Journal of Chromatography, 148 (1978) 413–427
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CHROM. 10,359

CONDITIONS FOR RAPID SEPARATIONS OF NUCLEOBASES AND NUCLEOSIDES BY HIGH-PRESSURE ANION-EXCHANGE CHROMATOGRAPHY

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(First received April 7th, 1977; revised manuscript received June 24th, 1977)

SUMMARY

The retention behaviour of nucleobases (purine and pyrimidine bases) and their nucleosides in systems consisting of water–ethanol mixtures with added electrolytes as mobile phase and a resin-type anion exchanger (Aminex A-28) as stationary phase has been systematically investigated. The effect of pH, type and concentration of the counterion, ethanol content of the mobile phase and temperature on the retention and column efficiency has been determined. The results obtained show that all of these parameters have a significant effect and have to be optimized in order to separate the nucleobases and nucleosides by isocratic elution. The separation of fourteen compounds has been achieved in *ca.* 30 min. The ability of the phase system is demonstrated by the analysis of a calf thymus DNA hydrolyzate.

INTRODUCTION

The analysis of nucleobases and their nucleosides in DNA and RNA hydrolyzates is of great importance[1] for characterizing the different DNA or RNA types. Alterations in the purine and pyrimidine metabolic pathways, caused by genetic diseases such as the Lesch–Nyhan syndrome, adenosine deaminase or nucleoside phosphorylase deficiencies, can sometimes be recognized from a quantitative analysis of the nucleobases and nucleosides in cell extracts[2–4]. Moreover, the measurement and metabolism of purine and pyrimidine analogues, used as theurapeutic agents in the treatment of cancer, provides valuable information[5]. For these applications, a large number of analyses is necessary in a relatively short time at low concentration levels. Therefore a rapid and sensitive method of determining these compounds is required.

Among other chromatographic techniques[6–11], liquid-phase column chromatography has been shown to be superior for the separation of nucleobases and nucleosides[12,13]. In particular, ion-exchange chromatography[14] and reversed-phase chromatography using chemically modified column packings[15] are preferred for analyzing these substances. However, most of these methods are time consuming, and gradient-

elution modes have been proposed in order to increase the speed of separation. In practice, this improvement is frequently "illusory" because of the time needed to bring the column back to the original condition after an analysis.

In this paper a systematic investigation is described in which the possibilities of optimizing the separation of nucleobases and nucleosides with respect to selectivity and speed of analysis are explored more extensively, using a resin-type anion exchanger and isocratic elution by high-pressure liquid chromatography.

EXPERIMENTAL

Apparatus

In all of the experiments a high-pressure liquid chromatograph (Siemens SP200, Karlsruhe, G.F.R.) was used together with a UV detector (Zeiss PMQ II) equipped with a home-made micro flow cell[16] (cell volume, 8 μl). The stainless-steel columns had an I.D. of 3 mm and a length of 250 mm. In order to minimize external peak broadening, modified Swagelok reducing unions fitted with 0.3 mm I.D. capillary tubing were used as column terminators. The eluent reservoir consisted of a glass vessel with a reflux condenser in order to prevent loss of ethanol at higher temperatures. The temperature of the eluent reservoir was kept 5° above the column temperature in order to prevent the development of air bubbles at the low-pressure side. The wavelength of the UV detector was set at 260 nm.

Materials

Aminex A-28 (8% crosslinking) (Bio-Rad Labs., Richmond, Calif., U.S.A.), having a particle size of 8–12 μm, was used as anion exchanger without any pretreatment. The nucleobases and nucleosides were obtained from commercial sources (Sigma, St. Louis, Mo., U.S.A.; Merck, Darmstadt, G.F.R.). The following abbreviations for these substances are used: uracil (Ura); cytosine (Cyt); thymine (Thy); adenine (Ade); guanine (Gua); hypoxanthine (Hyp); xanthine (Xan); uridine (Urd); cytidine (Cyd); adenosine (Ado); guanosine (Guo); Inosine (Ino); xanthosine (Xao); thymidine (dThd or dThy); 5-methylcytosine ($5CH_3$–Cyt).

Procedures

The capacity ratio (k_i') of a component i was determined from the retention time and that of an unretained compound (ethylammonium chloride, a solute that shows some UV absorbance at 260 nm). The selectivity factor (r_{ji}) of successively eluted compounds was calculated as the ratio of the capacity ratios.

In order to pack the column, the metal tube, closed by a metal frit at the bottom, was connected to a filling tube (300 mm × 10 mm I.D.). The analytical column was filled with water; the filling tube contained a suspension of Aminex A-28 (1.5 g) in 20 ml of the mobile phase (aqueous salt–ethanol mixture). This whole set-up was placed in a thermostat and equilibrated at the chosen working temperature. After equilibration, the mobile phase was pumped into the filling tube at a high flow-rate and an ultimate pressure of 80 bar, displacing the slurry into the column. After passage of 50 ml of eluent, the column was disconnected from the filling tube and placed in the chromatographic system. Fifty column volumes were required for obtaining constant capacity ratios.

The pH of the eluent was adjusted at ambient temperature, before ethanol was added, with a pH meter (Type PHM 22r; Radiometer, Copenhagen, Denmark). The salt and buffer concentrations reported were calculated with respect to the total volume of the water–ethanol mixtures. The samples were dissolved in eluent and injected using an injection valve (Valco CV-6-UHP$_a$-C20) equipped with a sample loop of 10 μl.

RESULTS AND DISCUSSION

The total distribution coefficient (D_N) of an amphoteric solute (NH), such as a nucleobase or nucleoside, between a resin-type anion exchanger and an aqueous solution containing a counter ion (A^{n-}) can be approximately described by

$$D_N = \sum_i \Delta K_i + \Delta K_e \qquad (1)$$

where ΔK_i represents the contribution of one of the distributions other than anion exchange, such as: (i) partition of the molecules between the mobile phase and the matrix; (ii) adsorption of the molecules at the matrix–mobile phase interface; (iii) exclusion of the positively charged ions (selective ion exclusion). In practice, it is almost impossible to distinguish between these different types of distribution mechanism. The magnitude of the individual ΔK_i terms is influenced by the eluent composition and temperature, but this influence is small compared to that of the counter ion concentration and pH on the ΔK_e term.

The ΔK_e term is related to the actual anion-exchange equilibrium between the solute and the counter ion. For a n-valent counter ion and a monovalent sample ion, ΔK_e can be expressed as a function of the pH and counter ion concentration by

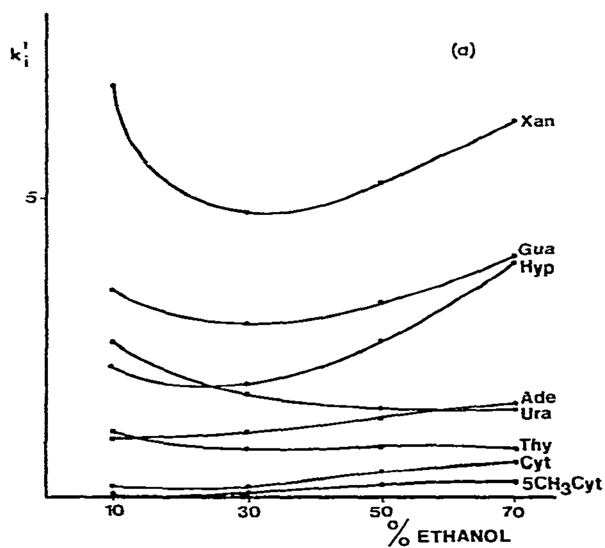
$$\Delta K_e = \frac{1}{[A^{n-}]_m^n} \cdot \frac{K_1}{(1 + K_N [H^+]_m)} \qquad (2)$$

where $[H^+]_m$ is the hydrogen ion concentration in the mobile phase, K_1 is the ion-exchange equilibrium constant (selectivity coefficient), K_N is the formation constant of the acid NH and $[A^{n-}]_m$ is the counter ion concentration in the mobile phase, which depends on the analytical concentration, the formation constants and the pH. Because of this strong and predictable dependence of ΔK_e on the pH and the counter ion concentration, the ΔK_e term can be distinguished from the other terms. The effect of the eluent composition and temperature on the distribution coefficient (and so on the capacity ratio) is in principle given by eqn. 1.

Optimal chromatographic conditions for the rapid separation of two substances can be calculated from the equations for the resolution and retention time. It has been shown that large selectivity factors, medium capacity ratios, small theoretical plate heights and large eluent velocities are required to increase the speed of separation[17]. In order to find such conditions for the separation of nucleobases and nucleosides by anion-exchange chromatography, the effect of the eluent composition and column temperature on the retention and plate heights has been investigated.

Factors affecting the selectivity

Ethanol concentration in the eluent. The addition of organic solvents to the



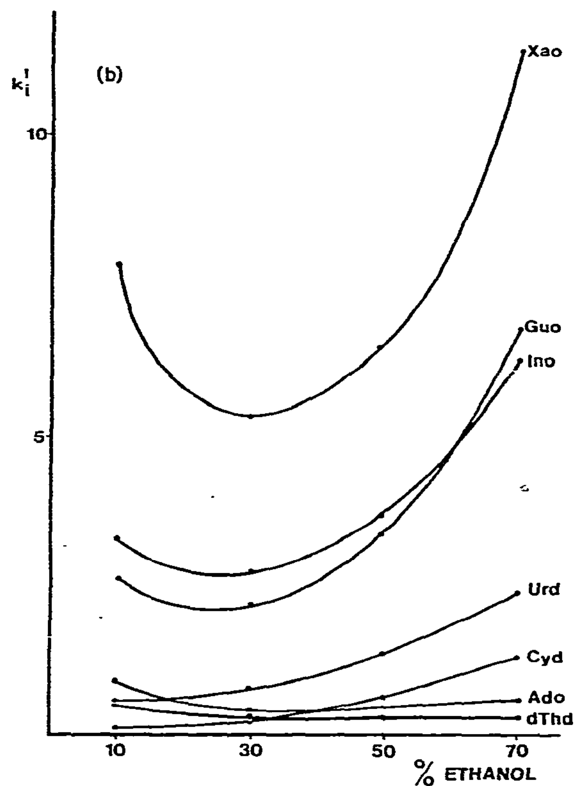


Fig. 1. Effect of the ethanol content of the mobile phase on the capacity ratio k'_i for some nucleobases (a) and nucleosides (b). Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-3}$ M citrate–$5 \cdot 10^{-2}$ M phosphate buffer (pH = 7.25); $T = 70°$; $\Delta P = 80$ bar; $\langle v \rangle = 1$–2 mm/sec.

aqueous mobile phase in ion-exchange systems often remarkably affects the retention of organic substances[18,19]. Ethanol is often used[20,21] for this purpose in the separation of nucleobases and nucleosides.

The capacity ratio of nucleobases and nucleosides was determined as a function of the ethanol content of the mobile phase, keeping the overall ionic strength and temperature constant (Fig. 1). In most instances the capacity ratio first decreases with increasing ethanol content, passes through a minimum at *ca.* 30% and then increases more or less sharply at larger ethanol contents. It is known that ethanol is adsorbed in the matrix of the resin. The mole fraction of ethanol in the interface was found to be larger than the mole fraction of ethanol in the eluent up to a mole fraction of 0.35 of ethanol in the eluent[18]. At larger mole fractions of ethanol in the eluent the mole fraction in the matrix is always smaller than in the eluent. These two regions coincide with those in which we observe a decrease and an increase, respectively, in the capacity ratio when increasing the ethanol content of the mobile phase. Interactions with other ions and solvation in both phases will strongly depend on the ethanol content in these phases; it is therefore reasonable to suppose that the specific shape of the distribution isotherm of ethanol mentioned above causes the minimum in the k_i' values. Furthermore, it is possible that partition is promoted at larger ethanol contents in the eluent. This effect might be detected by changing the counter ion and ethanol concentration, but this has not yet been investigated.

As can be seen from Fig. 1, the ethanol content is a very valuable parameter for adjusting the retention. In particular, the selectivity can be influenced remarkably. In all further investigations of the influence of the different parameters, an ethanol content of 50% was used.

Type of counter ion. The nature of the counter ion can affect the distribution of nucleobases and nucleosides in two ways: (i) it influences the ion-exchange equilibrium constant K_1 and therefore the ΔK_e term; (ii) it influences the nature of the matrix and consequently the ΔK_i terms. If ion exchange dominates, the degree of retention can be affected by the type of counter ion, but usually no significant changes occur in the selectivity factors. However, in those cases where the ΔK_i terms contribute significantly to the overall distribution coefficient, more significant changes in the selectivity factors can be expected.

The selected anions (sodium salts) were dissolved in the eluent, consisting of $5 \cdot 10^{-2}$ *M* phosphate buffer (pH = 7.25) and 50% ethanol, to a concentration of $5 \cdot 10^{-3}$ *M* (Fig. 2). As can be seen from Fig. 2, the capaciy ratios as well as the selectivity factors change considerably with the nature of the counter ion. Citrate and perchlorate showed the best selectivity factors and favourable capacity ratios for obtaining high-speed separations. In all further experiments, however, citrate was used as counter ion because with perchlorate as counter ion greater tailing of the peaks occurred.

Effect of the pH. The effect of pH on the distribution of an amphoteric compound can be partly predicted by means of eqns. 1 and 2. The pK_a (= $-pK_N$) values of nucleobases and nucleosides range from 5 to 12 (ref. 12). This means that ion exchange for these substances just occurs in a neutral or alkaline medium. For citrate, however, complete dissociation into the trivalent form is achieved only at pH > 7.25, and this complicates the pH dependence of the ΔK_e term at pH < 7.25. Eqn. 2 pre-

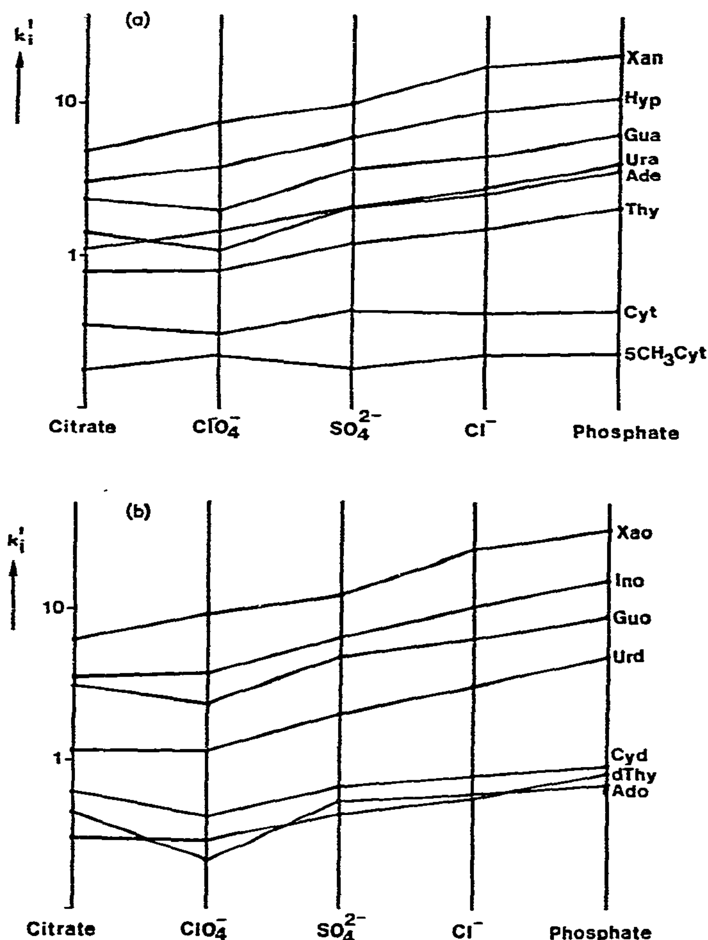


Fig. 2. Effect of the type of counter ion on the capacity ratio of some nucleobases (a) and nucleosides (b). Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-3}$ *M* anion indicated–$5 \cdot 10^{-2}$ *M* phospahte buffer (pH = 7.25)–50% ethanol; $T = 70°$; $\Delta P = 65$ bar; $\langle v \rangle = 2.00$ mm/sec.

dicts an increase of ΔK_e with increasing pH, reaching a constant value at pH $\gg$ pK_N. At low pH (*i.e.*, pH $\ll$ pK_N), ΔK_e will decrease to zero. It is noted that, at very high pH, ΔK_e decreases as the anion exchanger is no longer in the ionized form.

The effect of pH on the ΔK_i terms is more difficult to predict. The dependence of k_i' on the counter ion concentration as well as on pH was studied experimentally in order to obtain some insight into this effect. The capacity ratios of nucleobases and nucleosides were determined on Aminex A-28 with water–ethanol (1:1) as the mobile phase, containing $5 \cdot 10^{-3}$ *M* citrate and $5 \cdot 10^{-2}$ *M* phosphate buffers of different pH at 70° (Fig. 3). Most of the substances examined show a pH dependence in agreement with eqn. 2. The retention values of Cyt, 5CH$_3$–Cyt, Cyd and Ado are almost independent of pH because these compounds have pK_a values of *ca.* 12, precluding ion exchange in the pH range investigated. Since these retention values are essentially constant, we may assume that the ΔK_i values are not significantly affected by pH. At high

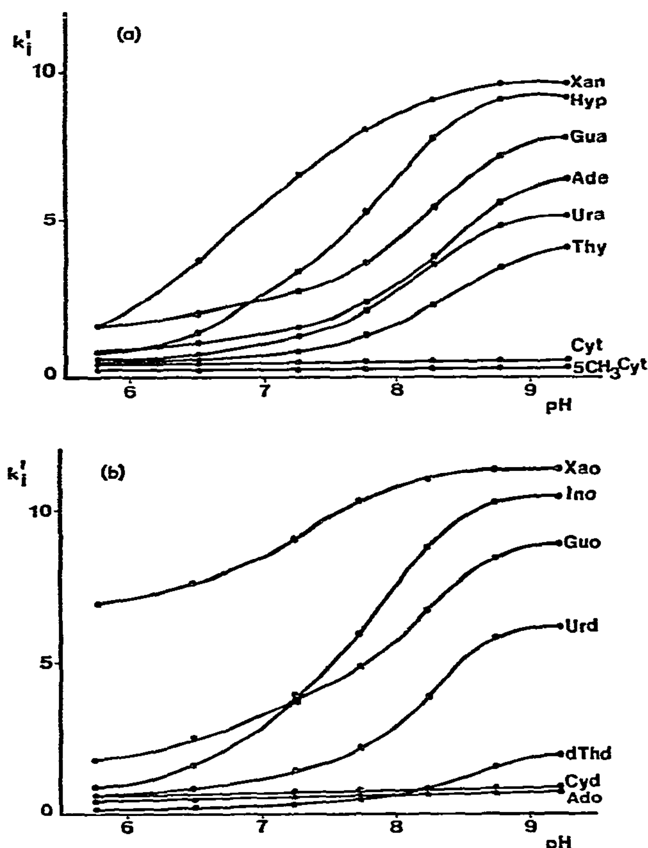


Fig. 3. Dependence of the capacity ratio on the pH of the mobile phase for some nucleobases (a) and nucleosides (b). Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-3}$ M citrate–$5 \cdot 10^{-2}$ M phosphate buffer–50% ethanol; $T = 70°$; $\Delta P = 70$ bar; $\langle v \rangle = 2.28$ mm/sec. For pK_a values see ref. 12.

pH the elution order of nucleobases and nucleosides is that of their pK_a values; compounds having high pK_a values are eluted first[12]. At low pH, where ion exchange is negligible for all of the compounds except Xao, the elution order changes. The capacity ratios also tend to converge to constant values.

Fig. 3 shows that pH is a very powerful parameter for adjusting the retention and selectivity.

Effect of the counter ion concentration. The effect of the counter ion concentration on the capacity ratio is given by eqn. 1. When ion exchange dominates a clear relation must exist between k'_i and the counter ion concentration. If the ΔK_i terms dominate, however, such a clear effect is not expected.

The effect of the counter ion concentration on k'_i was determined using Aminex A-28 and a phosphate-buffered water–ethanol (1:1) mixture at two pH values (7.25 and 9.00) and 70° (Figs. 4 and 5). At pH = 7.25 (Fig. 4), for most of the compounds examined k'_i first decreases with increasing counter ion concentration (citrate) and then becomes approximately constant or even increases at higher citrate concentrations. Significant changes in the selectivity factors can be detected from Figs. 4

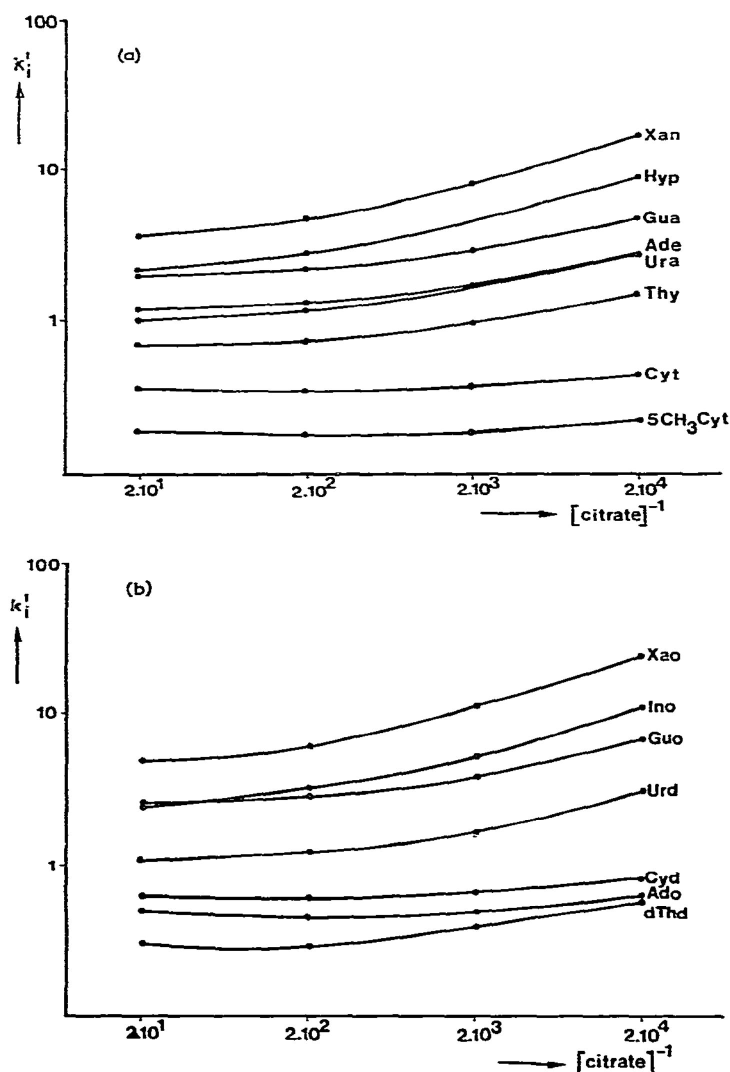


Fig. 4. Dependence of the capacity ratio on the anion concentration in the mobile phase at pH = 7.25 for some nucleobases (a) and nucleosides (b). Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-2}$–$5 \cdot 10^{-5}$ M citrate –$5 \cdot 10^{-3}$ M phosphate buffer (pH = 7.25)–50% ethanol; $T = 70°$; $\Delta P = 80$ bar; $\langle v \rangle = 2.55$ mm/sec.

and 5 for pairs of compounds such as Hyp–Gua, Ade–Ura, Ino–Guo and Ado–dThd. This must be attributed to a large contribution from the ΔK_t terms at this pH.

At pH = 9.00 (Fig. 5) the capacity ratio of all of the compounds shows a linear dependence on the reciprocal of the citrate concentration in the range $5 \cdot 10^{-2}$–$5 \cdot 10^{-4}$ M (larger amounts of citrate did not dissolve in the eluent). Because of the counter ion properties of the added phosphate used as buffer, there is no change in the capacity ratios when changing the citrate concentration from $5 \cdot 10^{-4}$ to $5 \cdot 10^{-5}$ M. Accordingly, at $5 \cdot 10^{-5}$ M citrate the capacity ratios of all of the compounds are

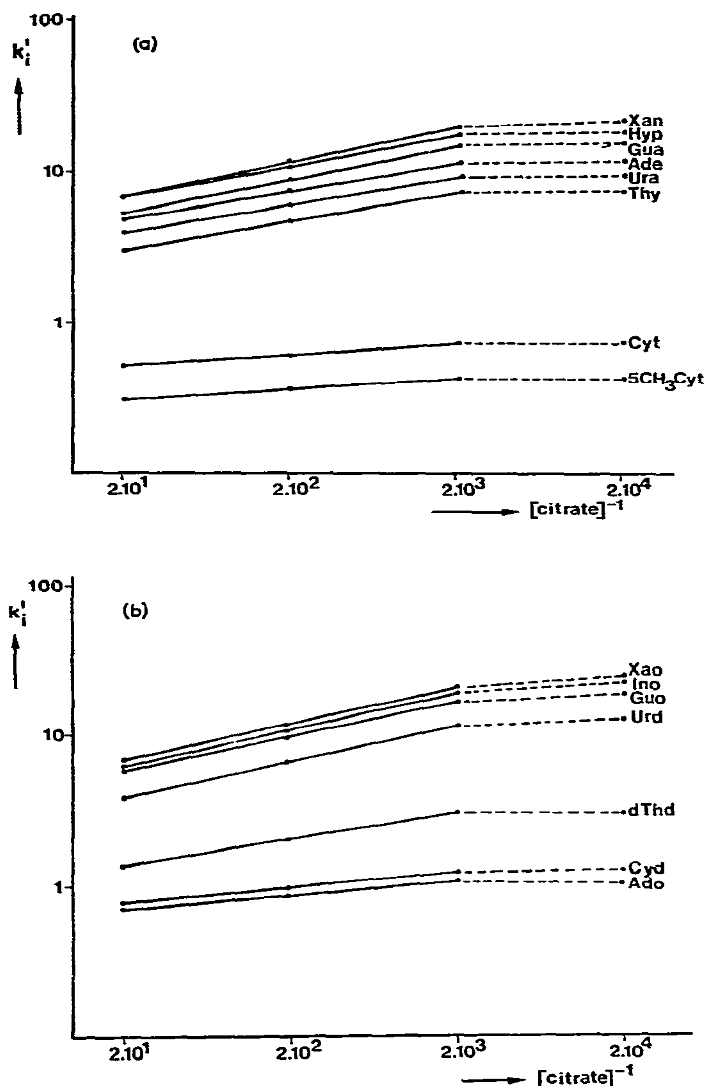


Fig. 5. Dependence of the capacity ratio on the anion concentration in the mobile phase at pH = 9.00 for some nucleobases (a) and nucleosides (b). $\langle v \rangle$ = 2.51 mm/sec. Other conditions as in Fig 4.

linearly related to the reciprocal of the buffer concentration; this effect was not found at pH = 7.25. At pH = 7.25 the capacity ratios slightly increase with increasing buffer concentration. As can be seen from Fig. 5, no remarkable selectivity changes occur at pH = 9.00 (except for Xan–Hyp) as could be expected when ion exchange dominates.

Thus the counter ion concentration in combination with the pH can be used to adjust the retention and selectivity of nucleobases and nucleosides.

Effect of temperature. The effect of temperature on the capacity ratio and selectivity factors of nucleobases and nucleosides was investigated at three different

temperatures on Aminex A-28 with water–ethanol (1:1) containing $5 \cdot 10^{-2}$ M phosphate buffer (pH = 7.25) as mobile phase and $5 \cdot 10^{-3}$ M citrate as eluent. The results are collected in Table I.

TABLE I

INFLUENCE OF TEMPERATURE ON THE CAPACITY RATIO (k_i') AND SELECTIVITY FACTOR (r_{ji})

Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-3}$ M citrate — $5 \cdot 10^{-2}$ M phosphate buffer (pH = 7.25) — 50% ethanol; T = 30°–70°; ΔP = 80 bar; $\langle v \rangle$ = 0.8 — 2.5 mm/sec.

Compound	*30°*			*50°*			*70°*		
	k_i'	r_{ji}	$(r_j - 1)\frac{k_i'}{k_i' + 1}$	k_i'	r_{ji}	$(r_{ji} - 1)\frac{k_i'}{k_i' + 1}$	k_i'	r_{ji}	$(r_{ji} - 1)\frac{k_i'}{k_i + 1}$
$5CH_3$-Cyt	0.25	—	—	0.21	—	—	0.18	—	—
Cyt	0.41	1.64	0.128	0.39	1 86	0.149	0.36	2.00	0.152
Thy	0.88	2.15	0.334	0.80	2.05	0.295	0.71	1.97	0.257
Ura	1.05	1 19	0.089	1.11	1.39	0.173	1.02	1.44	0.183
Ade	1.52	1.45	0.230	1.43	1.29	0.153	1.22	1.20	0.101
Gua	2.35	1.55	0.332	2.72	1.90	0.530	2.06	1.69	0.379
Hyp	3.24	1.38	0.266	2.72	1.00	0.000	2.29	1.11	0.074
Xan	4.92	1.52	0.397	4.35	1.60	0.439	3.84	1.68	3.459
dThd	0.50	—	—	0.35	—	—	0.30	—	—
Ado	0.95	1.90	0.300	0.70	2.00	0.259	0.49	1.63	0.145
Cyd	1.07	1.13	0.063	0.83	1.19	0.020	0.63	1.29	0.095
Urd	1.69	1.58	0.300	1.40	1.69	0.313	1.10	1.75	0.290
Guo	3 90	2.31	0.823	3.66	2.61	0.939	2.64	2.40	0.733
Ino	4.18	1.07	0.056	3.95	1.08	0.063	2.55	1.04	0.025
Xao	7.91	1.89	0.718	5.73	1.45	0.359	4.93	1.93	0.868

For all of the compounds examined the capacity ratio decreases with increasing temperature, as is usual, but to differing extents. According to Table I, adjustment of the column temperature can have marked, influence on a separation. For pairs of compounds such as Urd–Guo and Gua–Hyp the optimal column temperature lies between 30° and 70°, while for other pairs a monotonous change in selectivity factors with temperature is observed.

In terms of resolution, expressed as $R_{ji} = (r_{ji} - 1)\, [k_i'/(1 + k_i')](L/H_i)^{\frac{1}{2}}$, the effect of temperature on the selectivity factor has to be combined with the effect of temperature on k_i' and the theoretical plate height H_i. The effect of temperature on H_i is more or less uniform, whereas that on k_i' and r_{ji} is specific[22,23]. Therefore it is realistic to consider the effect of temperature on k_i' and r_{ji} combined (*i.e.*, the effect on $(r_{ji} - 1)\, [k_i'/(1 + k_i')]$) and then the effect of temperature on the plate height [*i.e.*, the term $(L/H_i)^{\frac{1}{2}}$].

Column efficiency

Effect of temperature on the theoretical plate height H_i. The favourable effect of increased column temperatures on the column efficiency of ion-exchange systems is well known[18]. The effect of temperature on H_i is mainly determined by the effect of temperature on the capacity ratio and the diffusion coefficients in the mobile and

stationary phase[23]. Usually the capacity ratios decrease with increasing temperature and this will affect H_i to an extent dependent on the fluid velocity and particle size[24]. The change of H_i with the value of the capacity ratio is particularly significant for capacity ratios of the order of $\leqslant 1$. Large diffusion coefficients always result (in practice) in smaller H_i values as these coefficients occur in the denominator of the significant plate-height terms[24].

Graphs of H_i *versus* $< v >$ were obtained at three different temperatures for a number of compounds having capacity ratios ranging from 0.37 to 4.00, (Fig. 6). At 30° the curves are very steep owing to a slow mass transfer. At 70° the H_i values are much smaller and the curves become approximately horizontal as a result of a great improvement in the mass transfer into the stationary phase. The larger H_i value of Cyt ($k_i' = 0.37$) compared to Ade ($k_i' = 1.50$) at 70° must be attributed to external peak broadening which is more pronounced at smaller capacity ratios.

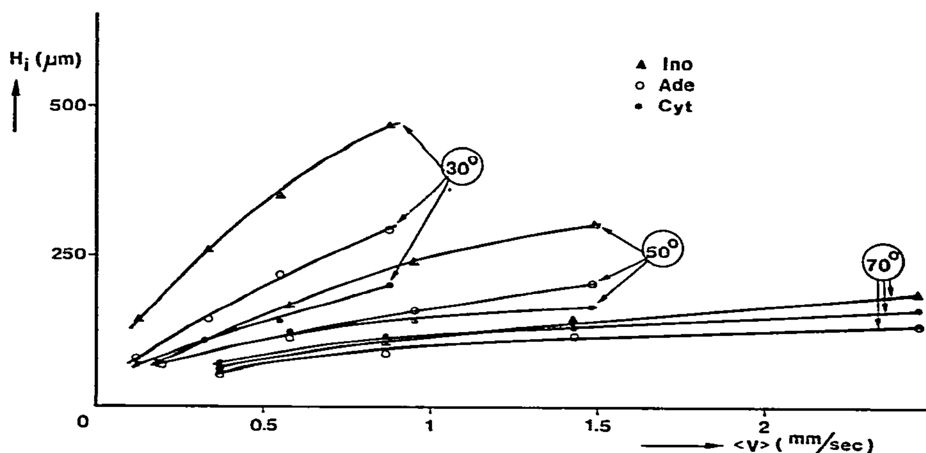


Fig. 6. Effect of temperature on curves of H_i *versus* $\langle v \rangle$. Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-3}$ *M* citrate–$5 \cdot 10^{-2}$ *M* phosphate buffer (pH = 7.25)–50% ethanol; ΔP = 80 bar.

From Table I and Fig. 6 it can be seen, particularly at high fluid velocities, that higher column temperatures considerably improve the resolution of two successively eluted compounds. Increased column temperatures also have favourable effects on the pressure drop.

Effect of pH on H_i. Apart from its effect on the retention and selectivity, the pH also exerts a strong influence on H_i of nucleobases and nucleosides as was noted previously[15].

Fig. 7 shows H_i measured at $\langle v \rangle = 2.5$ mm/sec as a function of the pH of the eluent, consisting of a water–ethanol (1:1) mixture containing $5 \cdot 10^{-3}$ *M* citrate and $5 \cdot 10^{-2}$ *M* phosphate buffer of different pH at 70°. The effect of pH on H_i is not uniform. For compounds such as Guo, Ade and Hyp the value of H_i reaches a maximum at a certain pH; for other compounds H_i decreases monotonously with increasing pH. The lowest H_i value occurs at high pH for all of the compounds. This is mainly caused by the increase of the capacity ratio with increasing pH, the diminishing contribution of external peak broadening with increasing capacity ratio and

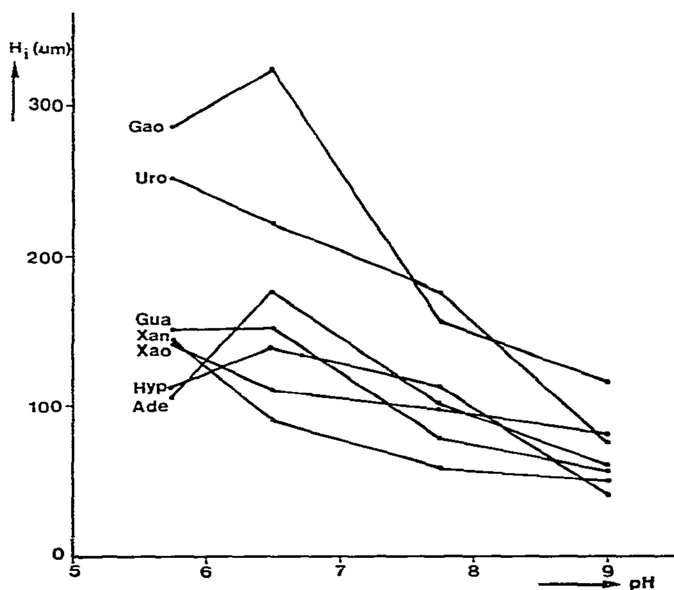


Fig. 7. Effect of the pH of the mobile phase on the theoretical plate height (H_i) at a constant linear velocity of 2.5 mm/sec. Stationary phase, Aminex A-28; Mobile phase, $5 \cdot 10^{-3}$ M citrate–$5 \cdot 10^{-2}$ M phosphate buffer–50% ethanol; $T = 70°$; $\Delta P = 80$ bar.

probably by the decrease of the rather slow non-ion-exchange distribution processes. A high pH seems to be favourable to column efficiency.

Effect of ethanol on H_i. The effect of the ethanol content of the eluent on the column efficiency was investigated by measuring H_i of some nucleobases and nucleosides, at the same linear velocity ($\langle v \rangle = 2.50$ mm/sec), on Aminex A-28 with water–ethanol mixtures containing $5 \cdot 10^{-3}$ M citrate and $5 \cdot 10^{-2}$ M phosphate buffer (pH = 7.25) at 70° (Fig. 8). The plate height decreases with increasing ethanol content, passes through a minimum at *ca.* 50% ethanol and then increases again at very large ethanol contents. From Figs. 1 and 8 it can be seen that the overall effect of ethanol addition on the resolution is obtained by combining the effects of ethanol on r_{ji}, k'_i and H_i.

Effect of salt concentration on H_i. Fig. 9 shows H_i, measured at constant linear velocity ($\langle v \rangle = 2.5$ mm/sec), as a function of the citrate concentration on Aminex A-28 with water–ethanol (1:1) and $5 \cdot 10^{-2}$ M phosphate buffer (pH = 7.25) at 70°. The H_i value of all of the compounds examined decreases with decreasing citrate concentration. This can be partly explained by the increase of the capacity ratio with decreasing citrate concentration and the diminishing contribution of external peak broadening. If H_i values of different solutes, having the same k'_i value but measured at different citrate concentrations, are compared then a systematical decrease of H_i with decreasing citrate concentration is found (dashed line in Fig. 9). This result, although not totally reliable because of the dependence of H_i on the individual solutes, indicates at least that effects other than those mentioned before are involved in the improvement of the column dispersion with decreasing citrate concentration.

Similar results, but to a lesser extent, were found for the effect of the buffer concentration on H_i. The plate height decreases with decreasing buffer concentration.

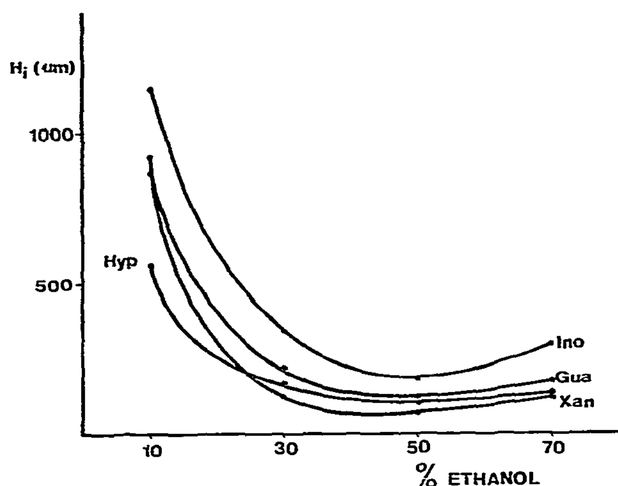


Fig. 8. Effect of the ethanol content of the mobile phase on the theoretical plate height (H_i) at a constant linear velocity of 2.5 mm/sec. Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-3}$ *M* citrate–$5 \cdot 10^{-2}$ *M* phosphate buffer (pH = 7.25); $T = 70°$; ΔP = 60–80 bar.

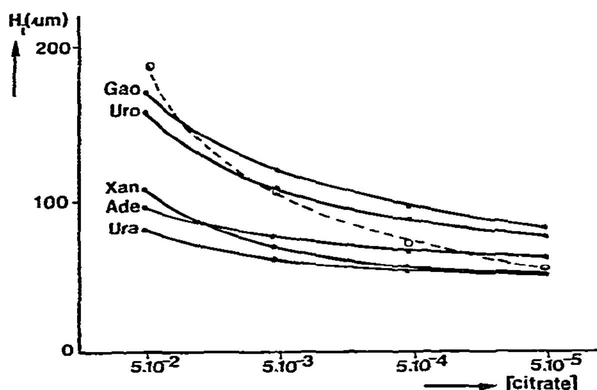


Fig. 9. Effect of the anion concentration of the mobile phase on the theoretical plate height (H_i) at a constant linear velocity of 2.5 mm/sec. Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-2}$–$5 \cdot 10^{-5}$ *M* citrate–$5 \cdot 10^{-2}$ *M* phosphate buffer (pH = 7.25)–50% ethanol; $T = 70°$; ΔP = 80 bar.

Choice of the phase system

The large number of parameters available hampers the choice of the optimal composition of the mobile phase with respect to speed and resolution. A pH of 7–9.25, a citrate concentration of $5 \cdot 10^{-3}$–$5 \cdot 10^{-4}$ *M*, a phosphate buffer concentration of $5 \cdot 10^{-2}$–$5 \cdot 10^{-3}$ *M*, an ethanol content of 40–60% and a temperature between 60° and 70° seem to be the optimal ranges with respect to selectivity, efficiency and speed of separation.

The choice of the eluent composition for a rapid separation of nucleobases and nucleosides is less critical. The eight nucleobases used in this study can be separated in less than 13 min by use of an eluent consisting of water–ethanol (1:1) containing $5 \cdot 10^{-3}$ *M* citrate and $5 \cdot 10^{-2}$ *M* phosphate buffer (pH = 7.25) at 70°, on a

250 mm column. On the same column, using 55% ethanol and at pH = 6.9, the seven nucleosides can be separated in *ca*. 23 min. For the separation of a mixture of nucleobases and nucleosides the parameter adjustment is very critical. Many conditions can achieve the desired result of complete or near complete separation. An example of a rapid isocratic separation of nucleobases and nucleosides is shown in Fig. 10. Some groups of compounds (Hyp, Guo, Xan and Xao) are not completely resolved in this phase system. The separation of these solutes, however, can easily be improved by changing the eluent composition to water–ethanol (11:9), $5 \cdot 10^{-3}$ *M* citrate, $5 \cdot 10^{-2}$ *M* phosphate buffer (pH = 7.75) and a temperature of 60°. By use of this eluent, these solutes are completely resolved but at the expense of other pairs such as Cyt–dThd and Ura–Ade. A further refinement, using known mathematical optimization procedures, can lead to a complete resolution of all of the compounds within 30 min. On the other hand, if one is just interested in a few nucleobases and nucleosides, as is often the case in chemotherapy, the phase system can easily be adapted so that these solutes are completely resolved from the other nucleobases and nucleosides.

The applicability of the described phase systems is demonstrated in Fig. 11 which shows the analysis of purine and pyrimidine bases in a calf thymus DNA hydrolyzate.

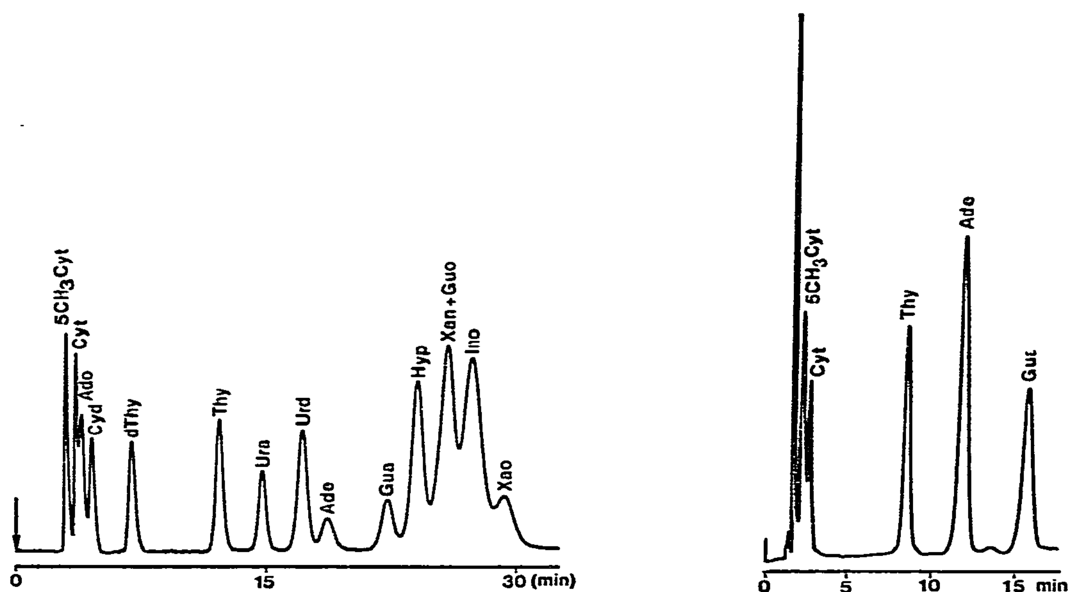


Fig. 10. Separation of a test mixture of nucleobases and nucleosides. Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-3}$ *M* citrate–$5 \cdot 10^{-2}$ *M* phosphate buffer (pH = 9.25)–55% ethanol; $T = 70°$; $\Delta P = 60$ bar; $\langle v \rangle = 1.81$ mm/sec.

Fig. 11. Analysis of purine and pyrimidine bases in a calf thymus DNA hydrolyzate. Stationary phase, Aminex A-28; mobile phase, $5 \cdot 10^{-3}$ *M* citrate–$5 \cdot 10^{-2}$ *M* phospahte buffer (pH = 9.25)–55% ethanol; $T = 70°$; $\Delta P = 80$ bar; $\langle v \rangle = 2.45$ mm/sec. Hydrolysis: calf thymus DNA Type I (Sigma) was heated for 3 h at 80° with 2 *M* HCl, diluted with 55% ethanol and 13 μl of the resulting solution were injected on to the column.

CONCLUSIONS

A rapid separation of nucleobases and nucleosides by high-pressure anion-exchange chromatography can be achieved in *ca.* 30 min by appropriate adjustment of the many parameters available which influence the retention and column efficiency. The main advantage of the described phase system is its simplicity since the separations can be performed under isocratic conditions. Future work will be devoted to the application of the described phase systems for the analysis of purine and pyrimidine analogues in blood.

ACKNOWLEDGEMENT

The authors are grateful to Dr. H. Poppe for his careful reading of the manuscript and to Dr. G. Westra of the Antoni van Leeuwenhoek Laboratory, Amsterdam for supplying the DNA hydrolyzate.

REFERENCES

1 A. L. Lehninger, *Biochemistry, The Molecular Basis of Cell Structure and Function*, Worth Publ., New York, 1972, p. 637.
2 J. B. Wijngaarden and W. N. Kelley, in J. B. Stanbury, J. B. Wijngaarden and D. S. Frederickson (Editors), *Gout, The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 1972, p. 911.
3 E. R. Giblett, J. E. Anderson, F. Cohen, B. Pollara and H. J. Meuwissen, *Lancet*, 2 (1972) 1067.
4 E. R. Giblett, A. J. Ammann, R. Sandman, D. W. Wara and L. K. Diamond, *Lancet* ,1 (1975) 1010.
5 S. H. Wan, D. H. Huffman, D. L. Azarnoff, B. Hoogstraten and W. E. Larsen, *Cancer Res.*, 34 (1974) 392.
6 J. MacGee, *Anal. Biochem.*, 14 (1966) 395.
7 C. W. Gehrke and C. D. Ruyle, *J. Chromatogr.*, 38 (1968) 473.
8 D. B. Lakings, C. W. Gehrke and T. P. Waalkes, *J. Chromatogr.*, 116 (1976) 69.
9 C. W. Gehrke and A. B. Patel, *J. Chromatogr.*, 123 (1976) 335.
10 K. Randerath and E. Randerath, *Procedures Nucleic Acid Res.*, 2 (1971) 796.
11 E. Lederer and M. Lederer, *Chromatography, A Review of Principles and Application*, Elsevier, Amsterdam, 1957.
12 W. E. Cohn, in E. Heftmann (Editor), *Chromatography*, 2nd ed., Reinhold, New York, 1967, Ch. 22.
13 R. P. Singhal, *Separ. Purif. Methods*, 3 (1974) 339.
14 C. Horvath in D. Glick (Editor), *High Performance Ion Exchange Chromatography with Narrow Bore Columns, Rapid Analysis of Nucleic Acid Constituents at the Subnanomole Level*, Vol. 21, Wiley, New York, 1973, pp. 79–154.
15 P. R. Brown, *High Pressure Liquid Chromatography, Biochemical and Biomedical Applications*, Academic Press, New York, 1973, pp. 130–137.
16 J. F. K. Huber, *J. Chromatogr. Sci.*. 7 (1969) 172.
17 J. C. Kraak and P. Bijster, *J. Chromatogr.*, 143 (1977) 499.
18 O. Samuelson, *Ion Exchange Separations in Analytical Chemistry*, Almqvist & Wiksell, Uppsala, 1963.
19 R. P. Singhal and W. E. Cohn, *Anal. Biochem.*, 45 (1972) 585.
20 R. P. Singhal, *Arch. Biochem. Biophys.*, 152 (1972) 800.
21 R. P. Singhal and W. E. Cohn, *Biochemistry*, 12 (1973) 1532.
22 J. F. K. Huber and J. C. Kraak, *10th International Symposium on Advances in Chromatography, Munich, November 3–6, 1975.*
23 J. C. Kraak, *Thesis*, University of Amsterdam, 1974.
24 J. F. K. Huber, *Ber. Bunsenges. Phys. Chem.*, 77 (1973) 179.