ELSEVIER

Journal of Chromatography A, 734 (1996) 205–212

JOURNAL OF CHROMATOGRAPHY A

Solute retention of lysozyme in hydrophobic interaction perfusion chromatography

A. Staby[1], J. Mollerup*

Department of Chemical Engineering, DTU, Building 229, DK-2800 Lyngby, Denmark

Abstract

Perfusion chromatography is a rather new HPLC concept using packing materials having large through-pores, which were developed to overcome the problems of stagnant mobile mass transfer without loss of separation speed and column capacity. The perfusion media have been developed for both analytical and preparative separations in a number of distinct HPLC modes including hydrophobic interaction chromatography (HIC) where the separation principle is based on the surface hydrophobicity of the solute, which is adsorbed to the mildly hydrophobic stationary phase in an aqueous mobile phase at high salt concentration and eluted at a lower salt concentration.

In this study, the retention behaviour of egg white lysozyme in semi-preparative hydrophobic interaction perfusion chromatography is examined using the BioCAD Workstation. Retention data of lysozyme are measured on four perfusive HIC media at different ammonium sulphate concentrations in the eluent in both isocratic and gradient mode at three different pH values representing more than 400 chromatographic runs.

The solute retention behaviour of lysozyme on the HIC columns is described by a model of the capacity factor, k', as a function of the mobile phase pH and the ionic strength. The capacity factor is a function of the protein activity coefficient in the mobile phase, modelled by a modified Debye–Hückel equation, and the protein activity coefficient on the stationary phase, expressed by a simple non-linear term. The model is capable of correlating $\ln k'$ from zero to high ionic strength.

Keywords: Perfusion chromatography; Retention behaviour; Retention models; Lysozyme; Proteins

1. Introduction

Hydrophobic interaction chromatography (HIC) and other chromatographic techniques like ion exchange, affinity, and gel filtration are used for large-scale separation of proteins and biopolymers because of the mild process conditions, which normally maintain the structure and activity of the molecules. The HIC separation principle is based on the surface hydrophobicity of the solute, which is adsorbed to the mildly hydrophobic stationary phase in an aqueous mobile phase at high salt concentration and is eluted either isocratically or with a decreasing salt gradient.

Perfusion chromatography is a rather new concept using packing materials having large through-pores, which carry sample molecules, typically proteins, rapidly into the interior of each bead by convective flow [1–3]. Inside the beads, there are many short pores that branch off the through-pores providing a large internal surface area for binding of the sample molecules. These chromatographic packing materials were developed to overcome the problems of stagnant mobile mass transfer and have the advantage of

*Corresponding author. e-mail: jm@kt.dtu.dk

[1] Present address: Novo Nordisk A/S, HAD 2.257, Hagedornsvej 1, 2820 Gentofte, Denmark.

0021-9673/96/$15.00
SSDI 0021-9673(95)01161-7

doing so without loss of separation speed and column capacity compared to silica supports, rigid organic macroporous resins, and very small non-porous particles. The perfusion media have been developed for both analytical and preparative separations in a number of distinct HPLC modes including HIC.

There have been several suggestions to correlate solute retention in HIC by simplified models. In 1977 Melander and Horváth [4] adapted the solvophobic theory of Sinanoğlu and Abdulnur [5] to describe isocratic protein retention in HIC, and later Melander et al. [6] expanded the theory to treat retention in gradient elution. Fausnaugh and Regnier [7] evaluated the isocratic retention model for several bird egg white lysozymes on a TSK-GEL phenyl-5PW column with ammonium sulphate as the salt at pH 6.0, 7.0, and 8.0, and with four different salts at pH 7.0. Recently, model work has been done on specific salt effects [8–10] and on the interplay of hydrophobic and electrostatic interactions [11,12]. Some of the data are given in figures [6–9,11–14], others in tables [10,15].

The aim of this study is to measure solute retention data on semi-preparative HIC matrices and correlate the data in order to model solute retention of proteins on HIC perfusion media, both isocratically and by salt gradients.

2. Experimental

2.1. Chemicals

SigmaUltra grade of ammonium sulphate 99.5%+ (A2939), monosodium phosphate 99.0%+ (S8282), and disodium phosphate 98%+ (S7907) were purchased from Sigma Chemical (St. Louis, MO, USA). Combi-Titrisol grade of hydrochloric acid (9911) and sodium hydroxide (9913) were obtained from Merck (Darmstadt, Germany).

Three times recrystallized, dialysed, and lyophilized chicken egg white lysozyme 95% (L6876) (EC 3.2.1.17) was supplied by Sigma.

2.2. Equipment

The BioCAD Workstation from PerSeptive Biosystems (Cambridge, MA, USA) was used throughout this study. The workstation was equipped with an automatic variable-volume injection valve with a 100-μl loop, pump heads for flows between 0.5 and 60 ml/min, six solvent channel lines with mixing of up to four solvents at a time, a Model 205 dual wavelength UV–Vis detector, and pH and conductivity monitors.

The chromatographic courses were monitored by an IBM compatible computer with BioCAD software version 1.22. The dual wavelength detector was operated at 280 and 254 nm.

2.3. Sample preparation

Lysozyme was dissolved in the 20 m*M* phosphate elution buffer used at the given pH (see below) to a concentration of 5.0 mg/ml and subsequently filtered through a 0.45-μm filter. All chromatographic runs were conducted with an injected amount of the lysozyme solutions of 50 μl.

2.4. Columns

The prepacked HIC columns Poros PH/M (phenyl), Poros PE/M (phenyl ether), Poros BU/M (butyl ether), and Poros ET/M (ethyl ether) were purchased from Perseptive Biosystems (Cambridge, MA, USA). The 20-μm particles were prepacked into 100×4.6 mm I.D. polyether ether ketone columns having a column volume (CV) of 1.66 ml.

All chromatographic elutions were performed at room temperature. The temperature was measured to be 24.7±1.0°C.

2.5. Mobile phase

Lysozyme was eluted isocratically and by gradient elution using three pairs of standard phosphate buffers prepared at a pH of 6.0, 7.0, and 8.0. To solvent channel A of the BioCad Workstation, a 20.0 m*M* phosphate buffer solution (10 m*M* monosodium phosphate and 10 m*M* disodium phosphate) with 3.0 *M* ammonium sulphate was connected and to channel B, a 20.0 m*M* phosphate buffer solution was connected. All buffers were filtered through a 0.45-μm filter.

The elution programs of the isocratic runs consisted of: a purge segment of 10 ml and a 5-CV column equilibration segment of the elution buffer at

the specified ammonium sulphate concentration, an injection and a 10–30 CV elution segment of lysozyme at the given ammonium sulphate concentration, and a 5-CV cleaning segment of the column at zero ammonium sulphate concentration. Similar elution programs were employed in the gradient runs where the elution segment comprises a 2.40-ml isocratic elution at the initial ammonium sulphate concentration followed by a linearly decreasing gradient elution to zero ammonium sulphate concentration during 1–20 CV, and eventually followed by an isocratic elution at zero ammonium sulphate concentration. A flow-rate of 5.00 CV/min corresponding to 8.30 ml/min or 50 cm/min was used in all cases.

3. Theory and model calculations

3.1. The capacity factor

The capacity factor k' is defined as the retention or residence time of the solute in the stationary phase, $t_s = t_R - t_m$, divided by the residence time of an unretained component, t_m. If equilibrium can be assumed, the number of moles of a solute in a phase is proportional to the residence time of the species in the phase.

$$k' = (t_R - t_m)/t_m = t_s/t_m = n_s/n_m \quad (1)$$

where n_s denotes the number of moles of solute in the stationary phase and n_m denotes the number of moles of solute in the mobile phase.

In a non-ideal solution we define the activity of a solute, denoted by a, by the equation

$$\mu = \mu_{oo} + RT \ln a \quad (2)$$

where μ_{oo} is the standard state chemical potential. Clearly, in an ideal mixture the activity of a solute equals the mole fraction of the solute. The activity of a pure substance equals one, but else it is a function of the solute and solvent properties. The activity coefficient γ, which accounts for the deviation from ideality, is defined as the ratio of the activity and the mole fraction x

$$\gamma = a/x \quad (3)$$

The activity coefficient is normalized so that $\lim \gamma = 1$ when $x \rightarrow 1$.

The mole fraction $x = n/N$, where n is the mole number of solute and N is the total number of moles in the phase. If the phase ratio in the column is constant, then $N = V/v$, where V is the total phase volume and v is the molar volume of the phase. When the column load is high the local phase properties may differ from the bulk properties of the phase.

When we apply Eq. 2 at zero ionic strength, denoted by subscript o, and at the ionic strength I, without subscript, then

$$\mu = \mu_o + RT \ln\frac{a}{a_o} = \mu_o + RT \ln\frac{x\gamma}{x_o\gamma_o}$$
$$= \mu_o + RT \ln\frac{n\gamma}{n_o\gamma_o} + RT \ln\frac{v}{v_o} \quad (4)$$

In this work we assume that the volume ratio in the last term on the right-hand side of Eq. 4 is equal to one for the stationary phase but not for the mobile phase where the mobile phase molar volume depends on the ionic strength. Since we are applying the same column load in all the experiments we can assume that the activity coefficients are functions of the ionic strength only. At equilibrium the chemical potential of the solute in the mobile phase equals the chemical potential of the solute on the stationary phase, that is

$$\mu^s = \mu^m \quad (5)$$

and

$$\mu_o^s = \mu_o^m \quad (6)$$

The capacity factor k' can thus be calculated as

$$\ln k' = \ln k'_o + \ln\frac{\gamma^m}{\gamma_o^m} - \ln\frac{\gamma^s}{\gamma_o^s} + \ln\frac{v^m}{v_o^m} \quad (7)$$

The first term on the right side of Eq. 7 is the capacity factor at zero ionic strength. The ratio of activity coefficients in the second term on the right-hand side of the equation is the ratio of the solute activity coefficient at finite ionic strength over the solute activity coefficient at zero ionic strength. This term is modelled by a Debye–Hückel term plus a linear term to assure a qualitative correct solubility behaviour with a salting-in and a salting-out region, that is

$$\ln\frac{\gamma^{m}}{\gamma_{o}^{m}} = -\frac{1.5}{a}\frac{\sqrt{I}}{1+1.6\sqrt{I}} + 0.15I \quad (8)$$

where $\gamma_o > \gamma$ corresponds to salting-in or lowering the chemical potential of the solute in the solution, and $\gamma_o < \gamma$ corresponds to salting-out or increasing the chemical potential of the solute in the solution. The constant a is a function of the mobile phase pH.

The last term in Eq. 7 is more difficult to access and in this context we use an empirical expression

$$\ln\frac{\gamma^{s}}{\gamma_{o}^{s}} = bI - cI^{3} \quad (9)$$

The capacity factor at zero ionic strength is assumed to be independent of the active group on the column and since all columns are based on the same supporting material we have assumed that k_o' is a function of the mobile phase pH only. The parameters in the model are estimated by a least square fit of the model to the experimental data. The numbers including the estimated standard deviations are shown in Table 1. The mobile phase molar volume ratio is correlated as a linear function of the ionic strength with a deviation of less than 1%. The correlation is included in Table 1. The experimental data used are from Ref. [16].

3.2. Gradient elution

The gradient elution comprises an injection segment of 0.08 min followed by a gradient segment with an isocratic elution at the initial ionic strength I_o of 2.40 ml at a flow-rate of 8.30 ml/min corre-

Table 1
Parameters in the model of the capacity factor, Eqs. 7–9

Column	Parameter	pH 6	pH 7	pH 8
	$\ln k_o'$	−1.30±0.10	−1.25±0.10	−1.18±0.10
	a	1.00	1.15	1.30
PE/M	b	0.143±0.009	0.173±0.004	0.192±0.010
	c	0.0281±0.0004	0.0291±0.0002	0.0303±0.0004
PH/M	b	0.234±0.011	0.263±0.030	0.273±0.011
	c	0.0308±0.0004	0.0316±0.0012	0.0326±0.0004
ET/M	b	0.359±0.011	0.408±0.011	0.452±0.014
	c	0.0198±0.0003	0.0209±0.0003	0.0222±0.0004
BU/M	b	0.490±0.007	0.551±0.012	0.562±0.029
	c	0.0208±0.0002	0.0221±0.0003	0.0222±0.0007
	$\ln\frac{v^{m}}{v_{o}^{m}} = 0.0.16I$			

Table 2
Comparison of experimental and calculated adjusted retention times t_R' of lysozyme on POROS PH/M in linear gradient elution mode from 1.8 M ammoniumsulphate

pH	Duration (min)	Experimental t_R' (min)	Calculated t_R' (min)	Deviation (min)
6.0	1.0	0.839	0.779	0.060
	2.0	0.972	0.898	0.074
	4.0	1.125	1.055	0.070
7.0	1.0	0.844	0.790	0.054
	2.0	0.964	0.918	0.046
	4.0	1.133	1.089	0.044
8.0	0.2	0.642	0.636	0.006
	1.0	0.875	0.813	0.062
	2.0	1.044	0.961	0.083
	4.0	1.260	1.166	0.094

Table 3
Comparison of experimental and calculated adjusted retention times t'_R of lysozyme on POROS PE/M in linear gradient elution mode from 1.8 *M* ammoniumsulphate

pH	Duration (min)	Experimental t'_R (min)	Calculated t'_R (min)	Deviation (min)
6.0	1.0	0.864	0.794	0.070
	2.0	0.994	0.920	0.074
	4.0	1.161	1.085	0.076
7.0	1.0	0.881	0.808	0.073
	2.0	1.045	0.945	0.100
	4.0	1.228	1.130	0.098
8.0	0.2	0.664	0.642	0.022
	1.0	0.917	0.829	0.088
	2.0	1.092	0.985	0.107
	4.0	1.314	1.204	0.110

Table 4
Comparison of experimental and calculated adjusted retention times t'_R of lysozyme on POROS BU/M in linear gradient elution mode from 2.25 *M* ammoniumsulphate

pH	Duration (min)	Experimental t'_R (min)	Calculated t'_R (min)	Deviation (min)
6.0	1.0	0.764	0.718	0.046
	2.0	0.861	0.800	0.061
	4.0	0.958	0.904	0.054
7.0	1.0	0.772	0.727	0.045
	2.0	0.889	0.820	0.069
	4.0	1.014	0.941	0.073
8.0	1.0	0.742	0.708	0.034
	2.0	0.833	0.781	0.052
	4.0	0.928	0.870	0.058

Table 5
Comparison of experimental and calculated adjusted retention times t'_R of lysozyme on POROS ET/M in linear gradient elution mode from 2.2 *M* ammoniumsulphate

pH	Duration (min)	Experimental t'_R (min)	Calculated t'_R (min)	Deviation (min)
6.0	1.0	0.797	0.748	0.049
	2.0	0.922	0.849	0.073
	3.0	0.994	0.923	0.071
	4.0	1.050	0.981	0.069
7.0	1.0	0.822	0.758	0.064
	2.0	0.966	0.870	0.096
	4.0	1.117	1.021	0.096
8.0	1.0	0.806	0.746	0.060
	2.0	0.922	0.846	0.076
	4.0	1.053	0.977	0.076

sponding to an isocratic elution time of 0.37 min. After 0.37 min the gradient reaches the column entrance. The gradient reaches the protein after another Δt min or when

$$\frac{x}{v_o} = \frac{0.37 + \Delta t}{1 + k'(I_o)} = \frac{\Delta t}{1 + k'_{salt}} \quad (10)$$

corresponding to a position x in the column of

$$x = \frac{v_o(0.37 + \Delta t)}{1 + k'(I_o)} \quad (11)$$

The velocity of the protein during gradient elution is

$$v = \frac{dx}{dt} = \frac{v_o}{1 + k'(I)} \quad (12)$$

where v_o is the velocity of an unretained species and I the ionic strength which is a function of the time t and the position x

$$I = I_o - \frac{dI}{dt}\left(t - \frac{x}{v_o}(1 + k_{salt}) - 0.37\right) \quad (13)$$

The capacity factor as a function of the ionic strength is given by the model Eqs. (7–9) with the parameters as shown in Table 1. A comparison of calculated and experimental adjusted retention times are shown in Tables 2–5.

4. Results and discussion

The experimental results are shown in Figs. 1–4 and Tables 2–5. Figs. 1–4 show the experimental capacity factors for the isocratic runs in the linear range, and Tables 2–5 show the experimental corrected retention times for the gradient elutions. The corrected retention times t'_R are calculated as

$$t'_R = (t_R - t_0) \quad (14)$$

where t_R is the measured retention time of lysozyme and t_0 is the retention time of an unretained component. t_0 is calculated as the sum of the supplier specified dead-volume of the column and the measured tubing volume from the injection valve to the detector divided by the flow-rate.

Previously, temperature changes between 0 and 40°C have shown to have essentially no influence on

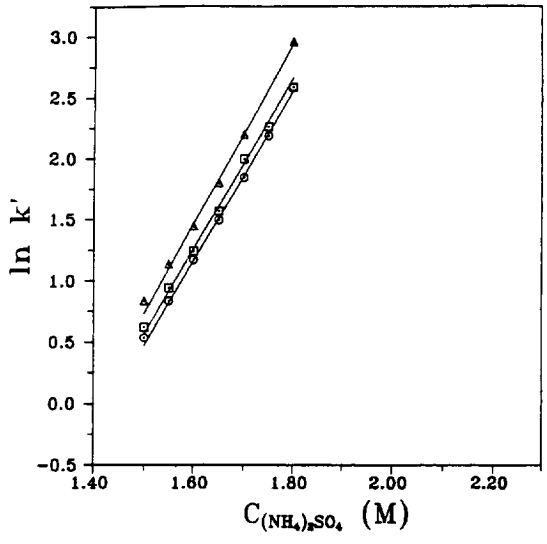

Fig. 1. The effect of pH on the capacity factors of hen egg white lysozyme as a function of the $(NH_4)_2SO_4$ concentration on the Poros PH/M HIC column. Experimental ○ = pH 6.0, □ = pH 7.0, △ = pH 8.0, compared to the linear model of Melander and Horváth (solid line).

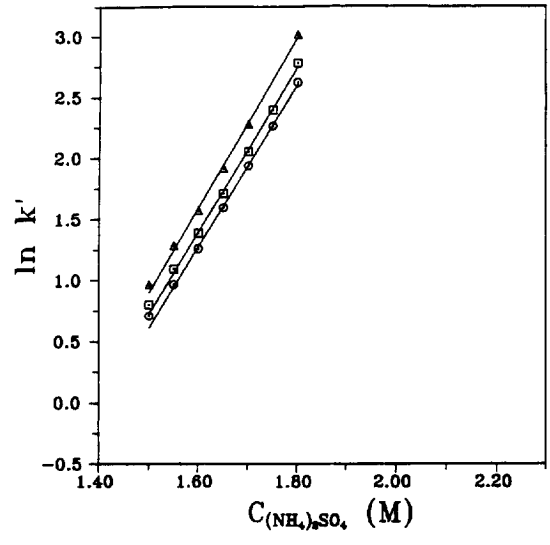

Fig. 2. The effect of pH on the capacity factors of hen egg white lysozyme as a function of the $(NH_4)_2SO_4$ concentration on the Poros PE/M HIC column. Experimental ○ = pH 6.0, □ = pH 7.0, △ = pH 8.0, compared to the linear model of Melander and Horváth (solid line).

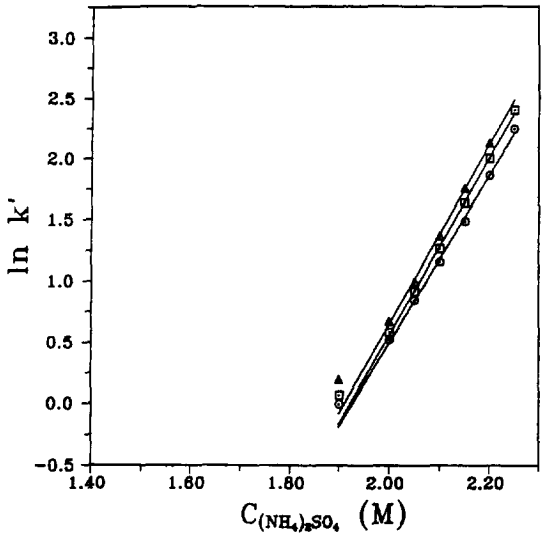

Fig. 3. The effect of pH on the capacity factors of hen egg white lysozyme as a function of the $(NH_4)_2SO_4$ concentration on the Poros BU/M HIC column. Experimental ○=pH 6.0, □=pH 7.0, △=pH 8.0, compared to the linear model of Melander and Horváth (solid line).

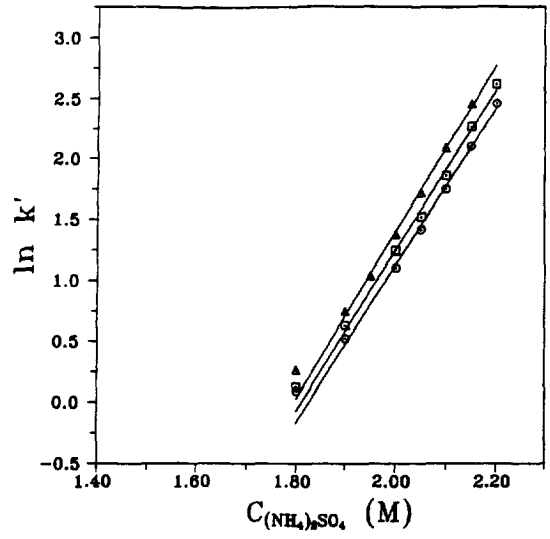

Fig. 4. The effect of pH on the capacity factors of hen egg white lysozyme as a function of the $(NH_4)_2SO_4$ concentration on the Poros ET/M HIC column. Experimental ○=pH 6.0, □=pH 7.0, △=pH 8.0, compared to the linear model of Melander and Horváth (solid line).

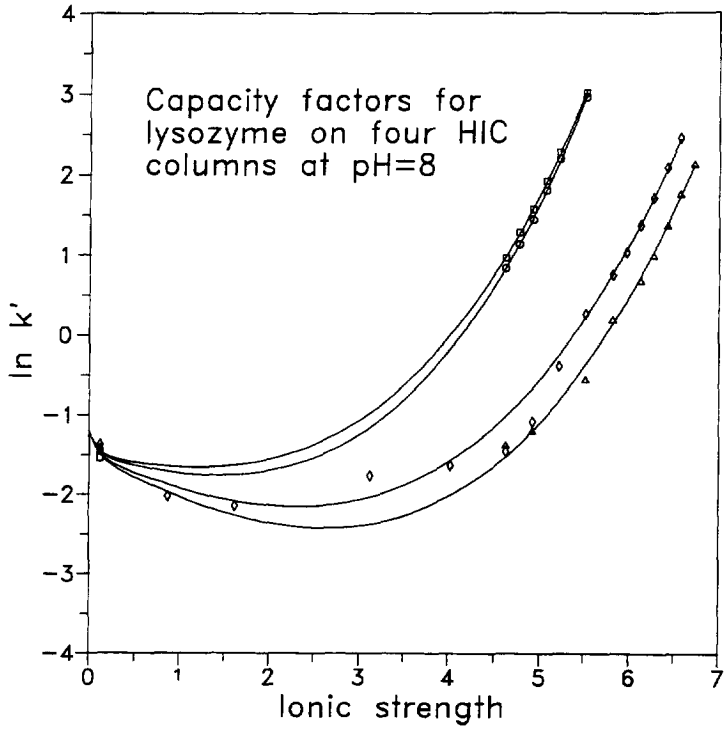

Fig. 5. Comparison of experimental and correlated capacity factors of hen egg white lysozyme as a function of the ionic strength at a pH value of 8.0 on the four Poros columns, □=PH/M, ○=PE/M, ◊=ET/M, △=BU/M. The model comprises Eqs. 7–9.

the retention behaviour of lysozyme in HIC [13], therefore the small temperature changes between the chromatographic runs are regarded as negligible to the retention of the solute.

Figs. 1–4 show the calculated capacity factors as function of the molar salt concentration. The capacity factors are calculated from the adjusted retention volumes using $t_m = 0.130$ min and a flow-rate of 8.30 ml/min. Also shown in the figures are fits of the data to the linear model of Melander and Horváth [4]. This model is capable of representing the data over a limited range in salt concentration but cannot be used to extrapolate to low salt concentrations. The model we have presented is, however, capable of representing the data over the whole concentration range. Fig. 5 shows a comparison of calculated and experimental results at a pH value of 8. As expected there is large scatter in the experimental data at low ionic strength due to the very small retention times at the high flow-rate used. The estimated capacity factors for the protein at zero ionic strength are in good agreement with a capacity factor measured for pure salt where it was found that $k'_{salt} = 0.45$, ($\ln k'_{salt} = -0.8$), which means that, as expected, the gel filtration is larger for the salt than for the large protein molecule.

The comparison of calculated and experimental adjusted retention times, Tables 2–5, shows that there is a systematic deviation in the calculated results. This may be due to the fact that we have not taken the time constant in the gradient mixer into account nor have we addressed the fact that the salt gradient in the column is dispersed due to mobile phase diffusion and gel filtration of the salt.

Acknowledgments

This work was supported by grant from the Danish Research Council (STVF).

References

[1] N.B. Afeyan, N.F. Gordon, I. Mazsaroff, L. Varady, S.P. Fulton, Y.B. Yang and F.E. Regnier, J. Chromatogr., 519 (1990) 1.
[2] N.B. Afeyan, S.P. Fulton and F.E. Regnier, J. Chromatogr., 544 (1991) 267.
[3] S.P. Fulton, N.B. Afeyan, N.F. Gordon and F.E. Regnier, J. Chromatogr., 547 (1991) 452.
[4] W. Melander and Cs. Horváth, Arch. Biochem. Biophys., 183 (1977) 200.
[5] O. Sinanoğlu and S. Abdulnur, Fed. Proc., 24(2) 1965 12.
[6] W.R. Melander, D. Corradini and Cs. Horváth, J. Chromatogr., 317 (1984) 67.
[7] J.L. Fausnaugh and F.E. Regnier, J. Chromatogr., 359 (1986) 131.
[8] A. Katti, Y.-F. Maa and Cs. Horváth, Chromatographia, 24 (1987) 646.
[9] L. Szepesy and Cs. Horváth, Chromatographia, 26 (1988) 13.
[10] B.F. Roettger, J.A. Myers, M.R. Ladisch and F.E. Regnier, Biotech. Prog., 5 (1989) 79.
[11] W.R. Melander, Z. El Rassi and Cs. Horváth, J. Chromatogr., 469 (1989) 3.
[12] I. Mazsaroff, L. Vàrady, G.A. Mouchawar and F.E. Regnier, J. Chromatogr., 499 (1990) 63.
[13] S.C. Goheen and S.C. Engelhorn, J. Chromatogr., 317 (1984) 55.
[14] P. Štrop, J. Chromatogr., 294 (1984) 213.
[15] N.T. Miller and B.L. Karger, J. Chromatogr., 326 (1985) 45.
[16] CRC Handbook of Chemistry and Physics, 64th ed., CRC Press, Boca Raton, FL, 1983–1984, p. D-227.