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Use of plate numbers achieved in capillary electrophoretic protein separations for characterization of capillary coatings

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Abstract

The number of theoretical plates achievable in capillary electrophoresis has been proposed for the characterization of the quality of capillary coatings. It is shown that, as predicted by theory, the efficiency is independent of capillary inner diameter and increases with increasing field strength as long as dispersive effects can be neglected. On the other hand, in contrast to chromatography, there is no linear range within which the plate numbers are independent of the amount or volume of sample injected (from aqueous solutions). By optimization of the injection technique (use of dilute aqueous protein solutions, electrokinetic injection techniques, short injection times), up to $3 \cdot 10^6$ theoretical plates can be achieved. The plates generated by injection of similar amounts of sample from buffer solutions are lower by a factor of 20.

1. Introduction

The tremendous increase in capillary electrophoretic (CE) applications is partly due to the high efficiency achievable by this method. Extremely narrow peaks can be obtained even with high-molecular-mass solutes. One reason is the plug-shaped flow and migration profiles [1]. In pressure-driven liquid chromatography, the parabolic Hagen–Poiseuille flow profile is the main reason for peak dispersion, especially when the profile cannot be flattened owing to small diffusion coefficients. These effects have been described by the various equations for peak broadening in chromatography correlating peak dispersion, flow velocity and capillary diameter (particle diameter), such as the Taylor–Aris equation [2,3], the Golay equation for capillary gas chromatography [4], the Van Deemter equation for packed column chromatography [5] and the Giddings universal plate height equation [6].

Because no parabolic flow profile is present in CE, the equation for plate number contains only the longitudinal diffusion term [7] and can be reduced to

$$N = \frac{\mu V}{2D}$$

where N is the plate number, D the solute diffusion coefficient in the buffer, V the applied voltage and μ the overall mobility (electrophoretic and electroosmotic). The validity of this equation for CE has already been proved. Giddings [6] has also shown that in the usual range of voltages applied in CE (0–35 000 V) and 1–10 elementary charges for the solutes, the equation for the plate number can be approximated to

$$N = 20zV$$

This means that up to $10 \cdot 10^6$ theoretical plates

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can be generated in CE, a range extending that found in chromatographic systems. With DNA fragments in anti-convective systems such as gels these figures have been already verified and even exceeded [8].

Because of the absence of the mass transfer term in the mobile phase, the achievable plate numbers should be independent of capillary diameter and should increase with decreasing diffusion coefficient (increasing molecular mass) and increasing voltage. Observed plate numbers below those predicted by theory have been attributed to slow mass transfer and solute–wall interactions. Therefore, the plate numbers achievable with proteins in surface-coated capillaries have been proposed as a measure for characterizing coating quality. However, because of the extremely small volumes to be handled in CE, the injection technique has a significant influence on efficiency [9]. Additional contributions to peak broadening can be caused by overloading effects of the buffer, temperature effects [10–13] and mobility differences between buffer and analyte ions [14]. Because of on-column detection, the contribution of the detector to overall peak broadening can mainly be reduced to slow data conversion.

In this paper, the parameters governing efficiency in CE will be demonstrated for the separation of basic standard proteins. It will also be shown how the efficiency can be manipulated by applying the various injection techniques.

2. Experimental

2.1. Reagents and materials

Fused-silica capillary tubes were purchased from Polymicro (Laser 2000, Munich, Germany). Reagents for surface modification were purchased from different suppliers, such as acrylamide (Bio-Rad, Munich, Germany), ammonium peroxodisulfate and N,N,N′,N′-tetramethylethylenediamine (Electran, UK) and vinyltrichlorosilane (Fluka, Neu-Ulm, Germany). The proteins were obtained from different suppliers such as cytochrome *c* (Sigma, Deisenhofen, Germany), chymotrypsinogen (Serva, Heidelberg, Germany) and lysozyme and ribonuclease A (Fluka). All buffer components were obtained from Fluka.

2.2. Modification of fused-silica capillaries

Capillaries were coated with vinyltrichlorosilane and polacrylamide as described previously [15].

2.3. Apparatus

For all measurements a Beckman P/ACE System 2050 was used. Data acquisition was accomplished with Beckman Gold Software (V 7.12) and an IBM PS2 personal computer.

3. Results and discussion

3.1. Plates, capillary length and diameter

In chromatography, the plate numbers increase with decreasing particle (capillary) diameter. Decreasing diffusion coefficients always lead to smaller plate numbers, because the mass transfer term governs efficiency in liquid mobile phases. The plate numbers achievable are also dependent on the mobile phase flow-rate. The maximum plate numbers are achieved at a flow-rate depending on particle diameter and sample diffusion coefficient. Of course, the plate numbers also depend on the column length. This concept is only valid when all the other conditions, i.e., temperature, mobile phase composition, etc., are kept constant. In chromatography, the theoretical value is always the highest achievable. Owing to additional dispersive contributions (variance contributions from injection volume, connecting capillaries, detector cell volume, etc.), in reality smaller plate numbers are always obtained.

In CE, the plate numbers increase linearly with increase in effective capillary length as predicted by theory. This is shown in Fig. 1. It should be mentioned that for these measurements the field strength was kept constant; with

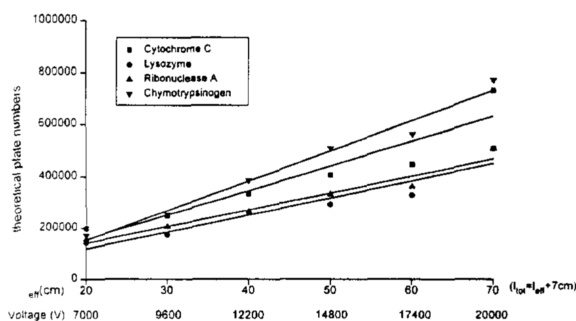

Fig. 1. Dependence of the number of theoretical plates on the effective capillary length with constant electrical field and injection plug length. Conditions: capillary, polyacrylamide–vinyltrichlorosilane, 75 μm I.D.; buffer, 50 m*M* phosphate (pH 3); field strength, 260 V/cm; samples dissolved in water; injection, pressure [$3.45 \cdot 10^3$ Pa (0.5 p.s.i.)], identical injection plug length in all capillaries (2.5 s with 60/67 cm capillary, 1 s with 20/27 cm capillary).

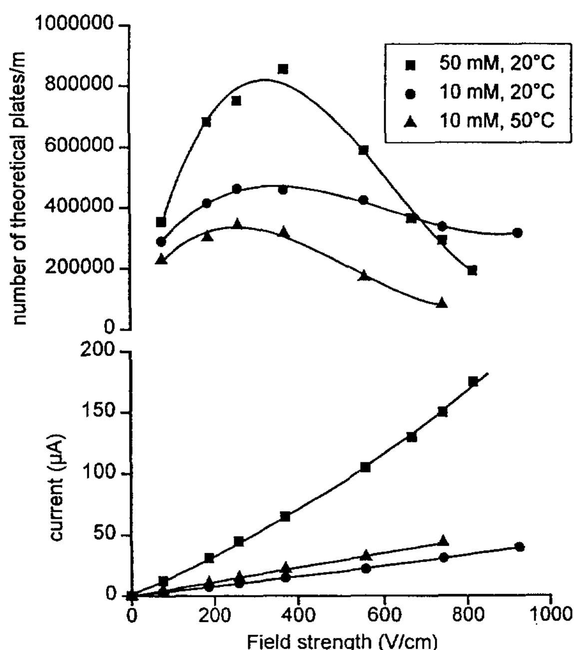

Fig. 2. Dependence of the number of theoretical plates on the applied electric field with different buffer concentrations and temperatures. Conditions: capillary, polyacrylamide–vinyltrichlorosilane, 20/27 cm × 75 μm I.D.; buffer, phosphate (pH 3); sample, lysozyme (100 ppm in water); injection, pressure [$3.45 \cdot 10^3$ Pa (0.5 p.s.i.)], 1 s with 50 m*M* buffer at 20°C and 2 s with 10 m*M* buffer at 20 and 50°C.

increasing capillary length the voltage was increased correspondingly. To exclude the contribution of the variance of the injection plug, the same portion of the capillary was always filled with sample. This means that the injection time was 1 s for the 20-cm capillary (total length 27 cm) and 2.5 s for the 60-cm capillary (total length 67 cm). With a polyacrylamide-coated capillary [15], between $6 \cdot 10^5$ and $1 \cdot 10^6$ plates per metre were achieved for proteins with molecular mass between 12 000 and 25 000. Surprisingly, but corresponding with theory, the protein with the highest molecular mass (α-chymotrypsinogen) gives the highest plate numbers.

The plate number should also increase linearly with increase in field strength at constant capillary length. However, this is only true if no additional zone dispersion due to secondary effects is present [10–13]. As can be seen in the upper part of Fig. 2, plate numbers increase with increase in field strength only if the latter does not exceed 300 V/cm. At higher field strengths, convective effects (temperature, viscosity and mobility gradients) caused by the Joule heat dissipation diminish the achievable plate numbers. This can be clearly seen for the 50 m*M* buffer. The lower part of Fig. 2, where the current is plotted vs. the field strength, shows that at field strengths above 400 V/cm deviations from Ohm's law occur. These deviations are caused by insufficient heat transfer from the capillary.

Fig. 2 also shows that with lower buffer concentrations the influence of insufficient heat dissipation on plate number is less important. With the 10 m*M* phosphate buffer identical plate numbers can be achieved between 200 and 600 V/cm. However, peak distortion due to heat transfer problems is noticeable when the capillary is used at elevated temperatures. This is caused by problems with heat transfer in the equipment in thermostating the capillary. Of course, at elevated temperatures diffusion coefficients are higher, lower plate numbers are generated and only lower field strengths can be applied. The advantage of capillaries with smaller inner diameters (<50 μm) is that higher field

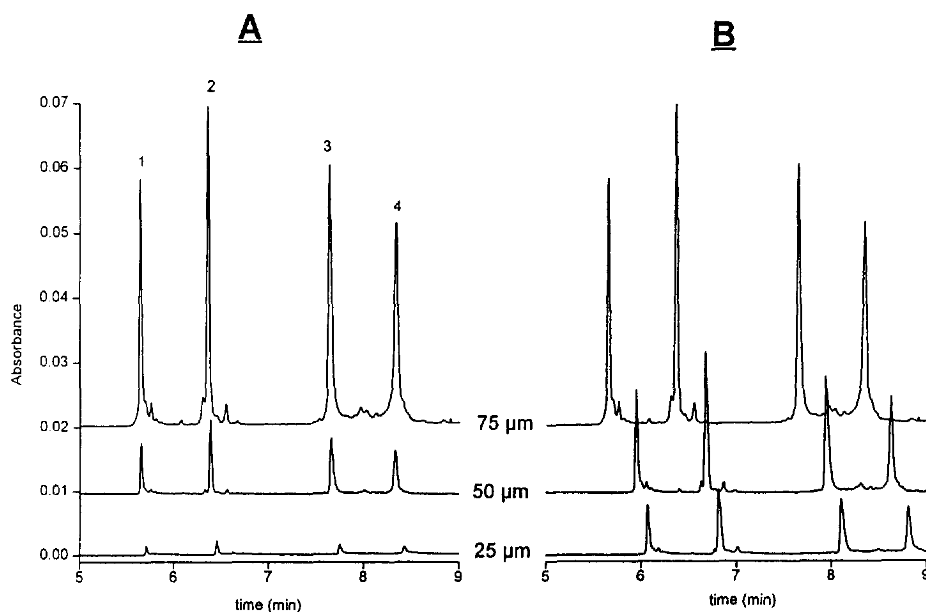

Fig. 3. Influence of the capillary inner diameter on the separation of basic standard proteins. Conditions: capillary, polyacrylamide—vinyltrichlorosilane, 20/27 cm; buffer, 50 m*M* phosphate (pH 3); field strength, 260 V/cm; samples, 1 = cytochrome c, 2 = lysozyme, 3 = ribonuclease A, 4 = chymotrypsinogen (100 ppm in water); injection, pressure [$3.45 \cdot 10^3$ Pa (0.5 p.s.i.)]; (A) identical injection time (2 s); (B) identical injection plug length (2 s with 75 μm I.D., 4.5 s with 50 μm I.D., 18 s with 25 μm I.D. = 0.45 cm).

strengths are applicable without a decrease in efficiency owing to more efficient heat dissipation.

Because only the longitudinal diffusion term contributes to peak broadening in CE, in theory the inner diameter of the capillary should not affect the plate numbers. In the right-hand part of Fig. 3 and in Table 1 it can be seen that plate numbers are independent of capillary inner diameter when the injection plug length is kept constant (contribution of injection volume variance to overall peak variance). A constant plug length of 0.45 cm was used, and consequently the injection time was varied between 2 s with the 75-μm capillary to 18 s for the 25-μm capillary. The error in the determination of plate numbers with these narrow peaks is up to 10%. As can be seen, it is possible to achieve $1 \cdot 10^6$ plates per metre without any problems. The decrease in peak height is due only to the decrease in the optical path length (Lambert–Beer law). The predictions of theory regarding

Table 1
Dependence of the number of theoretical plates per metre on the capillary inner diameter

I.D. (μm)	Injection (s)	Cytochrome *c*	Lysozyme	Ribonuclease A	Chymotrypsinogen
75	2	1 410 000	1 163 000	1 008 000	1 144 000
50	4.5	1 163 000	1 006 000	943 000	1 166 000
25	18	994 000	845 000	787 000	1 109 000
75	2	1 410 000	1 163 000	1 008 000	1 144 000
50	2	1 672 000	1 439 000	1 255 000	1 404 000
25	2	2 048 000	2 007 000	1 528 000	1 607 000

Conditions as in Fig. 3.

peak-broadening effects in CE are fulfilled: the plate number is independent of capillary inner diameter and increases with increase in field strength if secondary convection effects are negligible. Also, the number of plates is proportional to column length at constant field strength. It should be recalled that the variance contribution of the injection plug was identical in all cases.

On the other hand, when the same capillaries are used but the injection time is kept constant, of course in the 25-μm capillary only 11% of the amount is injected in comparison with the 75-μm capillary. As can be seen in the left-hand part of Fig. 3 and in Table 1, an increase in efficiency was observed when the capillary inner diameter was reduced from 75 to 25 μm. This is caused by the concomitant decrease in the amount of sample injected and the plug length (variance of injection volume). In comparing the two separations with the 25-μm capillaries, it can be seen that the efficiency can be varied by a factor of 1.5–2 just by changing the amount of sample injected. Of course, the plate number measured summarizes all contributions to peak broadening. Because of the extremely small volumes handled in CE, one should be very cautious about the contribution of dispersive effects other than the electromigration process. It should be stressed again that it is possible to compare and discuss efficiencies only when all parameters that contribute to peak broadening are kept constant. Because of the additional contributions of heat dissipation and the injection techniques of the various instruments (e.g., pressure profile in hydrodynamic injection), comparison of efficiencies of different capillaries can only be valid with the same instrument.

3.2. Overloading effects with hydrodynamic injection

So far either the plug length or the mass of the sample loaded to the capillary was kept constant. In Fig. 4, the influence of the injected sample concentration at constant plug length on efficiency is shown. As can be seen, the efficiency decreases tremendously when the mass of sample increases. No linear region as in chromatography can be observed. Therefore, the efficiency achievable cannot be a measure for capillary characterization. It should be mentioned that the sample was dissolved in water, so that the injected plug length is less important owing to the stacking effect that occurs. The observed triangular peaks at sample concentrations above 100 ppm indicate that the capacity of the 50 mM buffer is insufficient for these sample loads. On the other hand, the lower the sample concentrations the more efficient the sample stacking effect becomes. As can be seen, decreasing the sample concentration from 200 to 20 ppm results in an improvement in plate numbers by a factor of three.

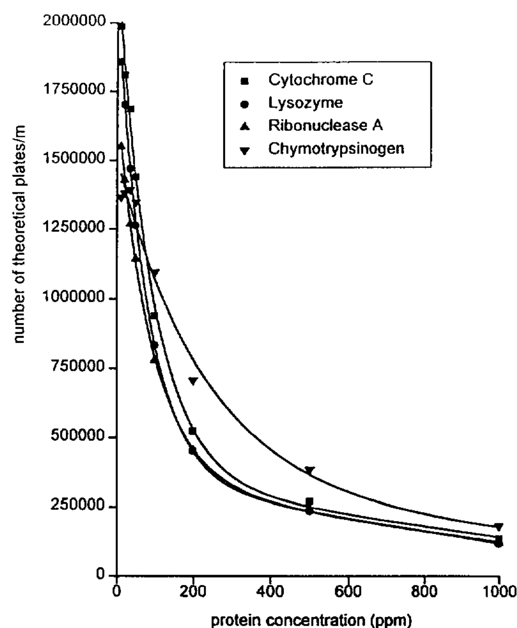

Fig. 4. Dependence of the number of theoretical plates on the protein concentration with a constant injection plug length. Conditions: capillary, polyacrylamide–vinyltrichlorosilane, 20/27 cm × 25 μm I.D.; buffer, 50 mM phosphate (pH 3); field strength, 260 V/cm; proteins dissolved in water; injection, 18 s pressure [$3.45 \cdot 10^3$ Pa (0.5 p.s.i.)].

When the protein sample is injected from water, volume overloading does not contribute to a certain extent to peak broadening because the sample stacking effect concentrates the sample at the borderline of the injection solvent and

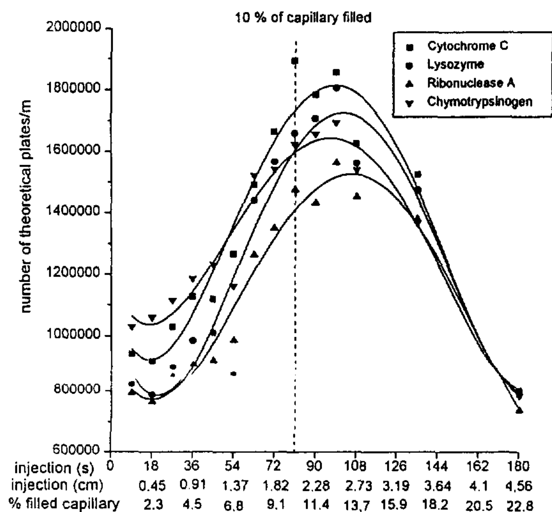

Fig. 5. Dependence of the number of theoretical plates on the injection plug length with constant injected sample mass. Conditions: capillary, polyacrylamide–vinyltrichlorosilane, 20/27 cm × 25 μm I.D.; buffer, 50 mM phosphate (pH 3); field strength, 260 V/cm; proteins dissolved in water; injection, pressure [$3.45 \cdot 10^3$ Pa (0.5 p.s.i.)], identical sample amount injected (18 s at 100 ppm = 180 s, at 10 ppm = 224 pg).

the buffer. As can be seen from Fig. 5, the plate number increases by a factor of around two when the plug length is increased from 0.45 to 2.73 cm and the mass of sample injected is kept constant. It should be mentioned that here 12% of the effective capillary length is filled with the sample solution. The increase in plate number is, of course, due to the lower sample concentrations injected (constant mass loaded), which improves the stacking effect. Only if the sample plug exceeds 12% of the capillary length is the volume variance no longer negligible and the plate numbers decrease again. The longer the part of the capillary filled with sample solution (water), the more the migration times increase because the field strength decreases mostly over the water plug [16].

The advantages of the stacking procedure are more clearly seen in Fig. 6, where this technique is compared with the conventional method, in which the sample dissolved in the separation buffer is injected. Comparable efficiencies are achieved only with the smallest plug length

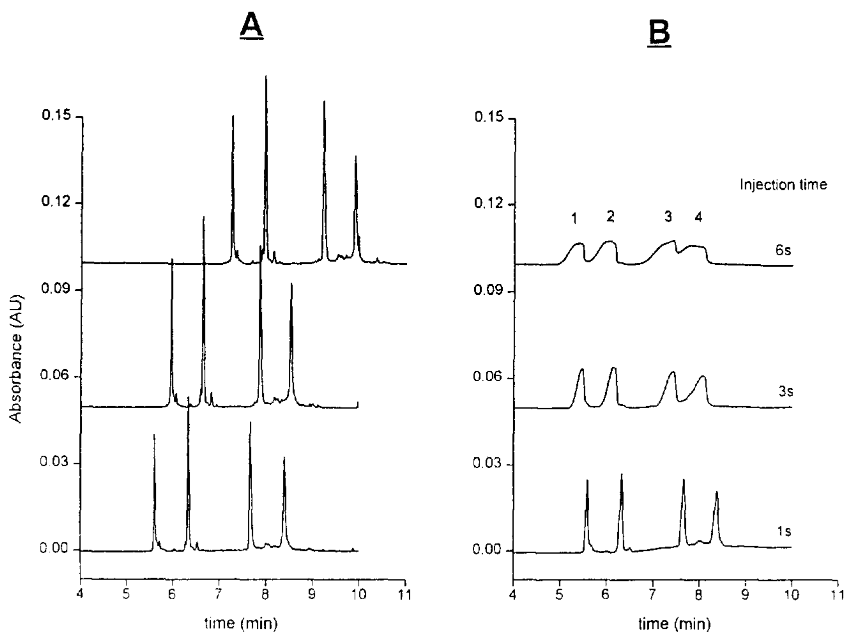

Fig. 6. Effect of injection plug length on peak performance for proteins from (A) aqueous solution or (B) separation buffer. Conditions: capillary, polyacrylamide–vinyltrichlorosilane, 20/27 cm × 75 μm I.D.; buffer, 50 mM phosphate (pH 3); field strength, 260 V/cm; samples, 1 = cytochrome c, 2 = lysozyme, 3 = ribonuclease A, 4 = chymotrypsinogen; injection, pressure [$3.45 \cdot 10^3$ Pa (0.5 p.s.i.)], identical sample amount injected (1 s at 200 ppm = 6 s, at 33.3 ppm = 224 pg).

injected (injection time 1 s). When longer plugs of the sample in buffer solution are injected (injection time >2 s), efficient and narrow peaks can no longer be observed. The peak width increases in proportion to the injection time because here the stacking effect is not working. On the other hand, with sample injection from water, highly efficient separations are observed even with an injection time of 6 s.

Stacking effects also play an important role in the case of mass overloading. However, when injected from buffer, generally fewer plate numbers are obtained and the efficiency is not greatly affected by increasing the sample concentration (constant plug length) with 50 m*M* phosphate buffer. However, the total plate number is only around 100 000 compared with 900 000 when the same amount (200 ppm) is injected from aqueous solution. Here one dilemma of CE becomes obvious: plate numbers are only defined when the separation conditions are kept constant, i.e., temperature, field strength and buffer composition. The use of the stacking procedure in combination with comparison of plate numbers is a mistake similar to that which one can make in calculating plate numbers in gradient elution. On the other hand, to achieve efficient and reproducible separations, the injection time cannot be chosen too short, and therefore stacking procedures are required for efficient protein separations. Only with very short injection times from buffer solutions is comparison of capillary efficiencies possible and permitted.

3.3. Electrokinetic injection

The most efficient injection technique for proteins in order to achieve high plate numbers is the electrokinetic injection technique. In Fig. 7 and Table 2 the electrokinetic injection technique from water and from buffer solution is compared with the already described hydrodynamic injection techniques. In the case of electrokinetic injection there is almost no difference between injection from buffer and water solutions. However, at a constant injection time a smaller amount is injected from the buffer solution, because the buffer components also

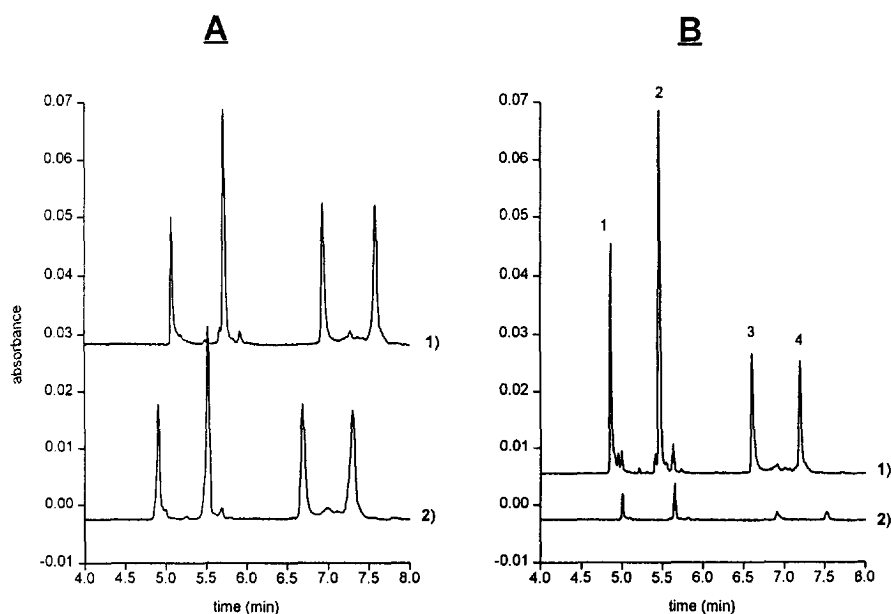

Fig. 7. Dependence of protein separation on injection technique. Conditions: capillary, polyacrylamide–vinyltrichlorosilane, 20/27 cm × 75 μm I.D.; buffer, 50 m*M* phosphate (pH 3); field strength, 260 V/cm; samples, 1 = cytochrome *c*, 2 = lysozyme, 3 = ribonuclease A, 4 = chymotrypsinogen; injection, (A1) pressure [$3.45 \cdot 10^3$ Pa (0.5 p.s.i.)], 1 s, samples in water; (A2) pressure (0.5 p.s.i.), 1 s, samples in buffer; (B1) 1 kV, 5 s, samples in water; (B2) 1 kV, 5 s, samples in buffer.

Table 2
Influence of the injection technique on the number of theoretical plates per metre

Solvent	Injection	Cytochrome *c*	Lysozyme	Ribonuclease A	Chymotrypsinogen
Water	1 s, pressure	977 000	782 000	792 000	784 000
Buffer	1 s, pressure	463 000	436 000	382 000	329 000
Water	5 s, 1 kV	1 350 000	979 000	1 180 000	1 315 000
Buffer	5 s, 1 kV	1 437 000	1 309 000	750 000	982 000

Conditions as in Fig. 7.

migrate into the capillary. As no electroosmotic flow was present in the capillary used, the discrimination of sample components according their mobility differences can clearly be seen by comparing the relative peak heights obtained with hydrodynamic and electrokinetic injection from the same solutions. Although from aqueous solution higher sample amounts are introduced in the capillary, the highest efficiencies are always obtained with the electrokinetic injection techniques. This can be explained by the plug-shaped electromigration of the sample molecules into the capillary.

3.4. How to achieve record plate numbers

In chromatography, there is a linear range within which the plate number is independent of sample size. In linear chromatography one usually works in this region. Only when this region is exceeded do plate numbers decrease with increasing sample size. In CE, however, there is no such region in the case of injection from water. Only when proteins are injected from buffer solutions is a linear region obtained, but the plate numbers achievable are much lower, only around 100 000–200 000. With the same capillary, plate numbers up to $3 \cdot 10^6$ can be achieved just by optimization of the sample injection techniques. Such a highly efficient separation of standard proteins is shown in Fig. 8. The same samples were used as in all demonstrations in this paper. Hence it is obvious that it is possible to achieve plate numbers predicted by theory just by optimization of injection techniques. It should be mentioned, however, that for real protein samples the application of the different injection techniques is limited. Therefore, it is hardly possible in the real world to achieve the plate numbers predicted by theory.

To obtain high plate numbers, the sample has to be injected elektrokinetically either from buffer or from water, or hydrodynamically from water with low sample concentrations and applying sample stacking techniques. Here either extremely short injection times should be used or the capillary should be filled up to 10% of its effective length with a very dilute aqueous sample solution. Generally the sample amount injected should be as low as possible. For reproducibility reasons it is better to inject a dilute solution for a longer time than a more concentrated solution for a relatively short period.

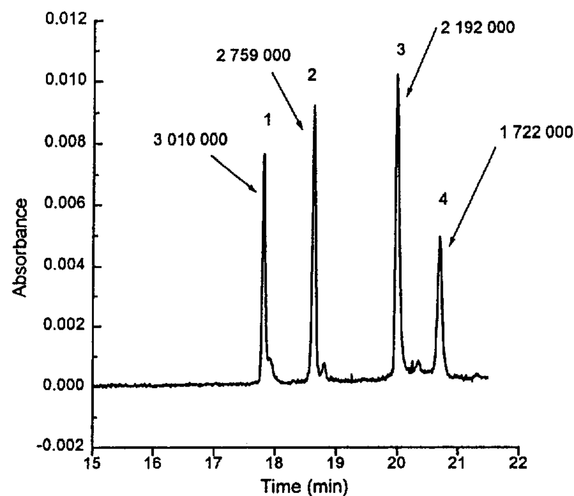

Fig. 8. Separation of basic standard proteins under optimized conditions. Conditions: capillary, polyacrylamide–vinyltrichlorosilane, 20/27 cm × 50 μm, I.D. buffer, 50 m*M* phosphate (pH 3); field strength, 260 V/cm; samples, 1 = cytochrome *c*, 2 = lysozyme, 3 = ribonuclease A, 4 = chymotrypsinogen (10 ppm each in water); injection, 33.8 s, pressure [$3.45 \cdot 10^3$ Pa (0.5 p.s.i.)].

Additionally, narrow capillaries (I.D. < 50 μm) have the advantage that higher field strengths can be used and zone dispersion by heat dissipation effects is negligible.

Consequently, plate numbers for proteins are not a good measure for comparing the efficacy of surface coatings in CE, and should be used only when all measurements are made under identical conditions with the same instrument.

References

[1] J.A. Taylor and E.S. Yeung, Anal. Chem., 65 (1993) 2928–2932.

[2] G.I. Taylor, Proc. R. Soc. London, Ser. A, 219 (1953) 196.

[3] R. Aris, Proc. R. Soc. London, Ser. A, 235 (1956) 67.

[4] M.J.E. Golay, in D.H. Desty (Editor), Gas Chromatography, Proceedings of 2nd Symposium (Amsterdam), Academic Press, New York, 1958.

[5] J.J. van Deemter, F.J. Zuiderweg and A. Klinkenberg, Chem. Eng. Sci., 5 (1956) 271.

[6] J.C. Giddings, Unified Separation Science, Wiley, New York, 1991.

[7] J.W. Jorgenson and K.D. Lukacs, Anal. Chem., 53 (1981) 1298–1302.

[8] A. Guttman, A.S. Cohen, D.N. Heiger and B.L. Karger, Anal. Chem., 62, (1990) 137.

[9] S. Terabe, K. Otsuka and T. Ando, Anal. Chem., 61 (1989) 251–260.

[10] J.H. Knox and K.A. McCormack, Chromatographia, 38 (1994) 207–221.

[11] S.L. Petersen and N.E. Ballou, Anal. Chem., 64 (1992) 1676–1681.

[12] E. Grushka, R.M. McCormick and J.J. Kirkland, Anal. Chem., 61 (1989) 241–246.

[13] J. Liu, V. Dolnik, Y.Z. Hsieh and M. Novotny, Anal. Chem., 64 (1992) 1328–1336.

[14] F.E.P. Mikkers, F.M. Everaerts and Th. P.E.M. Verheggen, J. Chromatogr., 169, (1979) 1–10.

[15] H. Engelhardt and M.A. Cuñat-Walter, J. Chromatogr. A, 716 (1995) 27.

[16] H.W. Zhang, X.G. Chen and Z.D. Hu, J. Chromatogr. A, 677 (1994) 159–167.