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Capillary zone electrophoresis of proteins with a dynamic surfactant coating

Influence of a voltage gradient on the separation efficiency

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ABSTRACT

In capillary zone electrophoresis of proteins, the adsorption of the proteins on the capillary wall is a considerable problem that seriously impairs the separation efficiency. The use of a dynamic surfactant coating is a possible way to diminish this adsorption. Highly efficient separations were achieved with a cationic fluorinated buffer additive as a dynamic surfactant coating in untreated fused-silica capillaries at neutral pH. The influence of a voltage gradient on the separation efficiency is discussed and a simple relationship is presented to calculate effective mobilities under voltage gradient conditions.

INTRODUCTION

Capillary zone electrophoresis (CZE) has become a widely used separation technique, especially for mixtures of biological compounds such as peptides, proteins and DNA fragments [1,2]. One of the main problems occurring with protein separations is the adsorption of these molecules on the surface of fused-silica capillaries. This phenomenon causes serious peak broadening, resulting in much lower separation efficiencies than theoretically predicted. To eliminate this protein adsorption, various methods have been described: (1) performing the separation under alkaline conditions [3,4]; if the buffer pH is higher than the isoelectric points (p*I*s) of the proteins, both capillary surface and proteins will have a net negative charge and adsorption is diminished by coulombic repulsion; (2) adding salts or zwitterions to the buffer, resulting in a competition between these buffer additives and proteins for the negative silanol groups on the silica surface [5,6]; (3) coating the capillary surface with a neutral hydrophilic compound by a chemical modification in order to shield the silanol groups [7–13]; (4) adding a surfactant to the buffer which forms a dynamic coating on the capillary surface, thus diminishing protein adsorption [14]. This last method has several advantages above the other methods. The buffer pH remains a freely adjustable parameter over a wide range to optimize selectivity and to avoid denaturation of the proteins. A buffer solution with a low ionic strength can be used to minimize Joule heating. No surface pretreatment is required so that the separation can be performed in untreated fused-silica capillaries.

Recently, Emmer *et al.* [14] reported a cationic fluorinated buffer additive as a dynamic surfactant coating. We used this approach for the separation of several proteins. The influence of the buffer pH on both the electroosmotic flow (EOF) and the sep-

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aration efficiency were studied. Further, the influence of a voltage gradient on the separation efficiency was examined.

THEORETICAL

In CZE, the velocity of a migrating component is given by

$$v = m_{app}E = m_{app}V/l_c \quad (1)$$

where v is the velocity of the component (cm/s), m_{app} the apparent mobility of the component ($cm^2/V \cdot s$), E the electrical field strength (V/cm), V the applied potential (V) and l_c the total length of the capillary (cm). Further,

$$l_d = vt \quad (2)$$

where l_d is the length from the injection to detection point (cm) and t is the migration time measured (s). From eqns. 1 and 2, the apparent mobility of a component can be calculated according to

$$m_{app} = \frac{l_c l_d}{tV} \quad (3)$$

The effective mobility, m_{eff}, of a component is given by

$$m_{eff} = m_{app} - m_{EOF} \quad (4)$$

where m_{EOF} is the "mobility" of the EOF, which can be calculated with the migration time of a neutral compound.

As reported by McCormick [7], the separation efficiency can be increased by using a programmed separation voltage, *i.e.*, the separation voltage is not applied immediately but is reached linearly in a certain programme time. In this instance, however, eqns. 2 and 3 cannot be used, as the separation voltage is not maintained constant during one experiment. In fact, longer migration times will be observed if the programme time is increased. The effective mobility of a component, however, will be constant with a given electrolyte system and can be used for identification [15]. It can be calculated from the measured migration times.

If the separation voltage is a function of time, eqn. 2 should be written as

$$l_d = \int_0^t v(t)dt \quad (5)$$

If the final separation voltage, V_p, is reached linearly in a programme time t_p, as shown in Fig. 1, combination of eqns. 1 and 5 leads to

$$l_d = \int_0^{t_p} m_{app} \cdot \frac{\alpha t + \beta}{l_c} \cdot dt + \int_{t_p}^{t} m_{app} \cdot \frac{V_p}{l_c} \cdot dt \quad (6)$$

where α and β are the slope of the voltage gradient and the initial voltage, respectively. The apparent mobility of a component now can be calculated according to

$$m_{app} = \frac{l_c l_d}{\frac{\alpha}{2} \cdot t_p^2 + \beta t_p + V_p(t - t_p)} \quad (7)$$

Note that for $t_p = 0$ this equation reduces to eqn. 3.

Combination of eqns. 3 and 7, with $\beta = 0$, leads to an expression for τ, the ratio of the migration time with and without a programmed separation voltage. The dimensionless parameter τ is a linear function of the programme time t_p:

$$\tau = \frac{t(t_p \neq 0)}{t(t_p = 0)} = 1 + \frac{1}{2} \cdot \frac{m_{app}V_p}{l_c l_d} \cdot t_p \quad (8)$$

To demonstrate the influence of a voltage gradient on the migration time, τ is shown in Fig. 2 as a function of t_p for different apparent mobilities.

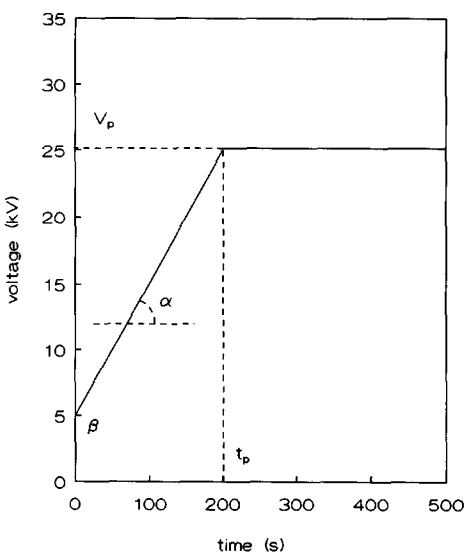

Fig. 1. Separation voltage *versus* time for a linear voltage gradient.

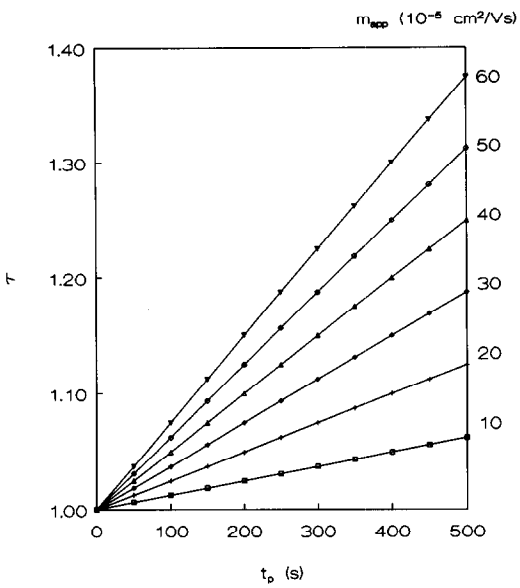

Fig. 2. Calculated graphs of τ *versus* the programme time, t_p, for different apparent mobilities shown on the right-hand ordinate. Capillary, $l_c = l_d = 100.00$ cm. Initial and final separation voltage 0 and 25 kV, respectively.

EXPERIMENTAL

Instrumentation

All experiments were carried out on a laboratory-built CZE apparatus. A computer-controlled high-voltage power supply was used (HCN 35-35000; F.u.G. Elektronik, Rosenheim, Germany). The cathode was positioned at the inlet side and the anode at the outlet side of the capillary, unless indicated otherwise. Capillaries could be flushed by applying vacuum on the outlet side. A Spectra 100 variable-wavelength UV–VIS detector was used (Spectra-Physics, San Jose, CA, USA), equipped with a flow cell for on-column detection. The length of the detection slit was about 0.1 cm. For all protein separations detection was carried out at 200 nm and for the EOF measurements at 254 nm. All experiments were carried out at ambient temperature. Both the high-voltage power supply and the detector were connected with a Tulip SX/AT personal computer via a laboratory-built Multilab-TS interface, to control the separation voltage and to register electropherograms. The laboratory-written data acquisition program CAESAR was used to analyse the electropherograms.

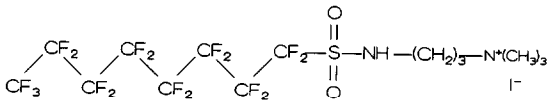

Fig. 3. Structural formula of the cationic surfactant FC135.

Materials and reagents

Fused-silica capillaries of 50 μm I.D.(Siemens, Mülheim, Germany) were used for all experiments. Lysozyme was obtained from Merck (Darmstadt, Germany) and all other proteins from Sigma (St. Louis, MO, USA). The cationic surfactant FC135 (Fig. 3) was kindly donated by 3M (Zoeterwoude, Netherlands). All other reagents were of analytical-reagent grade. Deionized water was used to prepare the buffers and all buffer and sample solutions were filtered through a 0.45-μm filter before use.

Methods

New capillaries were rinsed successively for 20 min with 0.1 *M* KOH, 20 min with water and 20 min with buffer solution before use. If buffer solutions were changed, the capillary was flushed with the new buffer for 10 min. All proteins were dissolved in the separation buffer with a final concentration of 0.1 mg/ml. Mesityl oxide was used as a neutral EOF marker. Injections were carried out by electromigration.

RESULTS AND DISCUSSION

Dynamic surfactant coating

If a sufficient amount of a cationic surfactant is added to the separation buffer, the charge of the capillary surface will be changed from negative to positive. This results in a reversal of the EOF, which will now be directed from the cathode to the anode. By selecting a buffer with a lower pH than the p*I* values of the proteins, both proteins and the capillary surface have a net positive charge and adsorption is diminished by coulombic repulsion. In this way a cationic surfactant can be applied as a dynamic coating in CZE of proteins. With this method, the pH range that can be used to optimize the selectivity is limited by the p*I* values of the proteins being analysed. However, basic proteins can be separated under neutral pH conditions, which is favourable with respect to denaturation. Because the proteins are migrating in the upstream mode, *i.e.*,

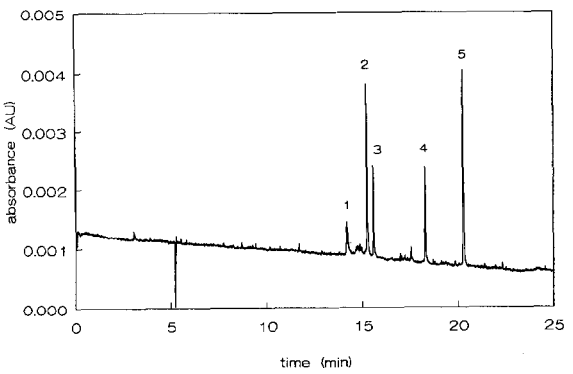

Fig. 4. Electropherogram for the separation of (1) mesityl oxide, (2) α-chymotrypsinogen, (3) ribonuclease A, (4) cytochrome *c* and (5) lysozyme. Capillary, l_c = 99.65 cm and l_d = 87.28 cm; injection, 5 s at 5 kV; separation voltage, 25 kV; buffer, 10 m*M* phosphate (pH 7.0) with 50 μg/ml of FC135 added.

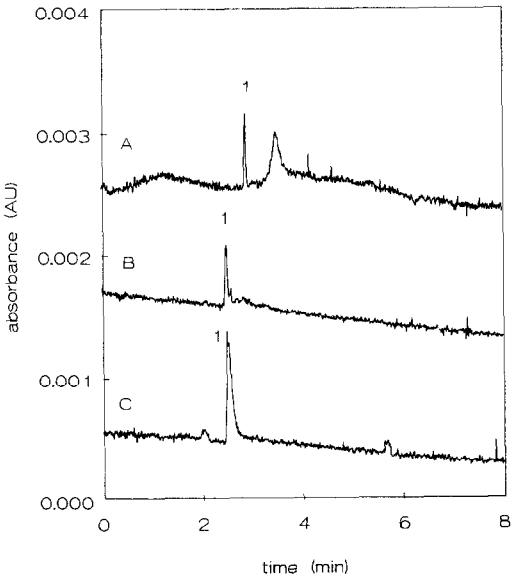

Fig. 5. Electropherograms obtained under conditions without a dynamic coating. Capillary, l_c = 42.15 cm and l_d = 34.60 cm. Anode at the inlet side and cathode at the outlet side, respectively. Injection, 5 s at 5 kV; separation voltage, 15 kV; buffer, 10 m*M* borate at (A) pH 11.0, (B) pH 9.0 and (C) pH 7.0. See Fig. 4 for the composition of the sample.

their electrophoretic mobility is opposite to the direction of the EOF, the EOF must be high enough to detect all components injected and to avoid long separation times. Further, the surfactant should not interact with the proteins, which occurs, for example, if cetyltrimetylammonium bromide (CTAB) is used [16].

Good results were obtained with the fluorinated cationic surfactant FC135 [14,17]. In Fig. 4 the electropherogram is shown of the separation of four basic proteins under neutral pH conditions. As can be seen from the results, listed in Table I, highly efficient separations ($N > 10^6$) can be achieved using this method.

To compare these results with separations under conditions without a dynamic coating, three experiments were carried out at different buffer pH values, without a surfactant added to the buffer. As can be seen from the electropherograms, shown in Fig. 5, at pH 11.0 only one peak for the proteins is observed, whereas at pH 9.0 and 7.0 no separation is obtained at all. The tailing of the peak at pH 7.0 is caused by one of the proteins co-eluting with mesityl oxide.

Influence of buffer pH

In order to study the influence of the buffer pH

TABLE I

ISOELECTRIC POINTS, p*I*, MOLECULAR WEIGHTS, MW, MEASURED MIGRATION TIMES, *t*, CALCULATED EFFECTIVE MOBILITIES, m_{eff}, AND THEORETICAL PLATE NUMBERS, *N*, FOR THE DIFFERENT SAMPLE COMPONENTS

Conditions as in Fig. 4.

No.	Component	p*I*	MW	*t* (min)	m_{eff} (10^{-5} cm/V · s)	$N \times 10^{-5}$
1	Mesityl oxyde			14.20	−40.83	1.94
2	α-Chymotrypsinogen	8.8	25 000	15.26	2.84	9.47
3	Ribonuclease A	8.7	13 500	15.59	3.64	12.43
4	Cytochrome *c*	10.8	12 200	18.30	9.15	11.13
5	Lysozyme	10.0	14 000	20.29	12.26	10.00

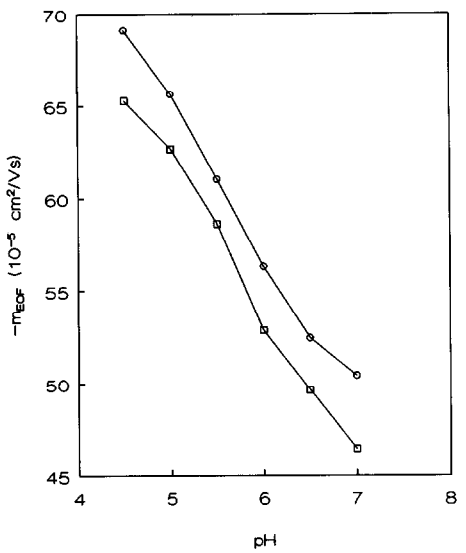

Fig. 6. Electroosmotic flow, m_{EOF}, *versus* buffer pH. Capillary, l_c = 66.32 cm and l_d = 53.45 cm. Injection, 5 s at 5 kV. Separation voltage, 25 kV. Buffer, 10 m*M* phosphate (pH 7.0) with (□) 25 μg/ml and (○) 50 μ/ml of FC135 added.

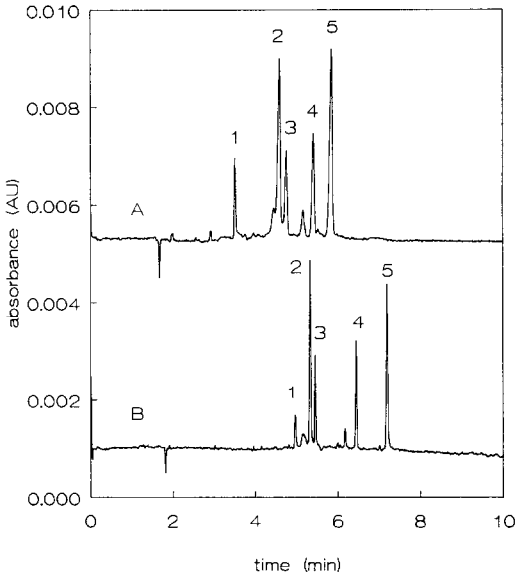

Fig. 7. Electropherograms for the separation of (1) mesityl oxide, (2) α-chymotrypsinogen, (3) ribonuclease A, (4) cytochrome *c* and (5) lysozyme. Capillary, l_c = 64.54 cm and l_d = 51.54 cm; injection, 5 s at 5 kV; separation voltage, 25 kV; buffer, 10 m*M* phosphate with 50 μg/ml of FC135 added at (A) pH 4.5 and (B) pH 7.0.

on the EOF, experiments were carried out at several pH values and two different surfactant concentrations. From the results, shown in Fig. 6, it can be seen that a higher EOF is obtained at a lower buffer pH and that an increase in the surfactant concentration leads to an increase in the EOF, suggesting that the capillary surface is not covered completely by the dynamic coating.

In Table II, the results are listed of separations at different buffer pH values. Owing to a higher net positive charge, the effective mobility increases with a decrease in buffer pH. At lower buffer pH values less efficient separations were obtained. Fig. 7 shows the electropherograms of separations at pH 4.5 and 7.0. This decrease in efficiency may be caused by adsorption on the uncovered ionized silanol groups at larger pH − p*I* differences and by denaturation. The most efficient and reproducible results were obtained at neutral pH.

TABLE II

AVERAGE EFFECTIVE MOBILITIES, m_{eff}, WITH STANDARD DEVIATIONS FOR THE DIFFERENT SAMPLE COMPONENTS AT DIFFERENT BUFFER pH VALUES (n = 5)

Conditions as in Fig. 7.

pH	m_{eff} ± S.D. (10^{-5} cm²/V · s)				
	Mesityl oxyde	α-Chymotrypsinogen	Ribonuclease A	Cytochrome *c*	Lysozyme
4.5	−64.67 ± 1.12	16.25 ± 1.01	17.94 ± 1.00	23.60 ± 1.10	15.86 ± 0.71
5.0	−56.43 ± 0.38	12.18 ± 0.13	13.91 ± 0.15	19.62 ± 0.34	23.37 ± 0.37
5.5	−53.62 ± 0.35	10.47 ± 0.13	12.12 ± 0.13	17.78 ± 0.16	21.51 ± 0.16
6.0	−49.19 ± 0.37	8.08 ± 0.12	9.54 ± 0.13	15.19 ± 0.17	18.85 ± 0.20
6.5	−44.78 ± 0.27	5.28 ± 0.07	6.40 ± 0.06	12.06 ± 0.13	15.55 ± 0.18
7.0	−44.18 ± 0.33	2.97 ± 0.04	3.90 ± 0.03	10.14 ± 0.07	13.69 ± 0.07

TABLE III

MEASURED MIGRATION TIMES, t, AND CALCULATED EFFECTIVE MOBILITIES, m_{eff}, FOR THE DIFFERENT SAMPLE COMPONENTS AT DIFFERENT PROGRAMME TIMES, t_p

Conditions as in Fig. 8.

t_p (s)	Mesityl oxide		α-Chymotrypsinogen		Ribonuclease A		Cytochrome *c*		Lysozyme	
	t (min)	m_{eff} (10^{-5} cm^2/V · s)	t (min)	m_{eff} (10^{-5} cm^2/V · s)	t (min)	m_{eff} (10^{-5} cm^2/V · s)	t (min)	m_{eff} (10^{-5} cm^2/V · s)	t (min)	m_{eff} (10^{-5} cm^2/V · s)
0	5.20	−42.65	5.59	2.98	5.71	3.81	6.72	9.65	7.47	12.96
10	5.21	−43.28	5.59	2.99	5.71	3.85	6.71	9.80	7.46	13.21
50	5.59	−42.84	5.96	2.86	6.09	3.77	7.10	9.67	7.85	13.02
100	6.04	−42.62	6.41	2.83	6.53	3.67	7.53	9.49	8.28	12.83
200	6.93	−42.11	7.31	2.83	7.43	3.65	8.45	9.43	9.19	12.64
300	7.79	−41.92	8.16	2.74	8.19	3.62	9.30	9.31	10.06	12.59
400	8.72	−41.19	9.09	2.65	9.22	3.50	10.22	8.98	10.98	12.18
500	9.61	−40.71	9.99	2.66	10.11	3.42	11.15	8.97	11.92	12.13
Average		−42.17		2.82		3.66		9.41		12.69
S.D.		0.81		0.12		0.14		0.29		0.36

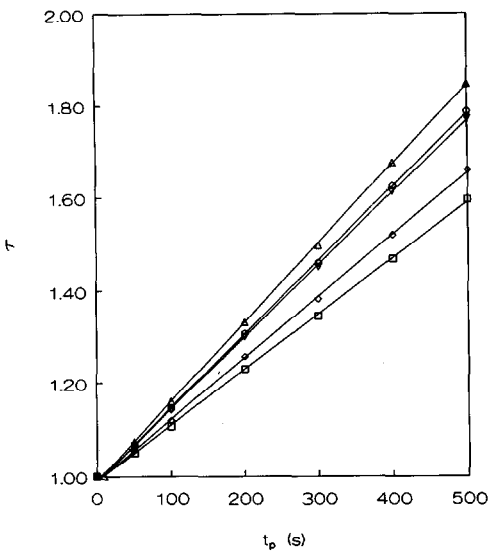

Fig. 8. Measured graphs of τ *versus* the program time, t_p, for (△) mesityl oxide, (○) α-chymotrypsinogen, (▽) ribonuclease A, (◇) cytochrome *c* and (□) lysozyme. Capillary, l_c = 64.54 cm and l_d = 51.54 cm; injection, 5 s at 5 kV; initial and final voltage, 0 and 25 kV, respectively; buffer, 10 m*M* phosphate (pH 7.0) with 50 μg/ml of FC135 added. For further explanation, see text.

Influence of a voltage gradient

To examine the influence of a programmed separation voltage, eight experiments were carried out with a linear voltage gradient and increasing programme times. The initial and final separation voltages were 0 kV (β = 0, eqn. 7) and 25 kV, respectively. As discussed in the theoretical section, the effective mobility of a component will be constant with a given electrolyte system and independent of the voltage gradient. In Table III, measured migration times, *t*, and effective mobilities, m_{eff}, calculated with eqn. 7 are listed. The slight decrease in the effective mobility with an increase in programme time can be explained by less Joule heating at longer programme times.

For the graphs of τ *versus* the programme time, t_p, almost linear plots were obtained, as shown in Fig. 8. From these graphs effective mobilities were calculated using eqn. 8, and are listed in Table IV. Using this calculation method, less Joule heating at longer programme times will lead to longer migration times and more positive slopes, which explains the higher values obtained for the effective mobilities.

The influence of the voltage gradient on the separation efficiency is illustrated in Fig. 9, where the peak variance, σ^2, is shown as a function of the programme time, t_p. A minimum in the peak variance is observed with an increase in programme time. This phenomenon was described by Bushey and Jorgenson [18], who used a stepped separation voltage to obtain a higher separation efficiency. If the Joule heating of the buffer causes an expansion of the buffer present in the capillary, a small part of the injection plug will be pushed back into the buffer vial. This results in a larger injection volume and broader peaks will be observed. If, however, a voltage gradient is applied at the beginning of the separation, the injected components have migrated further into the capillary when Joule heating becomes significant and smaller peaks are obtained. At longer separation times the peak variance due to diffusion increases, which explains the broader peaks obtained at longer programme times.

Huang *et al.* [19] pointed out that the peak variance in CZE is mainly affected by the injection length of the sample, longitudinal diffusion and

TABLE IV

CALCULATED EFFECTIVE MOBILITY, m_{eff}, SLOPE AND INTERCEPT (ARBITRARY UNITS) AND CORRELATION COEFFICIENT FOR THE GRAPHS SHOWN IN FIG. 8

No.	Component	Correlation coefficient	Slope	Intercept	m_{eff} (10^{-5} cm^2/V · s)
1	Mesityl oxide	0.99976	17.10	−0.009	−45.53
2	α-Chymotrypsinogen	0.99966	15.89	−0.011	3.23
3	Ribonuclease A	0.99970	15.57	−0.010	4.09
4	Cytochrome *c*	0.99957	13.29	−0.010	10.16
5	Lysozyme	0.99955	12.00	−0.009	13.58

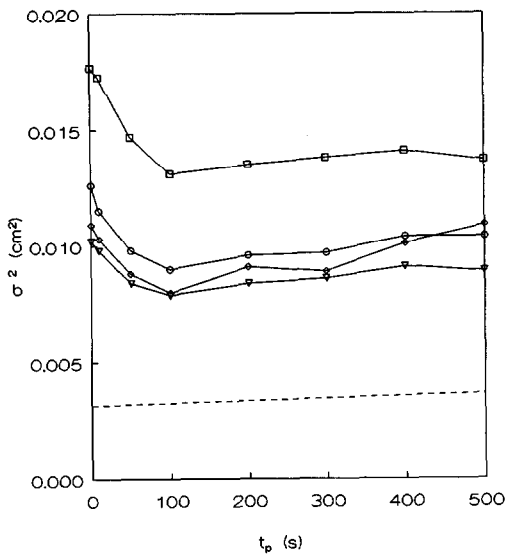

Fig. 9. Measured peak variances, σ^2, for (○) α-chymotrypsinogen, (▽) ribonuclease A, (◇) cytochrome *c* and (□) lysozyme, and calculated values (dashed line). Conditions as in Fig. 8. For further explanation, see text.

analyte–wall interactions. If the variance due to a finite detection path length is taken into account separately, the total peak variance, σ^2, can be written as

$$\sigma^2 = \sigma^2_{inj} + \sigma^2_{det} + \sigma^2{}_{diff} + \sigma^2_{ads} \quad (9)$$

where σ^2_{inj}, σ^2_{det}, σ^2_{diff} and σ^2_{ads} are the variances due to injection, detection, diffusion and adsorption, respectively. The first three terms in eqn. 9 are given by

$$\sigma^2_{inj} = l_{inj}/12 \quad (10)$$

$$\sigma^2_{det} = l_{det}/12 \quad (11)$$

and

$$\sigma^2_{diff} = 2Dt \quad (12)$$

where l_{inj} is the length of the injection plug (cm), l_{det} the length of the detection path (cm) and D the diffusion constant of the component (cm^2/s). In Fig. 9 calculated peak variances are shown (dashed line), assuming an apparent mobility of $35 \cdot 10^{-5}$ $cm^2/V \cdot s$ and a diffusion constant of $1 \cdot 10^{-6}$ cm^2/s [20]. Analyte–wall interactions were not taken into account. From Fig. 9 it can be concluded that the theoretical values are lower than the experimentally obtained values, irrespective of whether a voltage gradient is applied or not, indicating that other zone broadening effects such as adsorption and temperature effects still play a significant role.

CONCLUSION

From the foregoing experiments, it can be concluded that highly efficient separations of basic proteins can be achieved at neutral pH with the cationic fluorinated surfactant FC135 as a dynamic surfactant coating. With this method untreated fused-silica capillaries can be used for the separation of proteins.

The separation efficiency can be improved by applying a voltage gradient at the beginning of the experiment and effective mobilities under these conditions can be calculated with the method presented. The measured peak variances pass through a minimum with increasing programme times. These variances are, however, larger than the theoretically calculated values.

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