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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS ON SEPARON HEMA

I. THE EFFECT OF AN INITIAL SALT CONCENTRATION ON THE SEPARATION OF PROTEINS

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SUMMARY

The influence of an initial salt concentration, φ_0, on the gradient separation of proteins using hydrophobic interaction chromatography on Separon HEMA 1000 was investigated. The results obtained were compared with the retention times and peak widths calculated according to a mathematical model.

INTRODUCTION

High-performance hydrophobic interaction chromatography is a widely used technique for the separation of proteins. A number of reports[1–18] dealing both with the theoretical and the practical aspects of the method has been published. For the separation of proteins, generally a linear gradient of decreasing salt concentration, usually of ammonium sulphate in a low concentration of buffer at constant pH, is used. The resolution is inversely proportional to the square root of the gradient slope, g[17] (dimension M/ml). The gradient slope is usually changed by choosing various gradient times, t_g. The influence of the initial salt concentration on the resolution of protein peaks has been of little interest up to now[1,2,4,7]. The increased retention of proteins with increasing initial salt concentration and the effect on the recovery during separation were usually stressed.

Some other studies related to the gradient elution of proteins, mainly by reversed-phase chromatography, have been published recently[19–22].

The effect of the initial salt concentration at the gradient on the retention time, t_R, or on the peak width, σ_R^2, may be theoretically explained by applying, *e.g.*, the basic equation[23] describing the mass balance of the given component on the column at the point x and time t

$$\frac{\partial C(x,t)}{\partial t} + \frac{1-\varepsilon}{\varepsilon} \cdot \frac{\partial \bar{C}(x,t)}{\partial t} = D \cdot \frac{\partial^2 C(x,t)}{\partial x^2} - u \cdot \frac{C(x,t)}{\partial x} \tag{1}$$

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where u = the velocity of the mobile phase in cm/s, D = the axial dispersion coefficient in cm^2/s, ε = the fraction of the column void volume, $\bar{C}$ = the concentration of solute in the stationary phase in M of the stationary phase and C = the concentration of solute in the mobile phase in M. l is a distance reached by a pertinent component from the top of the column in a given time interval. l is an independent variable in the differential forms of eqns. 2 and 3.

In the case of equilibrium linear chromatography, the analytical solution of eqn. 1 exists, for the condition of the Dirac input signal. It has been published, *e.g.*, by Kučera[24] in the form of the relationships for the nth normalized statistical and central moments of the elution curve, for example for the first moment, μ'_1, and the second central moment, μ_2 where L = the column length and k' = the capacity factor.

$$\mu'_1 = t_R = \frac{L}{u}(1 + k') + \frac{2D}{u^2}(1 + k') \quad (2)$$

$$\mu_2 = \sigma_R^2 = 2D \cdot \frac{L}{u^3}(1 + k')^2 + \frac{8D^2}{u^4}(1 + k')^2 \quad (3)$$

Eqn. 2 in a differential form is used to compute the retention time, t_R, at gradient elution. Both k' and D are changed along the column during the movement of a pertinent component:

$$dt_R = \frac{1 + k'}{u} \cdot dl + \frac{1}{u} \cdot \frac{dk'}{dl} \cdot dl + 2\frac{1 + k'}{u^2} \cdot \frac{dD}{dl} \cdot dl + \frac{2D}{u^2} \cdot \frac{dk'}{dl} \cdot dl \quad (4)$$

$$1 \in \langle 0,L \rangle$$

Similarly for the computation of σ_R^2, eqn. 3 in a differential form is used:

$$d\sigma_R^2 = 2D \cdot \frac{(1 + k')^2}{u^3} \cdot dl + \frac{2l}{u^3}(1 + k')^2 \frac{dD}{dl} + \frac{4Dl}{u^3}(1 + k')\frac{dk'}{dl} \cdot dl + $$
$$+ \frac{16D}{u^4}(1 + k')^2 \frac{dD}{dl} \cdot dl + \frac{16D^2}{u^4}(1 + k')\frac{dk'}{dl} \cdot dl \quad (5)$$

If these equations are used for the description of gradient elution chromatography, the peak sharping has to be taken in account[25,26]. This occurs due to the difference in the rate of movement of the two peak wings. This correction of eqn. 5 may be expressed in differential form as:

$$d\sigma_{RC} = d\sigma_R - d\sigma_C \quad (6)$$

It is possible to evaluate the correction term $d\sigma_C$ by numerical solution of eqns. 4–6 (see below).

EXPERIMENTAL

The stainless-steel column (100 mm × 4 mm I.D.) was packed with Separon HEMA 1000, 10 μm (Tessek, Prague, Czechoslovakia). A Spectra Physics 8700 XL gradient pump (San José, CA, U.S.A.) was employed. Proteins were injected on the column always after the dwell time of the gradient system had elapsed (time necessary for the change in mobile phase composition to reach the top of the column, in our case 7.5 min at a flow-rate of 0.5 ml/min). All proteins used in this study (lysozyme, ovalbumin, transferrin, ribonuclease A, carbonic anhydrase, myoglobin and cytochrome *c*) were obtained from Sigma (St. Louis, MO, U.S.A.). Proteins were dissolved in 0.1 *M* phosphate buffer, pH 7.0, containing the initial concentration of ammonium sulphate chosen for a given gradient. Volumes of 20–60 μl of each protein solution were injected on the column. The UV detector Uvicord S 2138 (Pharmacia-LKB, Bromma, Sweden) was operated at 275 nm and connected to a 4290 Spectra Physics integrator.

The void volume was determined as the retention volume of pure phosphate buffer injected on the column washed with the solution of ammonium sulphate in phosphate buffer. The values obtained in this way were in good agreement with the retention volumes of non-retained hydrophilic proteins (cytochrome *c*, myoglobin) in sufficiently low concentration of salt, and also with results described earlier[27].

RESULTS AND DISCUSSION

In the case of isocratic elution of proteins, as in refs. 16 and 18, the logarithm of the capacity factor, log k', is usually proportional to the molar concentration of ammonium sulphate φ, see Fig. 1. The differences between the individual proteins

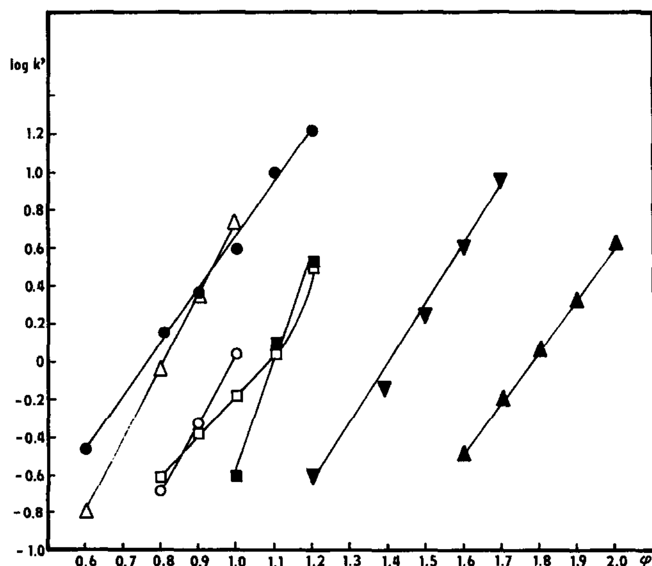

Fig. 1. The dependences of the logarithm of the capacity factor (log k') on the concentration of ammonium sulphate [$\varphi(M)$] in 0.1 *M* phosphate buffer pH 7.0 for the isocratic elution of proteins: ■, transferrin; □, ribonuclease A; △, carbonic anhydrase; ○, ovalbumin; ●, lysozyme; ▼, myoglobin; ▲, cytochrome *c*.

consist above all in the slope of the dependence of log k' *vs.* φ and in the concentration range in which this dependence can be investigated. Up to a certain salt concentration, the protein is not retained and is eluted with the void volume of column. When this salt concentration is exceeded, the elution volume of the protein increases but a pronounced increase in peak width occurs at the same time so that the number of theoretical plates decreases monotonously. At higher salt concentrations further measurement is impossible. The dependence for ribonuclease A suggests a pronounced non-linear increase in log k' in the concentration range 1.1–1.2 M $(NH_4)_2SO_4$. Further experiments were carried with lysozyme, ovalbumin and transferrin. These proteins differ in the relative molecular mass and in the slope of the dependence of log k' *vs.* φ.

The significant influence of the initial salt concentration on the course of the gradient elution is documented in Table I. First, a constant gradient time was chosen. In the range of $\varphi_0 = 0.9$–1.4 M, the gradient slopes (see values of g in Table I) increased by 50% due to the initial concentration. The resolution of the pair transferrin–ovalbumin was increased by a factor of three, whereas that of ovalbumin–lysozyme decreased by approximately 40%. The experiments given in lines 7 and 8 of Table I demonstrate that these changes in resolution are caused by the influence of the initial salt concentration. The gradient time was adjusted so as to obtain the same gradient slope as in lines 2 and 6. It is seen that, regardless of the same g value, the resolutions are different. It is usually thought that the steeper the gradient, the lower is the resolution. Comparison of lines 6 and 9 in Table I shows that the ratio of the slopes of the dependence of log k' *vs.* salt concentration can play an important rôle. Whereas the gradient slope, g, increased by a factor of three caused a decrease in the resolution of the pair transferrin–ovalbumin, on contrary the resolution of the pair ovabumin–lysozyme increased. Analogous results were obtained in Jandera's study on reversed-phase separation of pesticides[19]. The influence of the initial eluent composition is discussed in connection with the different slopes of the dependences of log k' *vs.* eluent composition. Chromatograms corresponding to the experiments in lines 1 and 6 of Table I are given in Figs. 2 and 3. It is interesting to follow the elution of an impurity contained in transferrin. While the t_R of this impurity does not change, its peak is eluted after the peak of transferrin when $\varphi_0 = 0.9$ M and before it when $\varphi_0 = 1.4$ M.

TABLE I

DEPENDENCE OF THE RESOLUTION OF TRANSFERRIN (1), OVALBUMIN (2) AND LYSOZYME (3) ON THE INITIAL CONCENTRATION OF AMMONIUM SULPHATE, φ_0

No.	φ_0 *(M)*	t_{grad} *(min)*	g *(M/ml)*	$R_S^{1,2}$	$R_S^{2,3}$
1	0.9	60	0.0300	0.45	1.78
2	1.0	60	0.0333	0.85	1.54
3	1.1	60	0.0366	1.04	1.71
4	1.2	60	0.0400	1.38	1.20
5	1.3	60	0.0433	1.44	1.00
6	1.4	60	0.0466	1.51	1.02
7	1.4	84	0.0333	1.45	1.09
8	1.0	43	0.0466	0.73	1.26
9	1.4	20	0.1400	0.92	1.62

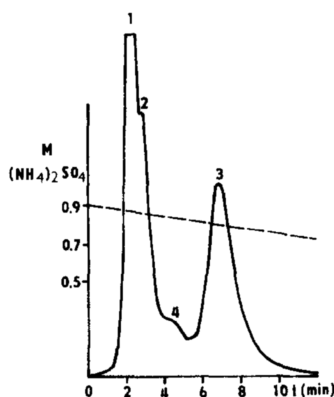

Fig. 2. The separation of transferrin (1), ovalbumin (2), and lysozyme (3) and the impurity in transferrin (4). Column: 100 mm × 4 mm I.D. packed with Separon HEMA 1000, d_p = 10 μm. Decreasing gradient of ammonium sulphate in 0.1 *M* phosphate buffer pH 7.0. Gradient time: 60 min. Flow-rate: 0.5 ml/min. Initial concentration of ammonium sulphate: 0.9 *M*.

For the practical utilization of isocratic dependences log k' = f(φ), the application of the model described in the Introduction may be advantageous. The relationships log k' *vs.* φ and D *vs.* φ (D was determined from the peak width using eqn. 3) measured in the isocratic mode for transferrin, ovalbumin and lysozyme were utilized for the numerical calculations of t_R and W ($W = 4\sigma$). The dependences log k' *vs.* φ and D *vs.* φ are described by eqns. 7 and 8 within the limits of the salt concentrations used during the experiments.

$$\log k' = A + B\varphi \quad (7)$$

$$D = a + b\varphi \quad (8)$$

Parameters A, B, a and b for the three proteins discussed were determined by linear regression. The results including the correlation coefficients are summarized in Table II.

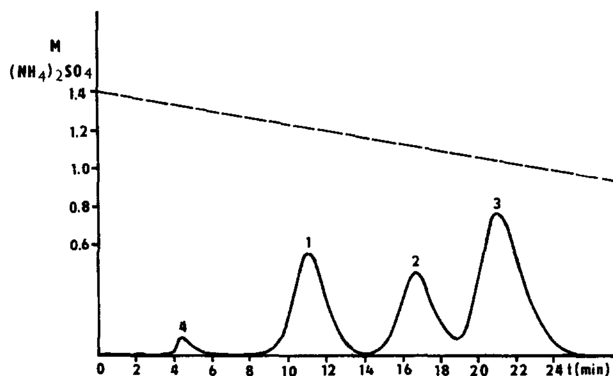

Fig. 3. Separation as in Fig. 2 but with initial concentration of 1.4 *M* ammonium sulphate.

TABLE II

PARAMETERS OF EQNS. 7 AND 8 FOR TRANSFERRIN, OVALBUMIN AND LYSOZYME DETERMINED FROM THE EXPERIMENTAL DEPENDENCES $\log k'$ *vs.* φ AND D *vs.* φ

Dependence	*Transferrin*	*Ovalbumin*	*Lysozyme*
$\log k' = A + B\varphi$			
A	−6.255	−3.598	−1.981
B	5.689	3.646	2.659
Correlation coeff.	0.9900	0.9988	0.9894
$D = a + b\varphi$			
a	−0.062	−0.038	−0.005
b	0.071	0.059	0.017
Correlation coeff.	0.9784	0.9999	0.9813

In the numerical solution the value of Δl was first chosen (in this case $\Delta l = L/100$). Then the values of t_R were computed step-by-step at Δl, $2\Delta l$, $3\Delta l$, ... $100\Delta l$ as the sums of corresponding values of Δt_{R_i} ($i = 1,2,3, \ldots 100$) (see eqn. 4). A given gradient is considered, of course. Similarly the values of $\sigma^2_{R_i}$ corresponding to Δl, $2\Delta l$, $3\Delta l$, ... $100\Delta l$ (see eqn. 5) are computed. These $\sigma^2_{R_i}$ correspond to the σ^2_R of a peak eluted from a column of length Δl, $2\Delta l$, $3\Delta l$, ... $100\Delta l$. However the correction is performed for each point with respect to peak focusing. For this purpose, σ_R (expressed in units of time) is converted into the peak width on the column, $\tilde{W}_l$ (expressed in units of length) utilizing eqn. 9

$$\frac{l - (\tilde{W}_l)_i}{t_{R_i} - 2\sigma_{R_i}} = \frac{1}{t_{R_i} + 2\sigma_{R_i}} \quad \begin{array}{l} l = i\Delta l \\ i = 1,2, \ldots, 100 \end{array} \tag{9}$$

which can be transformed into:

$$(\tilde{W}_l)_i = \frac{4l\sigma_{R_i}}{t_{R_i} + 2\sigma_{R_i}} \tag{10}$$

Then the speed of the rear edge (index r) and front edge (index f) of the peak is determined

$$v_{r(f)} = \frac{u}{1 + k'_{r(f)}} \tag{11}$$

and the correction of the peak width is calculated:

$$(W_c)_i = \Delta t_{R_i}(v_{r,i} - v_{f,i}) \tag{12}$$

The peak width on the column is defined more precisely acoording to

$$(W_l)_i = (\tilde{W}_l)_i - (W_c)_i \tag{13}$$

and used in eqn. 9 to calculate a more precise value of σ_{R_i}. The whole computation is repeated until $l = L$ ($i = 100$).

TABLE III

EXPERIMENTAL AND CALCULATED RETENTION TIMES, t_R (min), AND PEAK WIDTHS, W (ml), OF PROTEINS FOR VARIOUS INITIAL CONCENTRATIONS OF AMMONIUM SULPHATE, φ_0

t_{grad} = 60 min. First the experimental and then the calculated values are given.

φ_0 (M)	*Transferrin*		*Ovalbumin*		*Lysozyme*	
	t_R	W	t_R	W	t_R	W
0.9	2.12 1.96	0.70 0.61	2.80 2.74	0.80 0.95	6.89 5.85	1.50 1.14
1.0	2.53 2.33	0.0 0.75	4.23 3.82	1.30 1.23	9.09 8.38	1.85 1.56
1.1	3.47 3.46	1.16 0.92	5.93 5.77	1.20 1.64	11.53 11.59	2.08 2.02
1.2	5.25 6.00	1.20 1.19	9.67 8.65	2.00 2.11	14.79 15.14	2.25 2.45
1.3	7.81 9.59	1.60 1.20	13.51 13.09	2.35 2.11	18.15 18.25	2.30 2.36
1.4	11.14 13.34	1.70 1.15	16.79 16.89	2.05 2.02	21.14 22.76	2.20 2.22

The calculated values of t_R and W for a gradient time of 60 min and $\varphi_0 \in \langle 0.9, 1.4 \rangle$ are presented together with the experimental values in Table III. The simplified definitions $k' = 0$ for the lower φ values ($\varphi < 0.8$ for transferrin and ovalbumin and $\varphi < 0.6$ for lysozyme, see Fig. 1) and $k' \to \infty$ for $\varphi > 1.2$ certainly contribute to the differences between the experimental and calculated data, especially in the case of transferrin.

The differences between the computed values of t_R and W (see Table III) and the same values obtained by numerical solution of eqn. 1[28] for gradient elution were not greater than 10%. However, the computing time needed was 100 times shorter in the case of the data in Table III.

If the relationship for t_R, known from the literature[19], was applied values two times smaller than the experimental data were obtained. The reason for the unsuccessful application of the analytical relationship for t_R is probably the limited range of the salt concentration in which log k' is a linear function of this parameter.

On the other hand, if the correction to peak focusing was not considered, the values of W calculated as described above were two times greater than experimental results.

The agreement between the experimental data and the values calculated according to the model used here is quite good. From the standpoint of resolution, the difference between the computed and the experimental data is, of course, more pronounced. However, the qualitative trend is maintained; thus the computation corresponds to the conclusions concerning the influence of φ_0 revealed by Table I.

CONCLUSION

Both the experimental results and the model used confirm that the resolution of protein peaks in hydrophobic interaction chromatography on Separon HEMA 1000 depends mainly on the choice of the initial concentration of salt, φ_0, in the gradient elution. This is caused by the different functions of log k' *vs.* φ and D *vs.* φ of the proteins. The effect of different gradient conditions (starting composition, gradient steepness and gradient shape) may be predicted from the isocratic measurements.

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