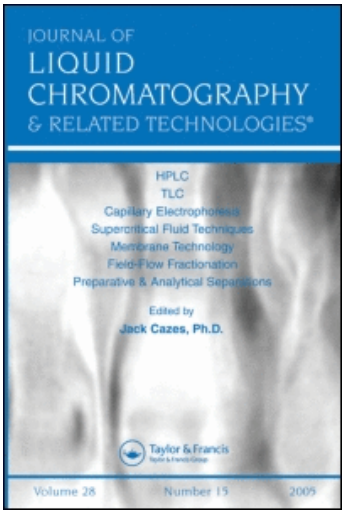

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Buffer Choice for Tuning the Selectivity in Reverse Phase Chromatography

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BUFFER CHOICE FOR TUNING THE SELECTIVITY IN REVERSE PHASE CHROMATOGRAPHY

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ABSTRACT

Retention behaviour of ionogenic species in high-performance liquid chromatography on reversed phase materials was studied, specifically dependence of buffer quality applied to mobile phases. The buffers' effect on retention of organic acids, amino acids and dipeptides is quantified by modelling capacity factors as a function of pH-values. At constant ionic strength, increasing capacity factors were observed going from phosphate to less polar citrate buffer, modification of accessible silanol groups of the stationary phase being responsible for this effect. Application of citrate buffer for separation of a seven-component mixture is demonstrated on the basis of a computerized search for optimum chromatographic performance. The evaluated factor levels (pH, methanol content and ionic strength) differ from those found using phosphate buffer-containing mobile phases.

INTRODUCTION

Selectivity in high-performance liquid chromatography may be affected by a variety of factors such as column length, particle diameter (1), and chemical

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modification of the stationary phase (2), or by changing easily variable factors as pH, ionic strength, temperature, elution strength or surface-active ion concentration (3).

Up to now, little attention has been paid to the variation of buffer quality as a discontinuous factor for tuning the selectivity in reverse phase chromatography (RPC). Melander and Horváth (4) have studied effects of different acidic amine phosphate buffers as eluents and found that cationic diammonium ions show similar hetaeron behaviour as is known for other ion pairing reagents based on ammonium cations. In addition, reduced peak tailing and improved resolution observed with these buffer systems (4) were attributed to masking of accessible silanol groups by the amine component of the buffers.

Less is known for effects that arise if buffer systems are varied with respect to the anion. Recently a very small effect was observed by Brugman et al. (5) who prepared buffers from acetate, chloroacetate, formate and propionate.

On the other hand, from practical applications it is well established that replacement of phosphate by citrate buffer may be necessary to get satisfactory separation (6). In order to derive a fundamental base for understanding such a behaviour, the aim of the present work is the investigation of the effect of different buffers on the separation of compounds such as amino acids, dipeptides and other organic acids by RPC. In addition, the experimental data can be used for numerical modelling of retention behaviour (7) in the presence of different buffer systems as a base for quantitative comparison of buffer quality as well as for locating optimum chromatographic performance.

EXPERIMENTAL

Chromatographic system, computations and materials

The chromatographic experiments were carried out using a WATERS 6000 M pump, and automatic injection system WATERS WISP 710A, a WATERS UV-detection (M 440) together with the WATERS DATA MODULE and the WATERS SYSTEM CONTROLLER 720. LiChrosorb RP-18 (MERCK, Darmstadt, F.R.G.), particle size 7 µm, was packed into a stainless steel column (150 x 3.2 mm I.D.). The column was operated at 22° C and a flow rate of 1 ml min^{-1}. The time equivalent to the void volume was estimated from injections of KBr solution to be t_o = 0.75 min.

All computations were carried out on an UNIVAC 1100 (Rechenzentrum Graz) and a HP-97 (Hewlett-Packard, Loveland, CO, USA) programmable calculator. The response surfaces were drawn from digitized data on a HP 9862A calculator-plotter interconnected to a HP 9830 digital computer (both, Hewlett-Packard).

Experimental design

In order to characterize the buffers' effect, the retention behaviour of L-leucyl-L-tyrosine, D-leucyl-L-tyrosine, L-tyrosine, anthranilic acid, m-aminobenzoic acid, p-aminobenzoic acid and phthalic acid as solutes has been studied as a function of pH of the mobile phase. According to buffer capacities, the following substances have been applied at pH values ranging from 2 to 7.5 for phosphate and citrate, 2 to 6 for acetate, 2 to 4 for glycine and 2 to 6 for tartrate in steps of 0.5 pH units.

The order of evaluating the 47 mobile phases was randomized to minimize confounding of time and reproducibility of surface property of the stationary phase

with the effect of pH and buffer quality. As changes of the buffer type - a process to be found reversible - may favour alteration of surface quality of the stationary phase, repetitive buffer changes were kept at a minimum.

For modelling retention data as to dependence on three factors (pH, ionic strength and methanol content), a fractional factorial design (3^2x2^{1-1}) (25) with 9 mobile phases prepared from citrate buffer, was additionally used. The levels of the randomized design being pH-values at 2.0, 4.0 and 7.0, volume percent methanol at 10, 20 and 30%, and 0.1 and 0.2 M ionic strength.

Mobile phases

To quarantee that the ionic strength is constant at any mobile phase composition, contributions from ionized buffer species to the total ionic strength were calculated for the actual pH-values and corrected for by the added amounts of base or acid for adjusting the pH. The final ionic strength was obtained by addition of 1 M potassium chloride solution to the mobile phase.

The influence of methanol on pH-measurements was corrected for by the δ-values given by Bates et al. (8). Prior to use all mobile phases were aspirated through a 0.45 µm Sartorius 11306 membrane filter and ultrasonically degassed.

RESULTS AND DISCUSSION

pH-dependences

In order to evaluate the effect of buffer on all ionic forms of the solutes, the retention behaviour was studied over a wide pH-range. Figure 1 gives the

experimental results for all seven solutes in presence of phosphate, glycine, tartrate, acetate and citrate buffer.

In spite of the fact that no tremendous changes in retention can be expected by applying different buffer types, the capacity factors vary up to 40%, e.g. if one compares retention data of D-leucyl-L-tyrosine in phosphate and citrate buffer at pH 2.5 (Figure 1). Also, the effect is high enough to reverse retention order of solutes, e.g. at pH 2.8 L-leucyl-L-tyrosine (k' = 13.60) elutes in citrate in front of anthranilic acid (k' = 14.80) but in phosphate buffer the peptide (k' = 17.40) is more retained than anthranilic acid (k' = 15.80).

For quantifying the buffers' effect the retention data were fitted to a mathematical model presented in equation 1 (cf. (3)).

$$k' = \frac{k_o + k_1 \frac{[H^+]}{K_{a1}} + k_{-1} \frac{K_{a2}}{[H^+]}}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}} \qquad (1)$$

where k_o, k_1 and k_{-1} are the capacity factors for species HS, H_2S and S, respectively, and K_{a1} and K_{a2} refer to consecutive dissociation constants.

Typical parameter estimates are given for L-leucyl-L-tyrosine, anthranilic acid and phthalic acid in Table 1. Inspection of experimental and computed data does not reveal any influence of the ionized forms of the solutes and/or of the buffer species on the mode of retention. Thus hetaeric effects based on ion pair formation cannot be responsible for moderated retention in presence of different buffers.

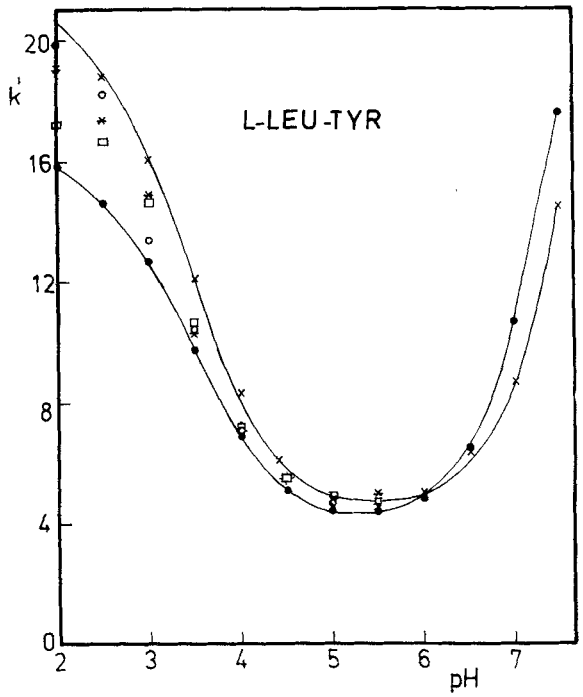

FIGURE 1

Effect of buffer on retention of solutes at different pH-values. x phosphate; o glycine; ✱ tartrate; □ acetate; ● citrate. 10 volume percent methanol; 0.1 M ionic strength; 15 mM buffer concentration.

(a) L-LEU-TYR - L-leucyl-L-tyrosine; (b) D-LEU-TYR - D-leucyl-L-tyrosine; (c) ANTHRA - anthranilic acid; MABA - m-aminobenzoic acid; PABA - p-aminobenzoic acid; (d) PHTHAL - phthalic acid.

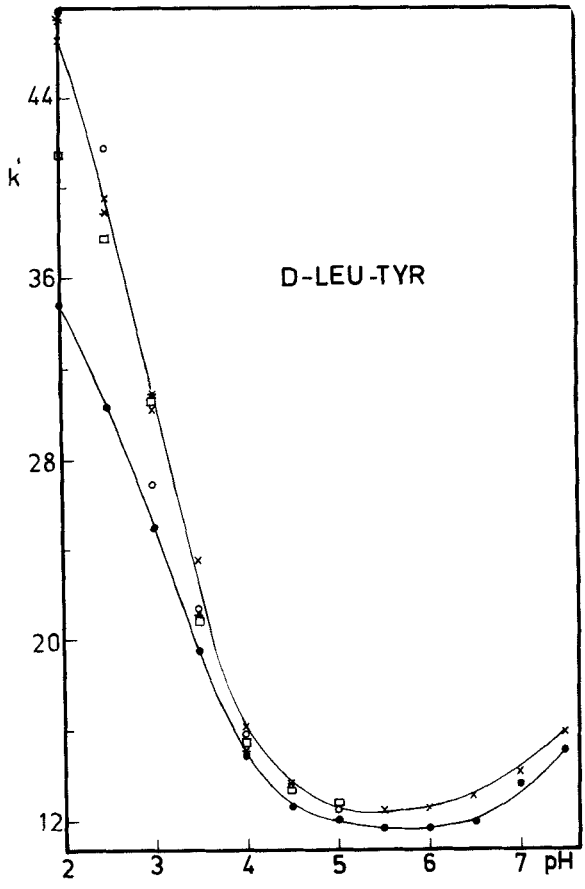

FIGURE 1B

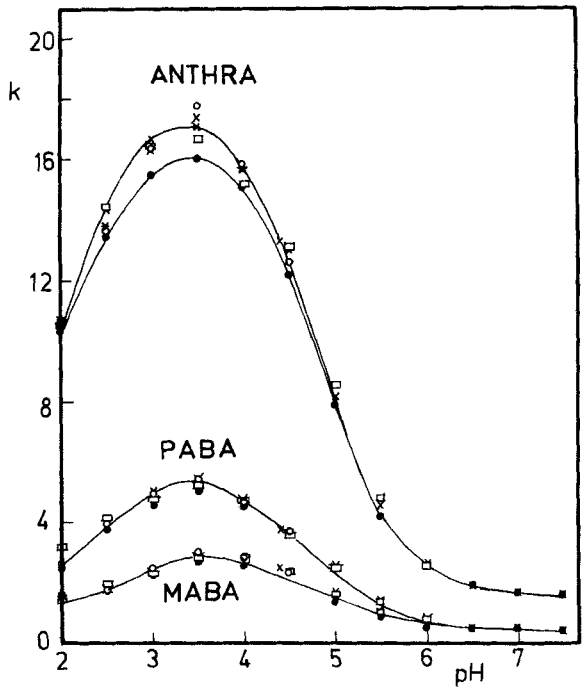

FIGURE 1C

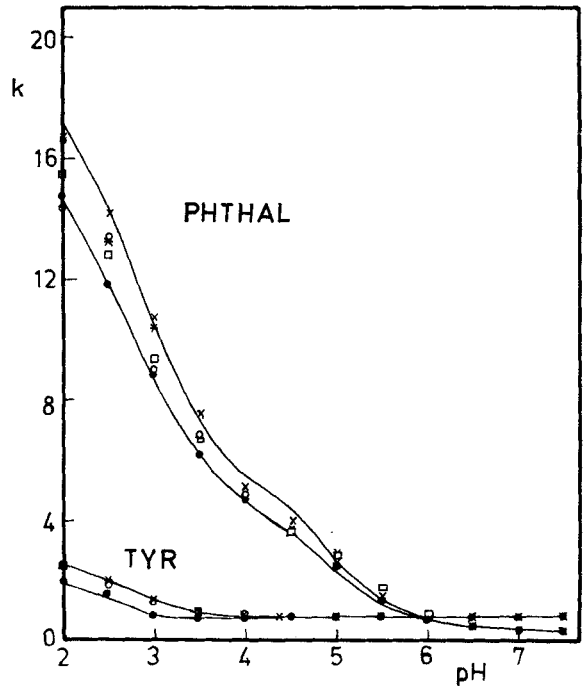

FIGURE 1D

TABLE 1

Parameter Estimates for Solutes by Use of Different Buffer Systems

Buffer	k_o	k_1	k_{-1}	K_{a1}	K_{a2}	s [a]
	L-leucyl-L-tyrosine					
phosphate	4.41	21.32	33.90	4.28×10^{-4}	1.75×10^{-8}	0.196
citrate	4.02	15.88	30.68	3.47×10^{-4}	3.26×10^{-8}	0.191
acetate	5.06	18.90	-	5.34×10^{-4}	(1.86×10^{-8})	0.300
tartrate	4.56	19.89	-	5.89×10^{-4}	(1.86×10^{-8})	0.155
glycine	4.50	20.54	-	6.51×10^{-4}	(1.86×10^{-8})	0.346
			lit.[9]	6.31×10^{-4}	1.86×10^{-8}	
	Anthranilic acid					
phosphate	18.41	3.89	1.58	8.39×10^{-3}	1.92×10^{-5}	0.191
citrate	17.31	6.18	1.53	5.98×10^{-3}	1.51×10^{-5}	0.131
acetate	17.37	1.46	1.67	1.86×10^{-2}	1.26×10^{-5}	0.268
tartrate	17.17	0.67	2.61	1.60×10^{-2}	1.35×10^{-5}	0.252
glycine	18.36	6.35	0.48	5.26×10^{-3}	1.25×10^{-5}	0.416
			lit.[19]	1.00×10^{-2}	1.63×10^{-5}	
	Phthalic acid					
phosphate	5.16	18.99	0.30	1.67×10^{-3}	1.17×10^{-5}	0.206
citrate	4.53	16.60	0.39	1.87×10^{-3}	1.17×10^{-5}	0.156
acetate	4.91	17.26	1.51	1.75×10^{-3}	1.56×10^{-5}	0.507
tartrate	4.03	18.08	0.92	1.35×10^{-3}	1.21×10^{-5}	0.336
glycine	4.49	17.50	0.21	1.75×10^{-3}	0.25×10^{-5}	0.385
			lit.[10]	1.78×10^{-3}	1.17×10^{-5}	

[a] - residuals

From comparison of retention data among different buffers, a general order follows for affecting capacity factors of the most retained ionic forms of solutes:

phosphate > glycine, tartrate, acetate > citrate.

Mechanism of buffer effect

From a chemical point of view, interactions of buffer species with non-polar stationary phase can be scarcely expected. Recent results of Horváth's group (11, 12), however, have shown that a dual retention mechanism has to be accounted for in RPC based upon solvophobic and silanophilic interactions. Masking of silanols as shown for ammonium cations (4, 12) may also occur with the studied buffer systems and may alter the capacity factors.

To prove this hypothesis, first, increasing buffer concentrations for phosphate and citrate were applied ranging from 10^{-3} to 0.1 M at pH 3.5 and an ionic strength of 0.1 M. As the result, retention data typically for both buffers were reproduced but no dependence on buffer concentration could be observed. Buffer concentrations lower than 10^{-3} M have not been used because of too low buffer capacity.

Secondly, a fresh stationary phase (all contaminations by buffer were eluted with the supporting electrolyte) was conditioned with phosphate or citrate and the amount of buffer in the eluate was measured titrimetrically or by monitoring it at 254 nm. In the initial 5 ml of eluate, no buffer substance was detectable, but after that, a typical breakthrough curve was found. By use of the breakthrough volume, V_b, the void volume, V_o, and the concentration of buffer in the mobile phase, c_m, the amount adsorbed onto the stationary phase (13)

was calculated, according to equation 2: 93 μM phosphate and 71 μM citrate.

$$q_{ads} = (V_b - V_o)\ c_m \qquad (2)$$

A rough estimate for the surface area within the 3.2x150 mm column reveals 100 m^2 (cf.(13)), from which a concentration of about 1 $\mu M/m^2$ of surface silanols is calculated that is affected by adsorption with buffer species. This coverage of silanols by the buffer has not reached maximum yet. For octadecyl-silica materials (C-18) from initially available silanol surface concentration of about 8 $\mu M/m^2$ (14, 15), only a concentration of 3.04 to 3.28 $\mu M/m^2$ (16) is covered by the hydrocarbonaceous ligates. Compared to alkyl silyl bonded phases of shorter alkyl chain length C-18 and C-22 show lower surface coverage than the shorter ones (16); this yields a higher number of accessible silanol groups. For this reason excessive peak tailing is mainly observed if long chain alkyl silyl bonded phases are used (17).

In order to specify such effects,i.e. dependence of the buffers' influence on peak symmetry upon buffer quality, was checked at low pH-values of mobile phases where non-ideal chromatographic peaks appear with dipeptides as solutes (7). In Table 2, peak asymmetries, measured for phosphate and citrate-containing phases, are presented. From the data, it is evident that modification of silanols by citrate buffer leads to better peak symmetry than is found with phosphate.

In conclusion, buffer quality is responsible for variation of selectivity as well as for the shapes of peaks and, therefore, for column efficiency. Similar effects were reported by Melander et al. (4) by comparing the influence of different phosphates on retention at surfaces where the coverage by the hydrocarbonaceous

TABLE 2

Peak Asymmetries+ for Cationic Dipeptides in Phosphate and Citrate Buffered Mobile Phases

Dipeptide	pH	Phosphate	Citrate
L-leucyl-L-tyrosine	2.0	3.90	2.50
	2.5	3.45	2.00
	3.0	1.90	1.27
	3.5	1.06	1.00
D-leucyl-L-tyrosine	2.0	6.55	5.71
	2.5	5.08	4.45
	3.0	4.24	3.57
	3.5	2.15	1.87

10 volume-percent methanol; 0.1 M ionic strength.

+ asymmetric refer to peak width ratios b/a measured at 10% peak height (26).

ligates was low (Partisil 10 ODS): upon changes from sodium to N,N,N',N'-tetramethylethylene diamine phosphate, variation in retention as well as peak sharpening was observed.

Masking of silanols by buffer type compounds is also known from thin layer chromatography (18) and from liquid chromatography with citrate buffered silicagel (19). In the case of the latter (19), batchwise citrate coated silicagel used for normal high-performance liquid chromatography, showed completely changed surface properties compared to untreated silica. Further modifications of surface silanols were achieved by

employing sodium sulphate, oxalic acid, tartrate and sodium phosphate (20).

The effect of changing retention data by variation of buffer type demonstrated in the present study is lower than found on untreated silica (19, 20) or with diamine phosphates (4). This is due mainly to solvophobic interactions that are operative in the water-rich hydro-organic mobile phases. By use of less polar mobile phases, silanophilic interactions will dominate and stronger dependences on buffer quality is to be expected as shown for methanol-rich mobile phases in RPC (11, 12) or for non-polar mobile phases applied to naked silica (19, 20).

Chemically, adsorption of buffers onto silanol groups is to be explained by hydrogen bond formation as described by Snyder (21, 22). Thus, interaction of silanol with an oxygen atom of the buffer molecules is thought to account for adsorption of buffers onto the stationary phase.

It should be mentioned that masking of accessible silanols by phosphate, tartrate, citrate, glycine and acetate was found to be reversible. This may be useful for tuning chromatographic selectivity by applying different buffer qualities or mixtures of buffers (6).

In general, a tendency may be deduced from the order of modification of stationary phase by buffers, i.e. the less polar the buffer the less are ionic species retained by the modified silanol groups.

To characterize the change in selectivity by varying buffer quality, separation of a mixture of all seven solutes was undertaken using citrate- and phosphate-containing mobile phases. As the separation should be compared under optimized conditions two additional factors - methanol content and ionic strength - had to be varied for citrate as previously carried out for phosphate

buffered eluents (7). Location of optimum chromatographic performance was done by computerized search for the global optimum based on a numerical description of retention data (7).

Three-factor numerical model for citrate

The model described recently (7) for fitting retention data in RPC in terms of dependence on pH, elution strength and ionic strength was used to describe the retention behaviour of all seven compounds in citrate buffer. The capacity factor of solute is expressed as follows:

$$k' = \frac{C_o(F_1 + F_2 e^{-K_3(\%M)})}{S} + \frac{C_1(F_3 + F_4 e^{-K_4(\%M)}) \frac{[H^+]}{K^o_{a1}P_1}}{S} + \frac{C_{-1}(F_5 + F_6 e^{-K_5(\%M)}) \frac{K^o_{a2}P_2}{[H^+]}}{S} \quad (3)$$

where $S = 1 + \frac{[H^+]}{K^o_{a1}P_1} + \frac{K^o_{a2}P_2}{[H^+]}$,

and F_1, F_3, F_5 are off-set terms; F_2, F_4 and F_6 stand for the capacity factors of the species HS, H_2S and S in absence of organic modifier, respectively; K^o_{a1} and K^o_{a2} represent the consecutive dissociation constants; K_3, K_4 and K_5 are constants characterizing the elution strength; C_o, C_1 and C_{-1} are corrections for ionic strength according to the Davies equation and P_1 and P_2 correct the dissociation constants with respect to ionic strength (Davies) and solvent influence by non-linear parameters K_6 and K_7; %M is the volume percent methanol in the mobile phase.

TABLE 3

Estimated Parameters for Modelling Capacity Factors on Variation of pH, Ionic Strength and Methanol Content

Parameter	Anthranilic Acid	m-Aminobenzoic Acid	p-Aminobenzoic Acid	L-Leucyltyrosine	D-Leucyltyrosine	Tyrosine	Phthalic Acid
F_1	5.95	3.25	2.55	-	-	1.16	-
F_2	46.88	2.19	10.45	8.19	30.51	-	4.03
F_3	-	-	-	1.30	1.58	0.46	3.36
F_4	7.66	0.59	0.71	55.70	124.2	2.25	42.25
F_5	-	-	-	-	-	-	0.16
F_6	1.16	0.42	0.30	92.61	34.47	-	-
pK^o_{a1}	2.27	2.51	2.28	3.13	2.92	2.02	2.89
pK^o_{a2}	4.77	4.53	4.73	-	-	-	4.43
K_3	14.34	24.24	12.77	6.86	9.92	63.33	3.92
K_4	7.46	-0.34	0.66	18.78	17.74	5.31	11.62
K_5	1.80	-0.85	-1.86	13.86	67.57	-	-
K_6	0.28	-4.55	-0.79	-	-	2.19	4.33
K_7	2.06	3.28	3.30	-	-	-2.10	0.47
s	0.159	0.203	0.214	0.329	0.412	0.308	0.214

The fitted parameters are given in Table 3. The low residuals (s) demonstrate the good agreement between experimental data and the model used. The numerical expression (Eq. 3) permits estimation of capacity factors of all seven solutes at pH-values from 2 to 7, at 10 to 30 volume percent methanol content and at ionic strength values from 0.1 to 0.2 M as a basis for the computerized search for optimum chromatographic selectivity.

Global optimum for citrate and phosphate buffer

Location of the global optimum was undertaken on the basis of the maximum of the smallest relative retention (α_{min}) under a given set of experimental conditions as optimization criteria. As relative retention for all possible pairs of the seven solutes are evaluated as a function of three chromatographic factors "window-diagrams" (23) that are multidimensional in nature (24) result.

A computerized grid search for minimum alpha values at about 7000 experimental conditions revealed as optimum α-value 1.43 corresponding to the mobile phase specified as follows: pH 3.30; 10 volume percent methanol and 0.1 M ionic strength. Minimum alpha plots at optimal ionic strength are given in Figure 2. The different step width for adjusting the experimental variables demonstrates how close the minimum alpha plot has to be described in order to account for all local optima that fall into the range of experimentation. The chromatogram in Figure 3 verifies the model-predicted capacity factors (cf. legend of Figure 3) showing good agreement between experimental and theoretical retention data.

In comparison, the optimum chromatographic performance in phosphate buffer was evaluated at pH 3.20, 14 volume percent methanol and 0.18 M ionic strength with optimum alpha value being 1.38 (7).

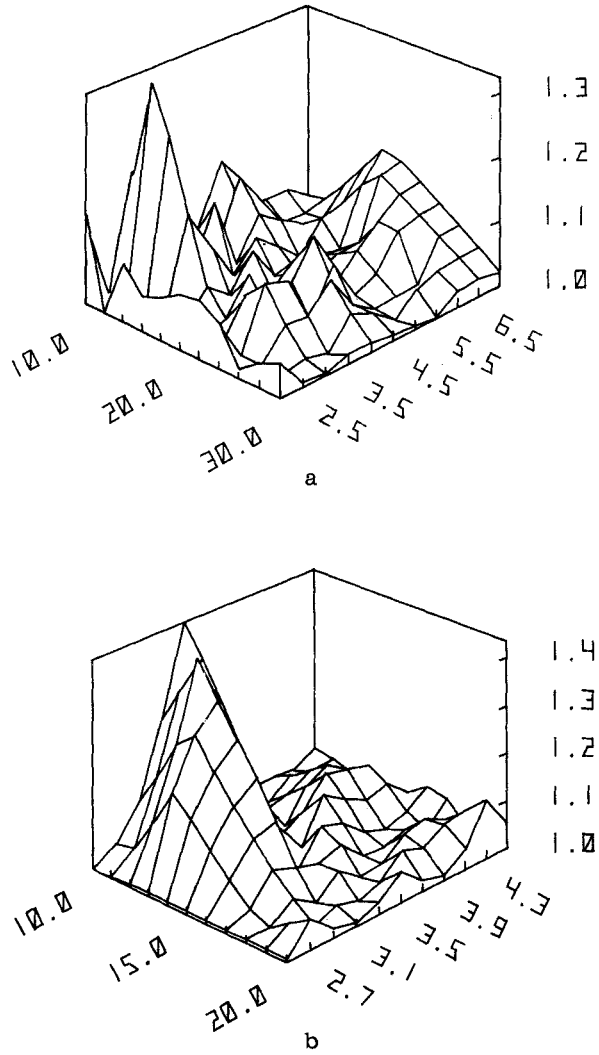

FIGURE 2

Computed minimum alpha plots for seven-component mixture in citrate buffer at optimal ionic strength (0.1 M) in dependence on volume percent methanol (left axis) and pH (right axis).

a. step width: pH = 0.5; %M = 2%
b. step width: pH = 0.2; %M = 1%

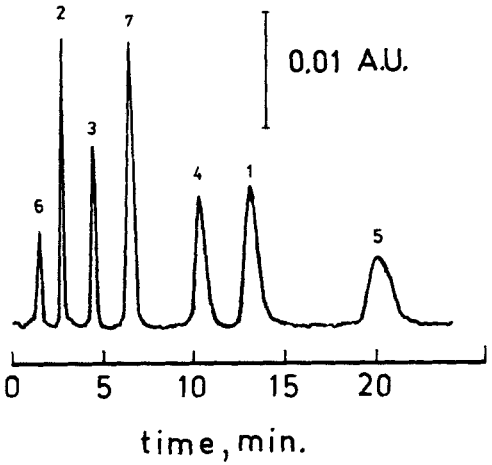

FIGURE 3

Chromatogram of seven-component mixture in citrate under optimized conditions.

The following retention data and standard deviations are from two chromatograms (model predicted capacity factors in brackets): 1 - anthranilic acid 16.75±0.05 (17.20); 2 - m-aminobenzoic acid 2.57±0.07(2.80); 3 - p-aminobenzoic acid 5.24±0.03(5.38); 4 - L-leucyl-L-tyrosine 13.27±0.40(13.50); 5 - D-leucyl-L-tyrosine 25.70±0.77(25.30); 6 - L-tyrosine 1.07±0.09 (1.03); 7 - phthalic acid 8.22±0.25(7.78).

Apart from high selectivity of separation by use of either phosphate (7) or citrate buffer (Figure 3), for practical application citrate buffer is to be preferred as at pH 3.20/3.30 the buffer capacity of phosphate is much lower than that of citrate enabling higher column loading with citrate buffered mobile phases.

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