Journal of Chromatography, 167 (1978) 1–16

CHROM. 11,400

RETENTION BEHAVIOUR OF CARBOXYLIC ACIDS IN REVERSED-PHASE COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

The retention behaviour of carboxylic acids in reversed-phase and reversed-phase ion-pair chromatography on hydrophobic alkyl-modified silica gel was investigated. In reversed-phase chromatography, the influence of ionic equilibria on the distribution processes was studied. Thermodynamically valid pH and pK values were used to interpret the ionic equilibria in aqueous–organic mixtures. Special attention was paid to the effect of added indifferent salts and a model describing the influence of foreign cations on the retention of carboxylic ions is proposed. The retention of ionized carboxylic acids can be increased considerably by adding a long-chain aliphatic amine. This counter ion is predominantly adsorbed on the surface of the alkyl-modified silica gel. The retention behaviour of carboxylic acids in this form of chromatography can be explained by assuming an ion-exchange mechanism.

INTRODUCTION

Until recently, the separation of ionogenic compounds, such as acids and amines, by liquid chromatography was carried out almost exclusively on ion-exchange columns[1]. The performance of these columns, however, is adversely affected by the properties of the polymer matrix of the ion exchanger. Mass transfer rates are relatively low; moreover, the compressibility of the soft gel does not allow for high pressure drops to be applied. Therefore, these materials are unsuitable for rapid separations.

Over the past few years, reversed-phase chromatography on silica gel with chemically bonded alkyl chains has proved to be a valuable alternative for separating polar ionogenic compounds. Two essentially distinct forms of reversed-phase chromatography are popular today. In the more conventional form an aqueous buffer, which may contain a highly water-soluble solvent, is used as a mobile phase. The

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retention behaviour of polar ionogenic compounds in this so-called "solvophobic" chromatography was investigated extensively by Horváth *et al.*[2,3]. It has been demonstrated that the retention of ionized solutes on chemically modified silica gel can be increased considerably by adding a lipophilic counter ion to the aqueous mobile phase[4]. This version of reversed-phase liquid chromatography is referred to as ion-pair chromatography[5,6], soap chromatography[7,8], solvent-generated ion-exchange chromatography[9], and recently hetaeric chromatography[10].

This idea followed that of ion-pair liquid–liquid partition chromatography, introduced by Schill and co-workers[11]. Here, the charged solute in the aqueous phase combines with the lipophilic counter ion and is extracted as an ion pair into an organic phase.

However, some doubt exists about the retention mechanism of reversed phase ion-pair chromatography on alkyl-modified silica gel. Ionic equilibria play an important role in both forms of reversed-phase chromatography. In this paper, we consider the influence of these equilibria on the retention behaviour of some carboxylic acids.

THEORETICAL

Reversed-phase chromatography of ionizable compounds

The overall distribution coefficient (K) of a weak organic acid between a buffered aqueous mobile phase and hydrophobic alkyl-modified silica gel can be considered as the sum of two different distribution processes:

$$K = \Delta K_{HX} + \Delta K_X \tag{1}$$

The term ΔK_{HX} describes the contribution of the undissociated acid to the overall partition between the mobile phase and the organic layer on the silica surface:

$$\Delta K_{HX} = \frac{K_{HX}}{1 + \frac{K_a}{[H^+]}} \tag{2}$$

$$K_{HX} = \frac{[HX]_{ads}}{[HX]} \tag{3}$$

$$K_a = \frac{[H^+]\,[X^-]}{[HX]} \tag{4}$$

where $[HX]$, $[H^+]$ and $[X^-]$ are the concentrations of the undissociated acid, the solvated proton and the dissociated acid in the mobile phase, respectively, $[HX]_{ads}$ is the concentration of the undissociated acid in the organic layer and K_a is the dissociation constant of the acid HX. Adsorption of the dissociated acid X^- is described by the term ΔK_X in eqn. 1. This adsorption should coincide with an increased concentration of cations from the buffered solution near the stationary surface layer in order to compensate for the negative charges of the anions X^-.

In aqueous buffered solutions, absorbed anions are partially associated with cations M^+ from the buffer salt[13]. If we denote the absorbed anion concentration

associated with M^+ by $[MX]_{ads}$, the total concentration of absorbed anions is given by $[X^-]_{ads,total} = [X^-]_{ads} + [MX]_{ads}$. Consequently, the contribution of the dissociated acid to the overall partition coefficient can be expressed as

$$\Delta K_X = \frac{K_{X-} + K_{MX}[M^+]_m}{1 + \frac{[H^+]}{K_a}} \quad (5)$$

where

$$K_{X-} = \frac{[X^-]_{ads}}{[X^-]_m} \quad (6)$$

$$K_{MX} = \frac{[MX]_{ads}}{[M^+]_m [X^-]_m} \quad (7)$$

It should be noted that variation of pH or $[M^+]$ is generally attended by variation of the total ionic strength of the buffer solution. With increasing ionic strength, activity coefficients should be introduced and eqn. 5 can be rewritten as

$$\Delta K_X = \frac{K_{X-} + \gamma_{M+} \cdot K_{MX} \cdot [M^+]_m}{1 + \frac{\gamma_{H+} \cdot [H^+]}{K_a}} \quad (8)$$

Combining eqns. 1, 2 and 8 and replacing distribution constants by capacity factors, we find

$$k' = \frac{k'_{HX}}{1 + \frac{K_a}{\gamma_{H+} \cdot [H^+]}} + \frac{k'_{X-} + \gamma_{M+} \cdot k'_{MX} \cdot [M^+]_m}{1 + \frac{\gamma_{H+} \cdot [H^+]}{K_a}} \quad (9)$$

Only if diluted buffer solutions ($<0.01\ M$) are used the influence of the activity coefficients can be neglected. A sigmoidal curve of K or k' *versus* pH will result, as has been pointed out by other workers[2,14], with limiting values $k' = k'_{HX}$ and $k' = k'_{X-} + \gamma_{M+} \cdot k'_{MX} \cdot [M^+]_m$ at $pH \ll pK_a$ and $pH \gg pK_a$, respectively.

With increasing cation concentration at $pH \gg pK_a$ a linear relationship between k' and $\gamma_{M+}[M^+]$ should be found.

Reversed-phase ion-pair chromatography

In the reversed-phase chromatography of ionized organic acids in columns packed with alkyl-modified silica gels the retention can be increased considerably by adding a suitable lipophilic counter ion, *e.g.*, a protonated long-chain amine or a quaternary ammonium salt. Two different mechanisms have been proposed for describing the distribution process in aqueous buffered eluents.

Kissinger[12] suggested that the lipophilic counter ions are absorbed on the surface of the hydrophobic packing material, which may act thereupon as a dynamically coated ion exchanger. A strong adsorption of a long-chain quaternary ammonium salt, trimethylcetylammonium bromide, has been reported by Knox and Laird[7]. The same mechanism was also proposed by Kraak *et al.*[9] to explain the retention behaviour of amino acids in buffered mixtures of water and organic solvents

containing anionic surfactants. These authors, however, did not investigate the correlation between retention and the amount of surfactant adsorbed, which could be the ultimate proof for the ion-exchange mechanism proposed. On the other hand, Horváth *et al.*[10] assumed that in the mobile phase ion pairs are formed between the ionized eluite and the lipophilic counter ion. Retention is due to the adsorption of these ion pairs on the apolar surface of the packing material. The main argument for this idea is that the binding of the counter ion to the surface is negligible. The adsorption of the counter ion was not measured directly but merely estimated from the relationship between capacity ratio and counter-ion concentration for the mobile phase. However, we observed strong adsorption of commonly used counter ions in a number of reversed-phase systems[15].

We felt that for these systems an ion-exchange mechanism could clearly be operative. In this study we have limited ourselves to neat aqueous solvents containing an ionized long-chain amine, Q^+, which is strongly adsorbed on the alkyl surface layer. The positive charge on the adsorbed amine is compensated for by an increased concentration of anions, B^{n-}, from the buffered eluent near the surface layer. With tracer ions, X^-, an ion-exchange equilibrium can be written as follows:

$$Q_nB_{ads} + nX^- \rightleftarrows nQX_{ads} + B^{n-} \tag{10}$$

The ion-exchange equilibrium is described by an equilibrium constant:

$$K_e = \frac{[QX]^n_{ads}\,[B^{n-}]}{[Q_nB]_{ads}\,[X^-]^n} \tag{11}$$

where $[QX]_{ads}$ and $[Q_nB]_{ads}$ are the surface concentrations of the ion-exchange groups in its X^- and B^{n-} forms, and $[B^{n-}]$ and $[X^-]$ are the bulk concentrations of the buffer anion and tracer ion, respectively. Knox and Laird[7] found that adsorption of a long-chain amine from an aqueous solution on a reversed-phase silica gel could be described by a Freundlich-type isotherm:

$$[Q_nB]_{ads} = K_\alpha[Q^+]^{\frac{n}{\alpha}} \tag{12}$$

where $[Q^+]$ is the concentration of the protonated amine in the mobile phase. If reversed-phase ion-pair chromatography is carried out at a pH at which all acids are totally ionized, the partition process may be thought of as the combination of the partition of the ionized solute and the ion-exchange mechanism mentioned above:

$$K = \Delta K_X + \Delta K_{QX} \tag{13}$$

with

$$\Delta K_{QX} = \frac{[QX]_{ads}}{[X^-]} \tag{14}$$

Combining eqns. 11–14 we find

$$K = \Delta K_X + \sqrt[n]{K_eK_\alpha} \cdot \frac{[Q^+]^{\frac{1}{\alpha}}}{[B^{n-}]^{\frac{1}{n}}} \tag{15}$$

Introducing capacity factors and using a logarithmic scale, eqn. 15 can be rewritten as

$$\log (k' - \Delta k'_X) = \text{constant} + \frac{1}{\alpha} \cdot \log [Q^+] - \frac{1}{n} \cdot \log [B^{n-}] \quad (16)$$

A linear dependence should be found between $\log (k' - \Delta k'_X)$ and $\log [Q^+]$ or $\log [B^{n-}]$ with slopes of $1/\alpha$ and $1/n$, respectively.

pH and pK measurements

Influence of ionic strength. As the pK value plays an important role in eqn. 9, the influence of the ionic strength on the pK value cannot be neglected. For low ionic strength, I, a modified Debye–Hückel equation, the Davies equation[16], can be used to evaluate the dependence of pK_a on I (ref. 17):

$$pK'_a = pK^0_a - \frac{(2n + 1) AI^{1/2}}{1 + I^{1/2}} + 0.1 (2n + 1) I = pK^0_a - \Delta pK_a \quad (17)$$

where pK'_a and pK^0_a are the pK values at a given ionic strength and when the ionic strength is zero, respectively, for the nth stage of the ionization of the acid; A is a constant and is known to be 0.512 at 25°. For the separation of weak organic acids with average pK_a values of 3–4 in ion-pair chromatography, the buffering capacity must be derived from acids with p$K_a > 5$. The second protolysis of an acid is generally used, for instance monophosphate–diphosphate. Fig. 1 shows a plot of ΔpK_a *versus* I for the first and second protolyses of the phosphate buffer in aqueous solutions according to eqn. 17.

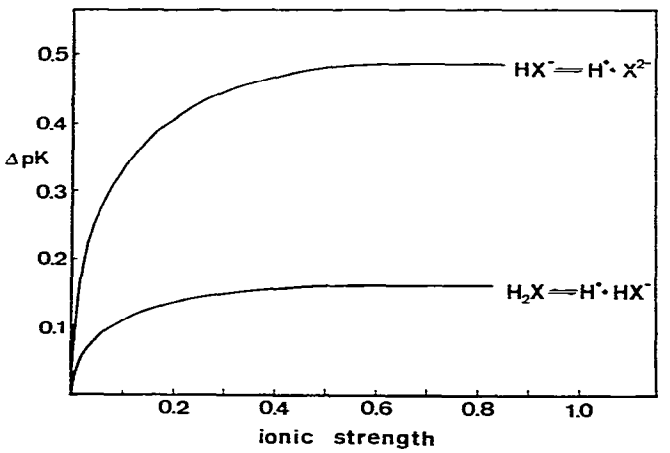


Fig. 1. Influence of ionic strength on the pK_a values of weak acids. The ΔpK_a value is given for the first and second protolyses of phosphate according to the Davies equation[16].

In methanol–water mixtures, a more complicated equation should be used, as given by Gronwall *et al.*[18].

Influence of methanol. The thermodynamically valid pH is defined by [19]

$$pH = - \log m_{H+} \gamma_{H+} \quad (18)$$

where m_{H+} is the proton molality and γ_{H+} is the proton activity coefficient. In general, the operational pH definition is used:

$$pH_X = pH_{st} + \frac{E_X - E_{st}}{0.05916} \tag{19}$$

where pH_X and pH_{st} are the unknown pH value and the known pH value of the test and standard buffer solutions, respectively, and E_X and E_{st} are the e.m.f. values measured in the test and standard buffer solutions.

In methanol–water mixtures the same procedure is applicable[20]. The thermodynamically valid pH is defined by

$$pH^* = -\log m_{H+}\gamma^*_{H+} \tag{20}$$*

If standard buffer solutions of the same solvent composition as the aqueous–organic test solvent are available, the same operational definition (eqn. 19) can be used. pH_X and pH_{st} are replaced by pH^*_X and pH^*_{st}. As the preparation of standard buffer solutions for different aqueous–organic mixtures is time consuming, aqueous standard buffer solutions can be used[21]:

$$pH_X^{app} = pH_{st} + \frac{E_X - E_{st}}{0.05916} \tag{21}$$

where pH^{app} is the measured pH value of the aqueous–organic mixture referred to an aqueous standard solution. As there should be a correction for differences between liquid-junction and glass-electrode potentials in aqueous and methanolic solvents, the thermodynamically valid operational definition is given by

$$pH^*_X = pH_{st} + \frac{E_X - E_{st}}{0.05916} - \frac{E^*_{j,X} - E_{j,st}}{0.05916} + \frac{E^{0\,*}_{gl,X} - E^0_{gl,st}}{0.05916} \tag{22}$$

where $E^*_{j,X}$ and $E_{j,st}$ are the liquid-junction potentials across the interface of the test and standard solutions and the aqueous satured potassium chloride solution of the reference electrode, and $E^{0\,*}_{gl,X}$ and $E^0_{gl,st}$ are the standard potentials of the glass electrode referred to methanol–water and neat aqueous solvents. Comparison of eqns. 21 and 22 gives a correction factor:

$$\delta = pH_X^{app} - pH^*_X = \frac{E^*_{j,X} - E_{j,st}}{0.05916} - \frac{E^{0\,*}_{gl,X} - E^0_{gl,st}}{0.05916} \tag{23}$$

If δ values are known, aqueous standards can be used for thermodynamically valid pH determinations in methanol–water mixtures. Experimentally evaluated δ values of Bates *et al.*[22], de Ligny and Rehbach[23] and Gelsema *et al.*[21] are given in Fig. 2. The last term on the right-hand side of eqns. 22 and 23 appeared to be within experimental error[24].

* Symbols marked with an asterisk refer to water–methanol mixtures to distinguish them from the same symbols referred to neat aqueous standard solutions.

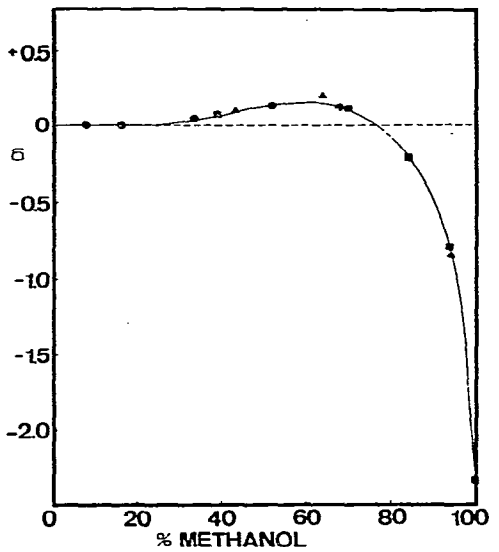


Fig. 2. Plot of correction factor, $\delta = pH_X^{app} - pH_X^*$, against methanol content of solvent mixture. Values are taken from: ●, Bates *et al.*[22]; ■, de Ligny and Rehbach[23]; ▲, Gelsema *et al.*[21].

Once a pH* scale is available, thermodynamic pK_a^* values can be determined by a potentiometric method. As aqueous standard solutions are used, the mixed-mode dissociation constant pK_a^m is measured[25]. With the appropriate δ value pK_a^{m*} is determined. The activity–dissociation constant pK_a^{0*} can be calculated by using the Gronwall *et al.* equation[18] or the experimental values of activity coefficients in water–methanol mixtures at different ionic strength cited in the literature[26].

EXPERIMENTAL

Apparatus

The chromatographic equipment consisted of a Waters M-6000 A dual piston pump, a Chromatronix HPSV-20 injection valve with a 15-μl sample loop and a Zeiss PMQ II UV spectrophotometer. The adsorption isotherm was measured with a recycle system[27], consisting of an Orlita AE 10-4 reciprocating pump, column and mixing reservoir. The total volume of the recycle system was about 5 ml. A more detailed description will be given elsewhere[28]. Titration curves were measured with a Mettler DV/DK titration system. Isotachophoretic analyses were performed on equipment developed by Everaerts *et al.*[29].

Chemicals

Columns (15 cm × 4.6 mm I.D.) were packed with the non-polar chemically bonded stationary phase LiChrosorb RP-18, with a mean particle size of 10 μm (Merck, Darmstadt, G.F.R.) Distilled water and pro analisi grade methanol (Merck) were used as the mobile phase. Orthophosphoric acid (85%) and Titrisol-grade 1 *N* sodium hydroxide solution (Merck) were used to establish the desired pH. Hexylamine (Fluka, Buchs, Switzerland) was used as a cationic extractant. The sample

solutes were purchased from Sigma (St. Louis, Mo., U.S.A.), Aldrich (Beerse, Belgium) and Merck. In Table I all sample solutes used are listed together with the abbreviations used.

Procedures

Capacity factors were evaluated from the retention time of the solute, t_R, and the retention time of an unretarded component $t_{R,0}$. A buffer solution with a concentration slightly different to that of the mobile phase was used to measure $t_{R,0}$. With eluents of low ionic strength water was used. Throughout this study phosphate was used as a buffer component. Even for the measurements of k' *versus* pH in the pH* range 3–5, where the buffer index of phosphate systems is unfavourably low, phosphate was used as different buffers will influence the capacity factors also. pK_a^{0*} values were determined by evaluation of titration curves. Activity coefficients in methanol–water mixtures were taken from Oiwa[26] in accordance with the Gronwall *et al.* equation. Activity coefficients in aqueous solutions were calculated according to Bromley[30] and Davies[16].

Amine concentrations before and after recycling were determined by isotachophoresis[29], using a cation operational system at pH 5. Qualitative information was derived from step-height measurements and quantitative information from step-length measurements.

RESULTS AND DISCUSSION

Reversed-phase chromatography of ionizable compounds

Non-polar stationary phases are eminently suitable for the separation of carboxylic acids with buffered water–methanol solutions as the mobile phase. The capacity factors can be shifted by changing either the pH or the methanol content of the eluent. The dependence of k' on pH* at different methanol contents was measured for the solutes listed in Table I and some results are given in Fig. 3.

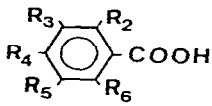
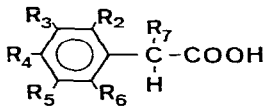
The mobile phase consisted of 0.05 *M* phosphate solution kept at a constant ionic strength of 0.2 with sodium sulphate. Owing to the properties of the siliceous support the pH* range is limited and no complete sigmoidal curve was found for compounds with $pK^* < 3.5$. However, in general the curves follow the ionic dissociation curve, as is found elsewhere[2,14], and obey eqn. 9.

With increasing methanol content the capacity factors decrease, as generally occurs in reversed-phase chromatography. Fig. 4 shows plots of k' *versus* methanol content for some acids. If a pH* is established around the pK_a^* of a solute, with increasing methanol content the decrease in k' cannot be accounted for only by differences in eluent parameters. The influence of the methanol content on the protonation constant cannot be neglected. A decrease in the degree of ionization due to an enhancement of the pK_a^* values is an opposite effect. In Table II, chromatographically evaluated pK_a^* values and pK_a^* values determined from titration curves are compared. In both methods an increase of at least 0.5 in the pK value is found.

Increasing concentrations of buffers or indifferent salts may influence the ionic equilibria involved. First the increasing ionic strength influences the degree of ionization of the acids. This is illustrated in Fig. 5A, where the capacity factor is plotted against the ionic strength for a 0.1 *M* phosphate buffer at pH 2.2, with increasing

TABLE I

LIST OF CARBOXYLIC ACIDS

Compound	*Functional groups*			*Abbreviation*
	$R = H$	$R = OH$	$R = OCH_3$	
				
Benzoic acid	R_{2-6}			BA
Salicylic acid	R_{3-6}	R_2		SA
2,5-Dihydroxybenzoic acid	$R_{3,4,6}$	$R_{2,5}$		25-DOBA
3,5-Dihydroxybenzoic acid	$R_{2,4,6}$	$R_{3,5}$		35-DOBA
2,4-Dihydroxybenzoic acid	$R_{3,5,6}$	$R_{2,4}$		24-DOBA
2,4,6-Trihydroxybenzoic acid	$R_{3,5}$	$R_{2,4,6}$		246-TOBA
2,4,6-Trimethoxybenzoic acid	$R_{3,5}$		$R_{2,4,6}$	246-TMBA
Vanillic acid	$R_{2,4,6}$	R_4	R_3	VA
				
Mandelic acid	R_{2-6}	R_7		MA
4-Hydroxymandelic acid	$R_{2,3,5,6}$	$R_{4,7}$		MOMA
3,4-Dihydroxymandelic acid	$R_{2,5,6}$	$R_{3,4,7}$		DOMA
Vanillylmandelic acid	$R_{2,5,6}$	$R_{4,7}$	R_3	VMA
Homovanillic acid	$R_{2,5-7}$	R_4	R_3	HVA
Phenylacetic acid	R_{2-7}			PAC
3,4-Dihydroxyphenylacetic acid	$R_{2,5-7}$	$R_{3,4}$		DOPAC

amounts of sodium sulphate. For most acids no significant influence of the ionic strength was observed. However, for 25-DOBA k' decreases with increasing ionic strength. This behaviour can be explained as follows.

At pH = 2.2 most acids except 25-DOBA are completely protonated and $k' = \Delta k'_{HX}$. The pK_a^0 for 25-DOBA is about 2.9, so this acid will be partially ionized. The increase in ionic strength will cause an increase in ΔpK and therefore an increase in the degree of ionization and a decrease in k'. In Table III the capacity factor is given as function of the ionic strength for estimated values of $\Delta k'_{HX}$ and $\Delta k'_{X}$ of 25-DOBA. According to eqn. 9 a decrease in k' is found with increasing ionic strength.

Secondly, at pH 7.2 all acids are completely dissociated. Here a strong influence of the ionic strength on k' was observed. The ionic strength was increased either by increasing the buffer concentration (Fig. 5B) or by addition of sodium sulphate to 0.1 *M* phosphate buffer. In all instances the pH was adjusted to 7.2. With increasing ionic strength, the cation concentration, $[Na^+]$, increases as well and adsorption of the anion on the organic silica surface will be facilitated. At low ionic strength a deviation from a linear relationship is found owing to the influence of the activity coefficients in accordance with the given theory.

In Figure 6, k' is plotted against $\gamma_{Na^+}[Na^+]$ for homovanillic acid and mandelic acid. Activity coefficients for Na^+ were calculated according Bromley[30]. The linear relationship is in accordance with eqn. 9. The height of the positive in-

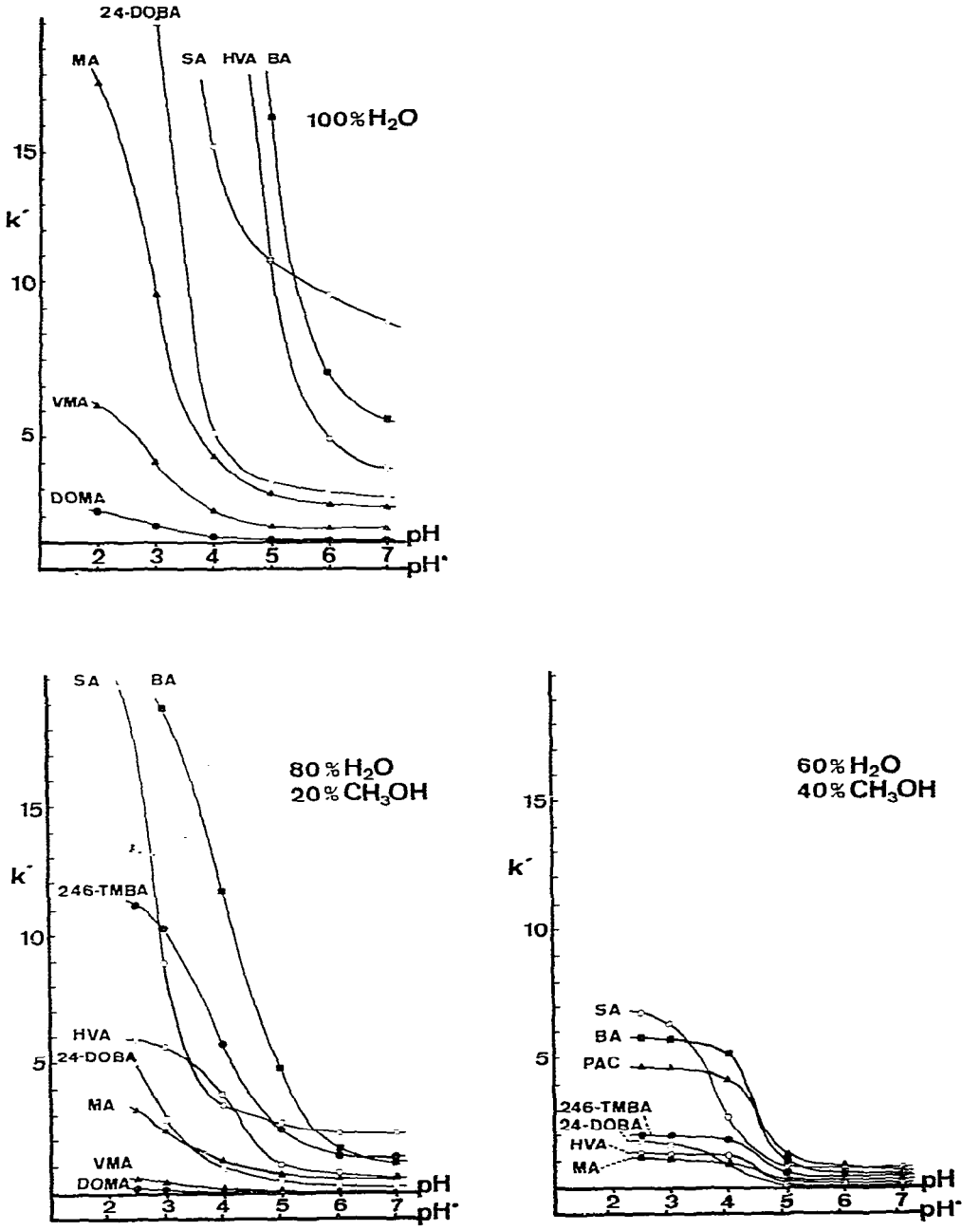


Fig. 3. Effect of pH on the capacity factors of different carboxylic acids for several eluent compositions. The upper pH scale is referred to an aqueous standard solution, and the lower pH* scale is the thermodynamically valid pH* scale referred to a standard solution of the same solvent composition. Column, LiChrosorb RP-18, 10 μm; eluent, 0.05 M phosphate solution, with Na_2SO_4 kept at $I = 0.2$.

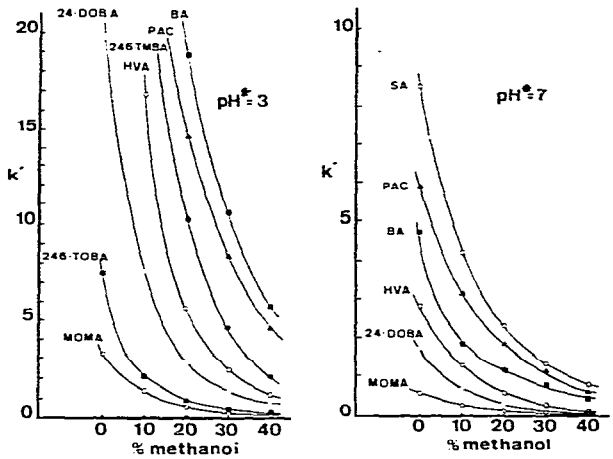


Fig. 4. Effect of methanol content on the capacity factor of some carboxylic acids at constant pH*. Conditions as in Fig. 1.

TABLE II

pK_a VALUES OF CARBOXYLIC ACIDS IN WATER–METHANOL MIXTURES OBTAINED BY TITRATION AND CHROMATOGRAPHIC EVALUATION.

Compound	*Water:methanol ratio*					
	100:0		*80:20*		*60:40*	
	pK_a^0 *(titr.)*	pK_a^0 *(chrom.)*	pK_a^{0*} *(titr.)*	pK_a^{0*} *(chrom.)*	pK_a^{0*} *(titr.)*	pK_a^{0*} *(chrom.)*
Benzoic acid	4.20	—	4.37	4.4	4.78	4.8
Salicylic acid	3.00	—	3.17	3.2	3.38	3.6
Mandelic acid	3.30	—	3.69	3.5	4.03	4.4
2,4-Dihydroxybenzoic acid	3.25	3.2	3.44	3.3	3.75	3.9

tercept is not yet understood; results cited elsewhere show the same relationship[2]. For partially ionized solutes (carboxylic acids at pH 4) an analogous increase in k' is found with increasing ionic strength. Our results at pH 2 do not fit the mentioned model, however.

Reversed-phase ion-pair chromatography

In the theoretical section a model was given that describes the retention mechanism in the presence of long-chain amines. The influences of the amine concentration and the phosphate concentration in the mobile phase were measured successively. The influence of the amine concentration, $[Q^+]$, on the capacity factor was measured in 0.05 *M* aqueous phosphate buffer (pH 7.0) and the results are shown in Fig. 7. An increase in the amine concentration gives an increase in the capacity factors, as generally occurs[7,9,10]. The maximum amine concentration is limited owing to the solubility in the mobile phase (*ca.* 10 mmole/l). The increase in the capacity factor is not linear but resembles a Freundlich adsorption isotherm.

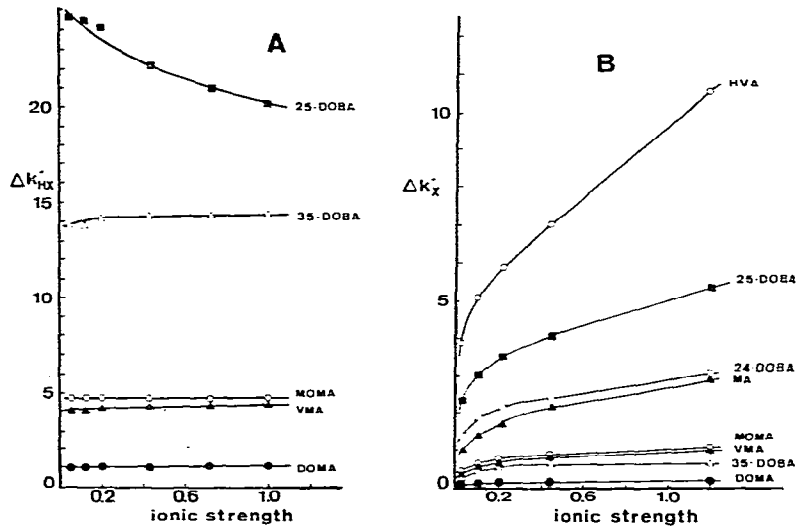


Fig. 5. (A) Plot of capacity factors of undissociated acids against ionic strength. Column, LiChrosorb RP-18, 10 μm; eluent, 0.1 M aqueous phosphate buffer at pH 2.2, ionic strength increased by addition of Na_2SO_4. (B) Plot of capacity factors of dissoliated acids against ionic strength. Column, LiChrosorb RP-18, 10 μm; eluent, aqueous phosphate buffer at pH 7.2, ionic strength increased by increasing phosphate concentration.

TABLE III

INFLUENCE OF IONIC STRENGTH ON CAPACITY FACTOR AT LOW pH

Ionic strength	pK_a	$\Delta k'_{HX}$	$\Delta k'_X$	k'
0	2.9	30	1	25.2
0.1	2.8	30	2.5	24.4
1.0	2.7	30	4	23.6

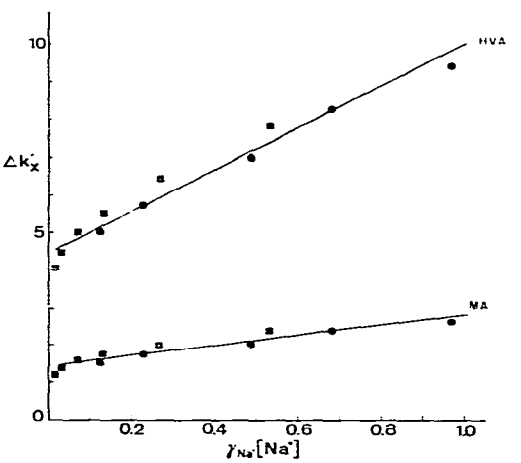


Fig. 6. Influence of cation concentration on the capacity factors of mandelic acid and homovanillic acid with correction for the activity coefficients. Column, Lichrosorb RP-18. Eluent: ●, 0.1 M sodium phosphate buffer at pH 7.2, ionic strength increased by addition of Na_2SO_4; ■, sodium phosphate buffer at pH 7.2, ionic strength increased by increasing sodium phosphate concentration.

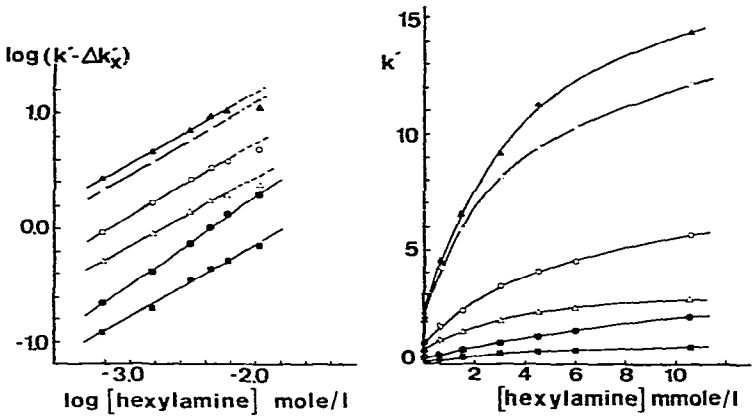


Fig. 7. Effect of hexylamine concentration in the mobile phase on the capacity factors of carboxylic acids: ■, $NaNO_3$; ■, DOMA; △, 35-DOBA; ○, MOMA; □, 24-DOBA; ▲, MA. Column, LiChrosorb RP-18, 10 μm; eluent, 0.05 *M* aqueous phaosphate buffer at pH 7 with increasing amine concentration.

To confirm the first part of eqn. 16, $\log (k' - \Delta k'_x)$ was plotted against $\log [Q^+]$, as shown in Fig. 7. A linear relationship was obtained, with a mean slope of 0.65 ± 0.05.

We measured the adsorption isotherm of hexylamine; known amounts of a hexylamine solution and stationary phase were brought into equilibrium in a recycle system. A 0.02 *M* phosphate concentration was used because of isotachophoretic limitations.

A logarithmic plot of the amine concentration in the stationary phase against the amine concentration in the mobile phase is shown in Fig. 8. A linear

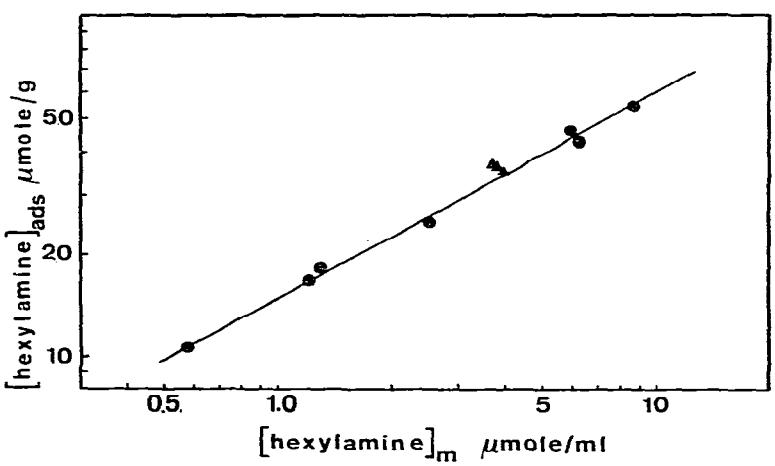


Fig. 8. Logarithmic plot of the adsorption isotherm of hexylamine (●). Column, LiChrosorb RP-18; eluent, 0.02 *M* aqueous phosphate buffer at pH 7 with increasing amine concentration. ▲, Effect of the phosphate concentration on the distribution of hexylamine between the mobile and stationary phases (see Table IV).

relationship is found, with a slope of 0.6 ± 0.05. This is in good agreement with the measured value of 0.65 for $1/\alpha$, derived from chromatographic experiments. The same type of plot was found by Knox and Laird for a non-buffered system[7]. With a fixed amount of amine in the recycle system the ratio between the amine concentrations in the mobile and stationary phases was measured at different phosphate concentrations. As shown in Fig. 8, the ratio is nearly independent of the phosphate concentration. In Table IV the amine concentrations in the mobile and stationary phases are given for different phosphate concentrations.

TABLE IV

INFLUENCE OF PHOSPHATE CONCENTRATION ON THE DISTRIBUTION OF HEXYLAMINE BETWEEN MOBILE AND STATIONARY PHASES

In Fig. 8 these points are denoted by ▲.

$[Phosphate]_m$ (μmole/l)	$[Hexylamine]_m$ (μmole/l)	$[Hexylamine]_{ads}$ (μmole/g)
47.9	3.65	37.1
37.7	3.72	36.7
17.3	3.95	35.4

If ion exchange is the predominant mechanism, the buffer concentration will have a considerable influence on the capacity factors, as shown by eqn. 16. The capacity factors of some carboxylic acids were measured as a function of the phosphate concentration in the eluent and the results are shown in Fig. 9. An increase in the phosphate concentration results in a decrease in the capacity factors. At high phosphate concentrations the influence becomes negligible. The same type of curve was found by Horváth *et al.*[10].

To evaluate the influence of the phosphate concentration on the ion-exchange mechanism, the capacity factor of the carboxylic anion, $\Delta k'_X$, should be subtracted. It should be noted, however, that $\Delta k'_X$ is not a constant but depends on the concentration of the sodium ion and the ionic strength of the buffer. Therefore, we estimated $\Delta k'_X$ values for the composition of the buffer used in this experiment, from the data presented in Fig. 5B. The results are given in Fig. 9. With increasing phosphate concentration, less hexylamine is available for ion-pair formation with the tracer ions and the contribution of $\Delta k'_X$ to the total capacity factor increases.

According to eqn. 16, $\log (k' - \Delta k'_X)$ *versus* $\log [B^{n-}]$ was plotted. The relationship is linear with a slope of 0.55 ± 0.05. Although the mobile phase will contain monophosphate and diphosphate ions, it is known from ion-exchange chromatography[31] and extraction experiments[32] that in general divalent ions are bonded more strongly than monovalent ions. Therefore, in eqns. 10–16 n can be taken as 2, and the theoretically derived slope of $\log (k' - \Delta k'_X)$ *versus* $\log [B^{n-}]$ will be 0.5. This is in good agreement with the value of 0.55, found above.

There are some practical consequences of the adsorption of the amine on the stationary phase. If an amine is added to the mobile phase, long equilibrium times are necessary before a stable system is achieved. More than 50 column volumes are needed before the capacity factors become constant. Analogously, over 50 column volumes are needed to wash out the adsorbed amine. Addition of methanol will

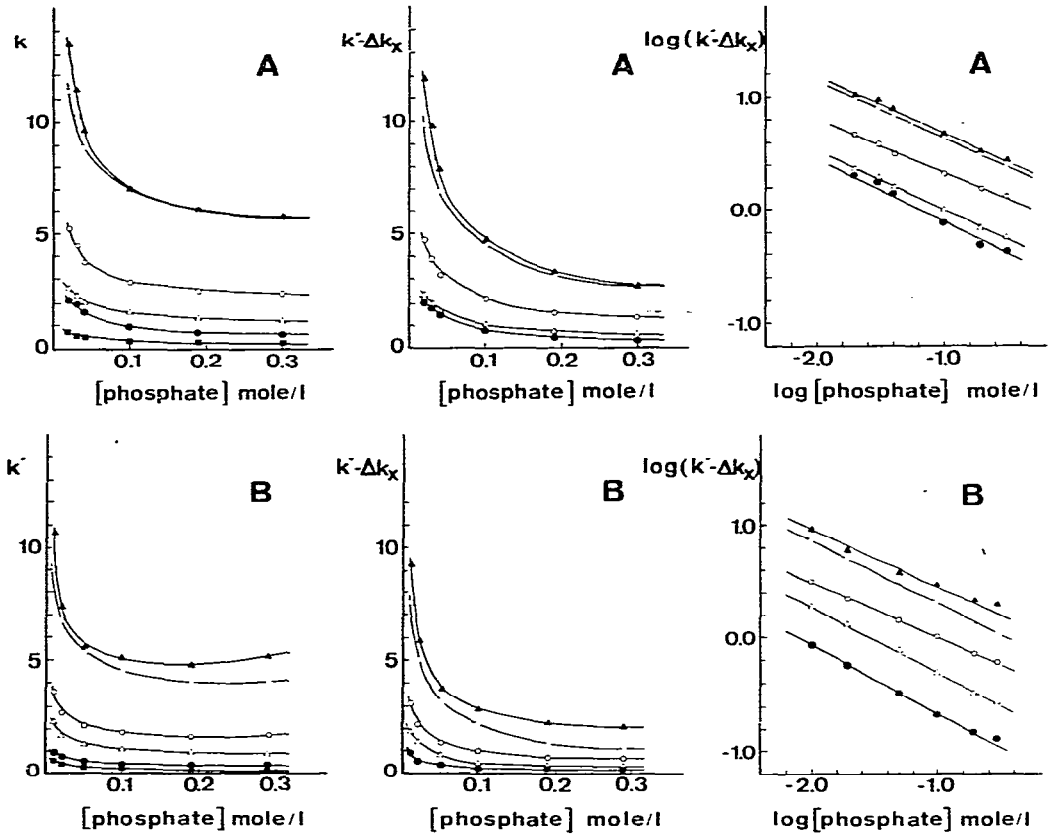


Fig. 9. Effect of phosphate concentration on the capacity factors of carboxylic acids in ion-pair chromatography: ■, $NaNO_3$; ●, DOMA; △, 35-DOBA; ○, MOMA; □, 24-DOBA; ▲, MA. Column, LiChrosorb RP-18; eluent, (A) 9.2 mmole/l hexylamine in aqueous phosphate buffer at pH 7 and (B) 1.6 mmole/l hexylamine in aqueous phosphate buffer at pH 7.

accelerate this process. If the column is washed out with distilled water the column can easily be damaged. The phosphate ions will be washed out first and, owing to the presence of the amines that remain, the pH in the column will increase. As the amines are washed out slowly the pH of the eluent will also increase. Table V gives the eluent pH at the column outlet if an amine–phosphate-loaded column is flushed

TABLE V

ELUENT pH AT COLUMN OUTLET IF AMINE-LOADED COLUMN IS WASHED OUT WITH DISTILLED WATER

Amount of water eluted (ml)	*pH*
0	7.0
30	7.6
60	8.7
90	9.1

with distilled water. As many ionic equilibria are involved in the retention mechanisms, the temperature should be kept constant within a certain range. A temperature increase from about 20° to 40° gave on average a two-fold decrease in the capacity factors.

ACKNOWLEDGEMENTS

The authors thank Ir. A. P. Konijnendijk and Mr. H. J. G. Beckers for their stimulating assistance. A grant from D.S.M. (Geleen, The Netherlands) to one of us (J. v.d. Venne) is gratefully acknowledged.

REFERENCES

1 P. Jandera and J. Churácek, *J. Chromatogr.*, 86 (1973) 351.
2 C. Horváth, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
3 C. Horváth and W. Melander, *J. Chromatogr. Sci.*, 15 (1977) 393.
4 D. P. Wittmer, N. O. Nuessle and W. G. Haney, Jr., *Anal. Chem.*, 47 (1975) 1422.
5 B. Franssen, K.-G. Whalund, I. M. Johansson and G. Schill, *J. Chromatogr.*, 125 (1976) 327.
6 I. M. Johansson, K.-G. Wahlund and G. Schill, *J. Chromatogr.*, 149 (1978) 281.
7 J. H. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1976) 17.
8 J. H. Knox and J. Jurand, *J. Chromatogr.*, 149 (1978) 297.
9 J. C. Kraak, K. M. Jonker and J. F. K. Huber, *J. Chromatogr.*, 142 (1977) 671.
10 C. Horváth, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.
11 S. Eksborg, P. O. Lagerström, R. Modin and G. Schill, *J. Chromatogr.*, 83 (1973) 99.
12 P. T. Kissinger, *Anal. Chem.*, 49 (1977) 883.
13 J. W. Galbraith, C. H. Giles, A. G. Halliday, A. S. A. Hassan, D. C. McAllister, N. Macaulay and N. W. Macmillan, *J. Appl. Chem.*, 8 (1958) 416.
14 J. J. Kippling, *J. Chem. Soc.*, (1948) 1483.
15 R. S. Deelder, J. H. M. van den Berg, J. L. M. van de Venne and H. A. J. Linsen, to be published.
16 C. W. Davies, *J. Chem. Soc.*, (1938) 2093.
17 D. D. Perrin and B. Dempsey, *Buffers for pH and Metal Ion Control*, Chapman and Hall, London, 1974.
18 T. H. Gronwall, V. K. LaMer and K. Sandved, *Phys. Z.*, 29 (1928) 358.
19 R. G. Bates, *Determination of pH*, Wiley, New York, 1964.
20 C. L. Ligny, P. E. M. Luykx, M. Rehbach and A. A. Wieneke, *Rec. Trav. Chim. Pays-Bas*, 79 (1960) 699 and 713.
21 W. J. Gelsema, C. L. de Ligny, A. G. Remijse and H. A. Blijleven, *Rec. Trav. Chim. Pays-Bas*, 85 (1966) 647.
22 R. G. Bates, M. Paabo and R. G. A. Robinson, *J. Phys. Chem.*, 67 (1963) 1833.
23 C. L. de Ligny and M. Rehbach, *Rec. Trav. Chim. Pays-Bas*, 79 (1960) 727.
24 W. J. Gelsema, C. L. de Ligny and H. A. Blijleven, *Rec. Trav. Chim. Pays-Bas*, 86 (1967) 852.
25 D. B. Rorabacher, W. J. MacKeller, F. R. Shu and M. Bonavita, *Anal. Chem.*, 43 (1971) 561.
26 I. T. Oiwa, *J. Phys. Chem.*, 60 (1956) 754.
27 J. C. Kraak, *Thesis*, University of Amsterdam, 1974.
28 J. L. M. van de Venne, *Thesis*, Eindhoven University of Technology, in preparation.
29. F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis — Theory, Instrumentation and Applications*, Elsevier, Amsterdam, 1976.
30 L. A. Bromley, *AIChE J.*, 19 (1973) 313.
31 F. Helfferich, *Ionenaustauscher: Grundlagen. Struktur, Herstellung, Theorie*, Band I, Verlag Chemie, Weinheim, 1959.
32 T. Sato, *J. Appl. Chem.*, 15 (1965) 10.