It was found that the plate number calculated from the alcohol peaks does not change as the concentration of sulphuric acid increases. The plate numbers calculated from the carbohydrate peaks almost doubled when the acid concentration of the eluent was increased from  $5 \cdot 10^{-4}$  to  $2 \cdot 10^{-3}$  M, e.g., from 1300 to 2790 for D-galactose and from 1370 to 2430 for D-mannose. The plate number changed in a different way for the carboxylic acids. Some typical data are given in Table I. As can be seen, the plate number is highly influenced by the degree of ionization of the acid. For the relatively strong carboxylic acids which are partially

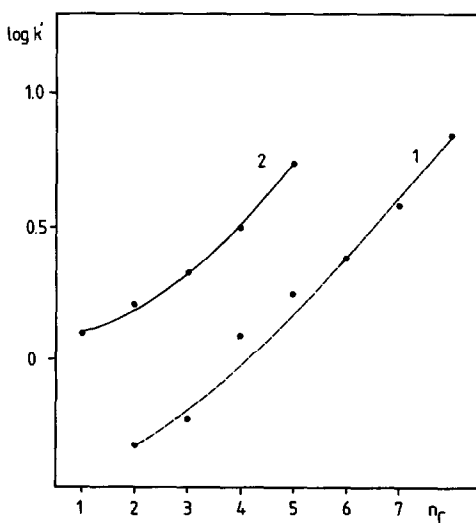


Fig. 5. Dependence of  $k'$  on the number of carbon atoms ( $n_C$ ) of acids: 1, dicarboxylic acids; 2, monocarboxylic acids. Eluent,  $10^{-3}$  M sulphuric acid;  $25^\circ\text{C}$ .

TABLE I  
COMPARISON OF THE COLUMN EFFICIENCIES

Variation of number of theoretical plates ( $N$ ) calculated from the peaks of acids with different sulphuric acid concentrations ( $5 \cdot 10^{-4}$ – $3 \cdot 10^{-3}$  M) in the eluent at 25°C.

Acid	$N$ /column			
	Sulphuric acid concentration			
	$5 \cdot 10^{-4}$ M	$1 \cdot 10^{-3}$ M	$2 \cdot 10^{-3}$ M	$3 \cdot 10^{-3}$ M
Oxalic	75	130	220	220
Citric	280	1000	1000	1000
Fumaric	1880	2980	3050	5120
Itaconic	3060	3670	5940	9780
Formic	3940	4800	12140	15000
Acetic	4160	4600	16500	16500
Crotonic	7400	8650	11600	11900

ionized at the pH of the eluent (oxalic, maleic and tartaric acids), the  $N$  values are low. For the acids with higher  $pK_a$  values, higher  $N$  values were obtained. As the concentration of sulphuric acid was increased the theoretical plate number increased for all the carboxylic acids studied. Using various acid eluents, Tanaka and Fritz<sup>5</sup>

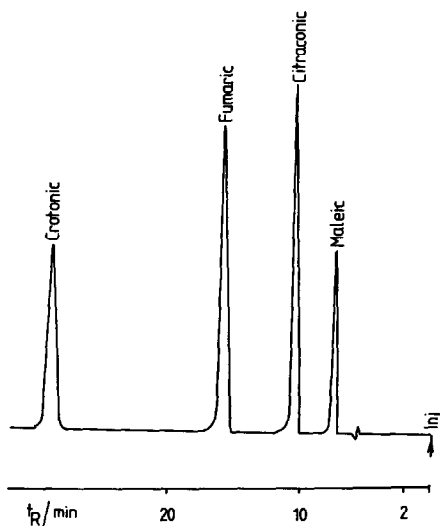
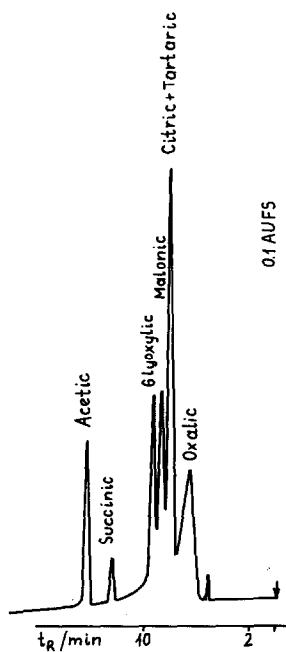


Fig. 6. Chromatogram of a synthetic mixture of acids. Eluent,  $2 \cdot 10^{-3}$  M sulphuric acid; UV detection, 0.1 a.u.f.s., 220 nm;  $10 \mu\text{l}$  of  $10^{-3}$ – $10^{-4}$  M carboxylic acids.

Fig. 7. Chromatogram of a synthetic mixture of acids. Eluent,  $3 \cdot 10^{-3}$  M sulphuric acid; UV detection, 0.1 a.u.f.s., 220 nm;  $10 \mu\text{l}$  of  $10^{-4}$ – $10^{-5}$  M dicarboxylic acids.

found that the  $N$  values obtained from the formic acid peaks were different. With water it was 130, in carbonic acid–water eluent 320 and in other acidic eluents (sulphuric, phosphoric, benzoic and salicylic acids)  $> 2300$ .

Figs. 6 and 7 show the chromatograms of two synthetic acid mixtures. The eluents were  $2 \cdot 10^{-3}$  and  $3 \cdot 10^{-3}$   $M$  sulphuric acid, respectively. The chromatograms of two different carbohydrate mixtures are shown in Fig. 8, obtained with  $2 \cdot 10^{-3}$   $M$  sulphuric acid as the eluent.

The retention of the compounds was also studied as a function of the column temperature at a constant  $10^{-3}$   $M$  acid concentration of the eluent. Similar experiments were made by Pecina *et al.*<sup>2</sup> with alcohols, aldehydes, ketons and carboxylic acids on an HPX-87H column in the range 40–80°C. The same type of column was used by Chiu<sup>6</sup> to study the change in retention of the various mono- and dicarboxylic acids in the range 20–60°C. Results obtained on the Catex-H column are similar to their results. The curves for some carboxylic acids indicate that only the retention of crotonic acid decreases slightly with increasing temperature (Fig. 9). No change was experienced in the retention of the carbohydrates. The plate number, however, calculated from the peak width increases slightly with increasing column temperature. Pecina *et al.*<sup>2</sup> observed a small increase in the retention of carbohydrates at higher temperatures.

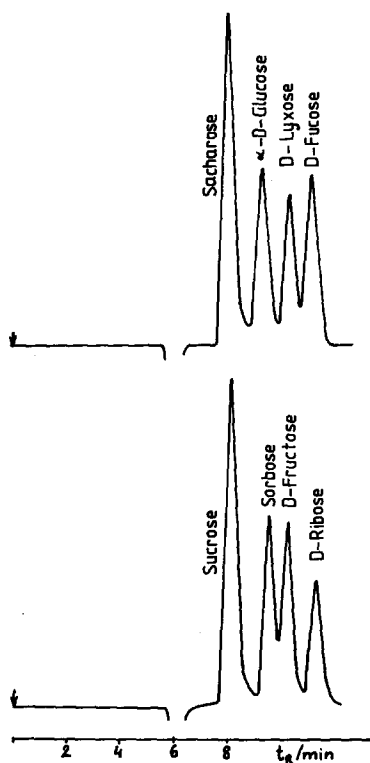


Fig. 8. Chromatograms of carbohydrates. Eluent,  $2 \cdot 10^{-3}$   $M$  sulphuric acid; refractive index detection,  $4 \times 10^{-5}$  refractive index units full-scale;  $10 \mu\text{l}$  of  $3 \cdot 10^{-3}$   $M$  each carbohydrate.

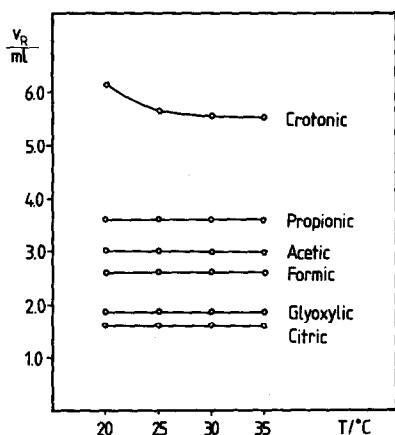


Fig. 9. Variation of retention volume ( $V_R$ ) of carboxylic acids with column temperature ( $T$ ). Eluent,  $10^{-3}$   $M$  sulphuric acid.

The retention of the alcohols increases in the range 20–35°C (Fig. 10). This change was not accompanied by a change in selectivity. Curves of various shapes were obtained by Pecina *et al.*<sup>2</sup> for the alcohols at higher temperatures, but the changes were not significant. In order to decrease the retention of strongly adsorbed compounds methanol<sup>5</sup> and acetonitrile<sup>5,19</sup> are used as organic modifiers in the IEC technique. The effect of the acetonitrile concentration in the eluent was also investigated for the three types of compounds studied. In an eluent containing  $10^{-3}$   $M$  sulphuric acid the concentration of the acetonitrile was varied between 0 and 10% (v/v). As the curves in Figs. 11 and 12 indicate, the changes are different for the various acids. The most significant decrease in retention was observed with crotonic acid. The same phenomena was observed by Kazuyoshi *et al.*<sup>19</sup> using a TSK gel SCX column. The decrease in retention clearly indicates that the crotonic acid is retained on the matrix of the cation-exchange resin by a hydrophobic interaction mechanism. The retention volume ( $V_R$ ) curves exhibit a maximum at *ca.* 2.5% acetonitrile concentration for the

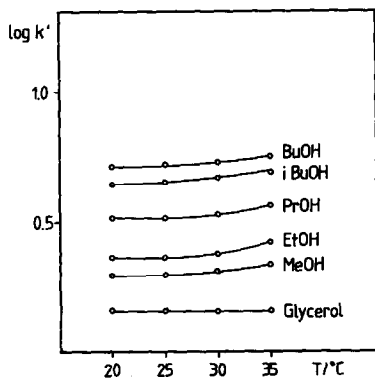


Fig. 10. Capacity factor of alcohols as a function of column temperature. Eluent,  $10^{-3}$   $M$  sulphuric acid.



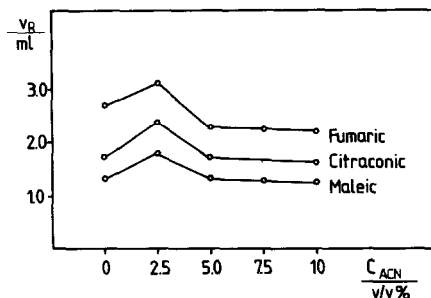
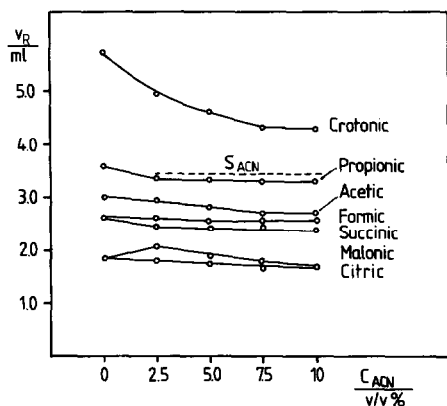


Fig. 11. Retention volume of carboxylic acids as a function of acetonitrile (ACN) concentration in the eluent. Eluent,  $10^{-3}$  M sulphuric acid + 0–10% ACN.

Fig. 12. Retention volume of relatively strong dicarboxylic acids as a function of acetonitrile (ACN) concentration in the eluent.

partially ionized carboxylic acids (fumaric, citraconic, maleic, malonic; Figs. 11 and 12). The retention enhancement observed at low acetonitrile concentration can be interpreted by the change in the ionization of the acid. A further increase in the acetonitrile concentration does not affect substantially the degree of ionization and the retention is not changed as can be seen from the curves in Fig. 12. An increase in the acetonitrile concentration strongly decreases the retention of the alcohols, as shown in Fig. 13. This indicates a partial hydrophobic retention mechanism for the longer carbon chain alcohols. The retention of the carbohydrates was found to be hardly influenced by the addition of acetonitrile to the eluent.

On the chromatograms of the alcohols and acids a disturbing system peak appears if the eluent contains acetonitrile and the injected samples are aqueous solutions (Figs. 14 and 15). The position of the system peak ( $S_{ACN}$ ) does not change but its height is proportional to the ACN concentration of the eluent. The system peak may

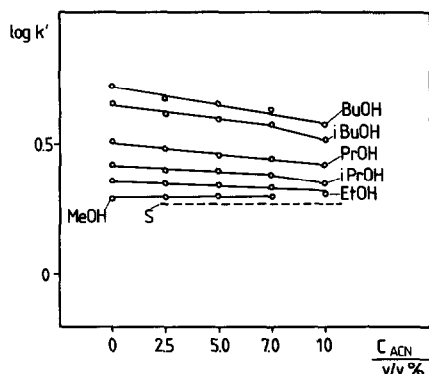


Fig. 13. Variation of capacity factor of alcohols with acetonitrile (ACN) concentration in the eluent.

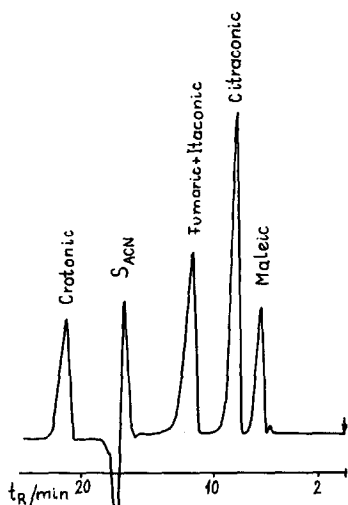


Fig. 14. Chromatogram of acids with an acetonitrile (ACN)-containing eluent. Eluent,  $10^{-3}$  M sulphuric acid + 10% ACN; UV detection, 0.05 a.u.f.s., 220 nm;  $10 \mu\text{l}$  of  $10^{-4}$ – $10^{-5}$  M acids;  $25^\circ\text{C}$ .

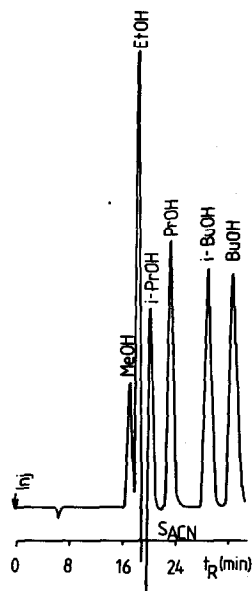


Fig. 15. Chromatogram of alcohols in acetonitrile (ACN)-containing eluent. Eluent,  $10^{-3}$  M sulphuric acid + 2.5% ACN; refractive index detection;  $25^\circ\text{C}$ .

disturb the evaluation of the chromatograms. As can be seen in Fig. 15, the determination of ethanol is not possible.

The column efficiency for the acids is strongly affected by the presence of acetonitrile in the eluent. Table II gives the calculated  $N$  values for eluents containing 2.5 and 10% acetonitrile. The plate numbers, however, did not change significantly for the carbohydrates and alcohols.

TABLE II

COMPARISON OF COLUMN EFFICIENCIES

Variation of number of theoretical plates ( $N$ ) calculated from the peaks of acids with different acetonitrile concentrations (2.5 and 10%) in the eluent.

Acid	$N/\text{column}$	
	Acetonitrile concentration	
	2.5%	10%
Citric	1650	740
Tartaric	1250	720
Maleic	1460	610
Fumaric	3460	1310
Acetic	4100	2500
Formic	4700	2900
Crotonic	6800	4100

## CONCLUSION

It may be concluded that the retention behaviour of the acids corresponds to the ion exclusion theory but some acids (oxalic, citraconic and fumaric) show significant deviations in the  $pK_a$  and  $\log k'$  relationship. Above three carbon atoms in the monobasic acids and above five carbon atoms at the dibasic acids the hydrophobic interaction effect between the solute and stationary phase prevails (*i.e.*, the  $\log k'$  vs.  $n_C$  curves are linear). The number of theoretical plates of the column is strongly related to the ionization state of the solute.

The system peak ( $S_{ACN}$ ) observed on the chromatograms (obtained with organic modifier-containing eluents) made possible a study of the retention mechanism in those system where the water concentration of the sample was higher than that of the eluent. The water peak of the sample appears at an eluent volume corresponding to the full permeability of the column ( $v_{S_{ACN}}$ ). Three regions can therefore be identified on the chromatogram. The first region extends from the injection point to the totally excluded volume (the small peak in front of the maleic acid in Fig. 14). The second region is from the totally excluded volume up to the totally permeated volume; the retentions of the solutes eluted in this region are governed by the Donnan and steric exclusion (*e.g.*, maleic, fumaric, acetic and propionic acid). When the retention volume of the solute is higher than the totally permeated volume, then the retention is dominated by the hydrophobic interaction between the solute and the stationary phase (*e.g.*, crotonic acid, monobasic acids with  $n_C > 3$  and dibasic acids with  $n_C > 5$ ).

For the separation of the *n*- and iso-alcohols the selectivity is increased on decreasing the sulphuric acid concentration of the eluent. The retention of alcohols can be decreased by the use of acetonitrile as modifier but the appearance of the system peak must be taken into consideration.

The retention behaviour of the carbohydrates proved to be almost independent of changes in the concentration of sulphuric acid, acetonitrile and column temperature in the ranges investigated.

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