*Journal of Chromatography*, 638 (1993) 187–193
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 863

# Experimental aspects of capillary isoelectric focusing with electroosmotic zone displacement

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ABSTRACT

The investigation of different parameters affecting separation and resolution in capillary isoelectric focusing (CIEF) with electroosmotic zone displacement is reported. Focusing is performed in an uncoated, open-tubular fused-silica capillary of length 50–100 cm and 75 $\mu$m I.D. An experiment proceeds by first filling the entire capillary with the catholyte containing the neutral polymer(s). Sample composed of carrier ampholytes and proteins is introduced at the anodic capillary end, the initial zone length being 10–50% of the effective capillary length. After application of power, two electrokinetic effects occur concurrently, the formation of a longitudinal pH gradient with the separation of proteins (isoelectric focusing) and, owing to the negative surface charge of untreated fused silica, the displacement of the entire zone pattern towards the cathode (electroosmosis). Basic proteins reach the detector prior to neutral and acidic compounds. The concentrations of the polymers, different protein solubilizing additives, carrier ampholytes, anolyte and catholyte, and also the initial sample zone length, the applied voltage, the capillary length and the rinsing procedures are shown to affect focusing in CIEF. A knowledge of the effects of these parameters leads to optimization of protein separation and resolution and provides an insight into the pros and cons of this method.

## INTRODUCTION

In the past few years increasing attention has been paid to protein isoelectric focusing (IEF) in capillaries with the ultimate aim of developing a fully instrumental approach to this high resolution method. First, free fluid focusing was studied in capillaries of rectangular cross-sections [1–5], in glass [6–8] or PTFE capillaries [9,10] of 200–500 $\mu$m I.D., and also in coated, open-tubular fused-silica capillaries of very small I.D. [11–16]. These approaches operated with minimized electroosmosis in which stationary steady-state zone patterns were established. Zones were detected either by the use of array detection or by visual inspection [1–5], and also by UV absorption measurement [6–15] or concentration gradient monitoring [16] towards one column end which required that after focusing the proteins had to be mobilized and swept past a stationary detector. Essentially two approaches for mobilization were studied. The first method consisted of the use of hydrodynamic flow which was applied after focusing was attained and without interruption of the current flow [6]. Second, electrophoretic mobilization was achieved through power interruption after focusing and replacement of one of the two electrode buffers prior to reapplication of current [6–15].

Recently, Mazzeo and Krull [17,18] and Thormann *et al.* [19] independently described two similar isoelectric focusing methods with electroosmotic zone displacement. In these studies it was discovered that small amounts of a neutral polymer (hydroxypropylmethylcellulose or methylcellulose) added to the buffer allowed IEF of proteins to be performed in untreated, open-tubular fused-silica capillaries, *i.e.*, in the presence of an electroosmotic flow along the separation axis. The added polymer provides a dynamic coating of the capillary surface which reduced

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0021-9673/93/$06.00 

both the protein–wall interactions and the electroosmotic flow. This, and also the plug flow characteristics of electroosmosis, were important prerequisites for low sample dispersion and therefore for efficient focusing. Electroosmotic zone displacement was shown to make mobilization after focusing obsolete. Mazzeo and Krull [17,18] described a configuration in which the column was initially completely filled with the sample, this requiring the further addition of a strong base to the sample in order to be able to detect basic proteins [9,14]. In our experiments a relatively short initial sample zone was employed, the remainder of the capillary being filled with catholyte. After power application the electroosmotic flow gradually displaced the developing zone pattern towards and eventually across the point of detection, making the addition of the strong base unnecessary. Further, fast-scanning multi-wavelength detection was shown to permit the simultaneous monitoring of proteins and carrier ampholytes, and the temporal behaviour of the current was found to provide information on the degree of focusing prior to sample detection at a specified location towards the capillary end [19].

In this paper, experimental data allowing the elucidation of factors affecting separation of proteins in our fully dynamic method of capillary isoelectric focusing (CIEF) are reported. Separation and resolution of model protein mixtures are shown to be dependent on instrumental variables, including applied power, capillary length, initial zone length and the capillary cleaning procedure, and on the chemical variables, such as the concentration of sample, polymer, carrier ampholytes, anolyte and catholyte.

## EXPERIMENTAL

### *Chemicals and sample preparation*

All chemicals were of analytical-reagent grade. Cytochrome *c* (CYTC) from horse heart ($M_r$ 12 384, p*I* 9.3), carbonic anhydrase (CA) from bovine erythrocytes ($M_r$ 31 000, p*I* 6.2), bovine serum transferrin (b-TF), iron free ($M_r$ 77 000, p*I* 5.2–5.8), human serum transferrin (h-TF), iron saturated ($M_r$ 79 570, p*I* 5–6), hydroxypropylmethylcellulose (HPMC) and methylcellulose (MC) with a viscosity of 4000 cP each (2% aqueous solution at 25°C) were obtained from Sigma (St. Louis, MO, USA), equine myoglobin (MYO) from skeletal muscle ($M_r$ 17 800, p*I* 6.8–7.3) from Serva (Heidelberg, Germany) and ampholines (pH 3.5–10, 4–6, 5–7 and 7–9) from Pharmacia–LKB (Bromma, Sweden). Proteins were dissolved in Ampholine solution at specified concentrations. Unless stated otherwise, HPMC and/or MC were added to the catholyte only.

### *Instrumentation*

Two different instruments were employed. Most of the experiments were performed on an ABI 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA). Typically, this instrument was equipped with a fused-silica capillary of 75 $\mu$m I.D. and effective and total separation lengths of 50 and 70 cm, respectively (Polymicro Technologies, Phoenix, AZ, USA). Electropherograms were recorded using a Model D-2000 chromato-integrator (Merck–Hitachi, Darmstadt, Germany). An automated ABI 270A-HT capillary electrophoresis system (Applied Biosystems), a similar instrument to that described above but featuring a larger autosampler, was also used. The capillary employed had effective and total lengths of 52 and 73.5 cm, respectively, and I.D. 75 $\mu$m (Polymicro Technologies). Data collection and evaluation were performed with a data acquisition system consisting of a PC Integration Pack (version 2.50) (Kontron, Zurich, Switzerland) together with a Mandax AT 286 computer system. This integration pack features automatic range switching and a dynamic sampling rate, allowing a data sampling rate of up to 100 Hz for quickly changing signals.

### *Standard running conditions*

Unless stated otherwise, 20 m*M* NaOH containing 0.06% (w/v) HPMC and 10 m*M* $H_3PO_4$ were used as catholyte and anolyte, respectively. Using the ABI 270A instrument, the applied voltage was 10 kV (initial currents 25–30 $\mu$A; minimum currents 1–2 $\mu$A; for explanations, see ref. 19) and the sample loading time (with

vacuum) was 20 s, providing an initial sample zone length covering about 20% of the effective column length. Ampholine was used at a concentration of 2.5% and the concentration of the proteins was about 0.16 mg/ml. Electropherograms were recorded at 200 nm (carrier ampholytes) and 280 nm (proteins) using the Model D-2000 chromato-integrator. Between runs, capillaries were rinsed with catholyte for 10 min.

## RESULTS AND DISCUSSION

A CIEF experiment is commenced with a sample zone at the anodic end covering typically <50% of the effective capillary length, the remainder being filled with catholyte. On application of power, isoelectric focusing is performed in an electroosmotic stream flowing towards the cathode. For the investigation of a range of parameters, model protein mixtures composed of CYTC, MYO and b-TF or CYTC, MYO and CA were used. Unless stated otherwise, experiments were performed in a 50-cm column (effective length) using the ABI 270A instrument, which features vacuum injection [20 and 5 in.Hg (1 in.Hg = 3386.4 Pa) for rinsing and sample loading, respectively]. Parameters examined on each electropherogram included protein separation, resolution, elution time and absorbance at 280 nm.

As reported previously [19], CIEF in a configuration without the addition of a polymer to the catholyte was found to be irreproducible. Consequently, HPMC concentrations of 0.15, 0.1, 0.06, 0.03 and 0.015% (w/v) were investigated in order to find the optimum conditions. The separation of CYTC, MYO and b-TF was fairly good in all instances. However, the HPMC concentration was found to affect the resolution of specific proteins (Fig. 1). The best results were obtained with 0.06 and 0.1% of HPMC. Further, conditioning of the capillary prior to sample application with catholyte was established to be essential for the performance of dynamic CIEF. Addition of the polymer to the sample was found to improve the separation of proteins or isoforms with close p*I* values (see below), whereas no changes in resolution were observed with HPMC in the anolyte (data not

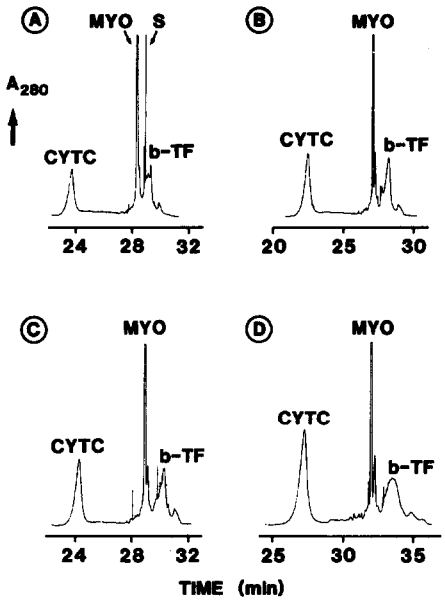


Fig. 1. Effect of HPMC concentration in the catholyte. CIEF of CYTC, MYO and b-TF with (A) 0.15, (B) 0.10, (C) 0.06 and (D) 0.03% of HPMC is shown under otherwise standard conditions using the ABI 270A instrument. The trace denoted by S is believed to be a spike originating from a particle (protein precipitate) that is transported through the detection cell.

shown). It appears that the loaded sample is indirectly influenced by the polymer concentration. With vacuum injection at a specified time interval it was observed that the lower the polymer concentration was, the more sample became introduced. The electropherograms shown in Fig. 1 clearly show this effect, particularly when the CYTC peaks are compared. With a longer initial sample zone less NaOH is present in the capillary at the beginning of the experiment, causing a smaller net electroosmotic flow and thereby increased retention times (see below).

MC at concentrations of 0.15, 0.1, 0.06 and 0.03% (w/v) were also investigated (data not shown). Compared with HPMC and in agreement with the method of Mazzeo and Krull [17,18], no significant differences were observed with regard to electroosmotic flow and protein interaction with the capillary walls. However, lower MC concentrations provided better resolution of the isoforms, particularly for b-TF. This effect could be attributed to a specific

protein–polymer interaction which differs from protein to protein. Good results were also obtained when a mixture of HPMC and MC at low concentrations (typically HPMC 0.03% and MC 0.015%) was used. Finally, an insignificant improvement of separation was noted with the addition of urea (1 *M*), glycine or glycylglycine [1% (w/v) each] to the sample (data not shown).

The effects of anolyte and catholyte concentrations were first analysed by keeping one solution at a constant concentration and varying the concentration of the other. CYTC, MYO and CA were used as sample proteins and MC (0.03%) was employed as the polymer in the catholyte. Variation of NaOH concentration was found to have a considerable effect on retention times, *i.e.*, on the electroosmotic flow. Retention became longer with increased catholyte concentration, permitting an improvement of the separation of certain isoforms. It was interesting that modification of the anolyte ($H_3PO_4$) concentration had an opposite effect, *i.e.*, a higher concentration led to shorter retention times. On increasing both the anolyte and catholyte concentrations while maintaining their ratio at $NaOH:H_3PO_4 = 2$, the retention times became larger. Typical electropherograms are presented in Fig. 2. It can be concluded that the catholyte (NaOH) essentially dominates the generation of the electroosmotic flow, thereby acting as the pump for mobilization and permitting the control of elution by selection of its concentration.

The concentration of the carrier ampholytes represents another parameter to be considered. In our investigation, Ampholine (pH 3.5–10) concentrations of 1, 2.5 and 5% (w/v) were employed. Under the chosen conditions with CYTC, MYO and b-TF as sample proteins and vacuum injection, 5% Ampholine was observed to provide faster elution than 2.5% carrier ampholyte. Under otherwise standard conditions, the elution time intervals of the three proteins were 18.4, 21.4 and 21.9 min (5%) and 24.1, 28.9 and 30.2 min (2.5%), respectively. Moreover, with 1% Ampholine, mobilization was even slower. In that case and with an applied voltage of 25 kV, CYTC eluted after 24.5 min and b-TF did not reach the detector within 45 min (data not shown). Again, it is assumed that with higher ampholyte concentrations a shorter initial sample zone length is obtained, thereby allowing for a longer NaOH zone, which induces increased electroosmotic pumping. The data depicted in Fig. 3 illustrate that, in addition to all the parameters mentioned above, pH gradient adjustment has to be employed to obtain the highest resolution of isoforms. For CIEF of b-TF and h-TF, a pH gradient ranging from pH 4 to 9 was found to resolve isoforms almost completely, a state which was not achieved with the broad

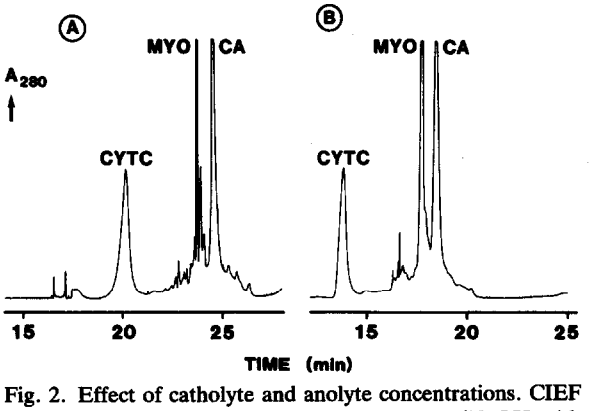


Fig. 2. Effect of catholyte and anolyte concentrations. CIEF of CYTC, MYO and CA having a catholyte (NaOH with 0.03% MC) and anolyte ($H_3PO_4$) of (A) 20 and 20 m*M* and (B) 10 and 5 m*M*, respectively, and using the ABI 270A instrument. All other parameters as given under standard conditions.

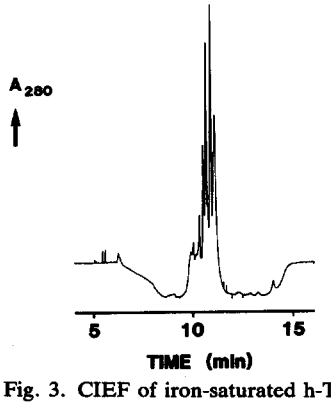


Fig. 3. CIEF of iron-saturated h-TF (0.4 mg/ml) employing the ABI 270A-HT instrument with 2.5% carrier ampholytes composed of Ampholine of pH 4–6, 5–7 and 7–9 (3:6:1, respectively), 0.03% MC in catholyte and sample, sample injection time 60 s (the initial sample zone length was about 50% of effective capillary length) and applied voltage 20 kV. All other parameters as given under standard conditions.

range (pH 3.5–10) preparation. The resolution shown in Fig. 3 is almost as good as that reported by Kilàr and Hjertén [11]. It is important to understand that the data in Fig. 3 were obtained with the addition of the polymer to both sample and catholyte.

With an ABI 270A instrument, most experiments were performed in capillaries of 70 cm total length (50 cm to the detector). In order to evaluate the effect of the electric power, experiments were executed with a constant voltage of 10, 15 or 20 kV. Not surprisingly, an increased voltage resulted in shorter retention times. While separation of the three test proteins was observed in all three instances, there was no resolution of isoforms at 20 kV (Fig. 4). Thus, with a given capillary length, achievement of the required resolution or speed of analysis depends on the proper selection of the ampholyte concentration and voltage. Further, for a given chemical configuration and at the expense of larger run times, longer capillaries were found to provide increased resolution. Investigations of the influence of the initial sample zone length on CIEF were executed via variation of the time interval of vacuum injection (10–30 s on the ABI 270A and up to 1 min on the ABI 270A-HT instrument with effective capillary lengths of 50 cm). Two major phenomena were observed (data not shown). First, with increased sample zone length a higher protein resolution is obtained. Second, the ampholyte front reaches the detector within a shorter time interval and the time required for detection of the entire gradient increases with longer sample zones. These effects are attributed to the increased sample zone length and a decreasing electroosmotic flow caused by the shorter part of the capillary filled with catholyte. Longer sample zones generate a flatter pH gradient so that the resolution of proteins with close p*I* values (isoforms) is increased.

Reproducibility was evaluated using CYTC, MYO and CA as test proteins. Typically for quadruplicates a relative standard deviation (R.S.D.) of 1.5–3% for retention times and 4–9% for peak areas was obtained (Fig. 5, Table

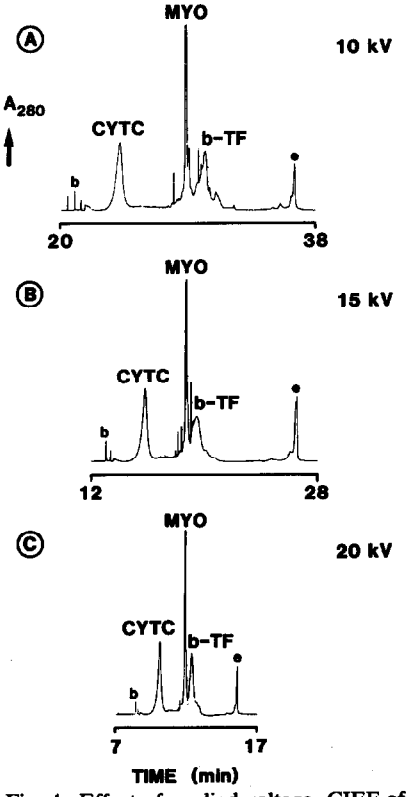


Fig. 4. Effect of applied voltage. CIEF of CYTC, MYO and b-TF using the ABI 270A at (A) 10, (B) 15 and (C) 20 kV and otherwise the specified standard conditions. The beginning and end of the detection of the carrier ampholytes are denoted by b and e, respectively.

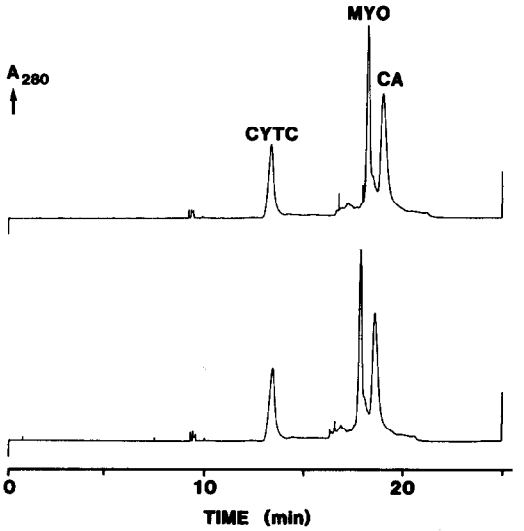


Fig. 5. Reproducibility. CIEF of CYTC, MYO and CA using the ABI 270A instrument and having 10 m*M* NaOH (with 0.03% MC) and 5 m*M* $H_3PO_4$ as catholyte and anolyte, respectively. All other parameters as given under standard conditions.

TABLE I

REPRODUCIBILITY DATA ($n = 4$) FOR TYPICAL CIEF RUNS

Data corresponding to the electropherograms shown in Fig. 5.

| Protein | Retention time | | Peak area | |
|---|---|---|---|---|
| | Mean (min) | R.S.D. (%) | Mean ($\mu$V s) | R.S.D. (%) |
| CYTC | 13.53 | 1.44 | 174 727 | 8.82 |
| MYO | 18.17 | 2.98 | 318 400 | 3.93 |
| CA | 18.89 | 2.97 | 350 545 | 5.44 |

I). In this approach, capillaries were first conditioned with catholyte (10 min). On working for longer periods with the same capillary it was observed that the conditioning of the capillary was not constant. In a series of ten experiments on the ABI 270A-HT instrument and using a similar protein mixture, a constant increase in retention time was observed, leading to poor reproducibility of both retention times and peak areas. Therefore, different conditioning procedures were investigated. Instead of rinsing the capillary for a long time with catholyte, the rinsing procedure was changed to a cleaning period with 0.1 or 1 *M* NaOH (destruction of conditioning) followed by a short dynamic conditioning with catholyte. The effect of an in-between rinse with distilled water was also evaluated. Typical data (CYTC peak only) for four different procedures are shown in Fig. 6. First, the capillary was rinsed with 0.1 *M* NaOH and catholyte for 3 and 10 min, respectively (Fig. 6A). Data obtained with sequential flushing using 0.1 *M* NaOH, water and catholyte (5 min each) are shown in Fig. 6B and those measured with a reversed sequence of water and 0.1 *M* NaOH in Fig. 6C. The fourth procedure (Fig. 6D) involved the use of 1 *M* NaOH for 1 min, followed by water and catholyte (5 min each). This approach clearly provided the best results with R.S.D. values of 2.7 and 4.0% for retention times and areas of CYTC, respectively. Corresponding values for procedures A, B and C were 4.6/17, 2.7/6.6 and 9.8/11.3%, respectively. Because of possible damage to the capillary with frequent use of 1 *M* NaOH, the conditions employed for Fig. 6B are recommended.

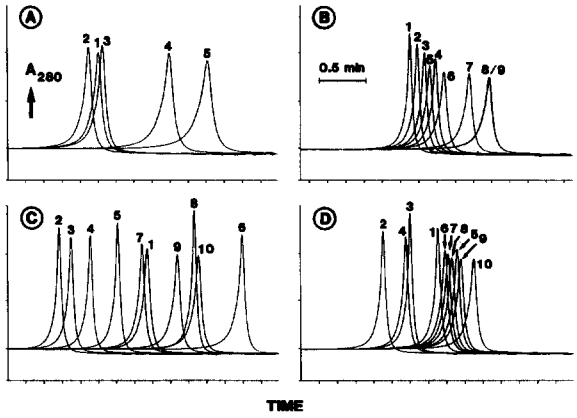


Fig. 6. Effect of capillary conditioning. CIEF of CYTC, MYO and CA (0.08 mg/ml each) employing the ABI 270-HT instrument with computerized data acquisition using the following rinsing procedures: (A) 0.1 *M* NaOH (3 min) and catholyte (10 min); (B) 0.1 *M* NaOH (5 min), water (5 min) and catholyte (5 min); (C) water (5 min), 0.1 *M* NaOH (5 min) and catholyte (5 min); and (D) 1 *M* NaOH (1 min), water (5 min) and catholyte (5 min). Sample injection time, 60 s (about 50% of the effective column length); applied voltage, 20 kV. Data for a 3-min time interval (CYTC peak only) are shown. The numbers in the four panels refer to the injections.

## CONCLUSIONS

In CIEF, a relatively short section of the capillary is occupied with the sample and isoelectric focusing is performed in one step, focusing and mobilization occurring simultaneously. Electroosmosis provides the flow for mobilization; hence in this technique this property plays a very central role. Generally, the electroosmotic flow is dependent on surface charge (pH), the electric field determined by current density and conductivity (composition of the solutions present in the capillary) and viscosity. In the described configuration, these three parameters are not constant during focusing. At any given time the sample is moving with a velocity defined as the mean of the electroosmotic velocities of the different longitudinally arranged fluid elements present. Its value changes with time, as (i) the catholyte is gradually leaving and the anolyte entering the column and (ii) the pH gradient is formed. As

the electroosmotic flow has a direct influence on retention, its control and reproducibility are of great importance, or else drastic changes can be observed from run to run. Optimized conditions for CIEF are shown to depend on a number of parameters, most notably the use of a neutral polymer in the catholyte, the concentrations of catholyte and carrier ampholytes, the applied voltage and capillary length, as well as the initial sample zone length.

The results show that different polymers (HPMC and MC) and combinations can be employed as additives to the catholyte. Further, higher resolution is obtained with the inclusion of the polymer in the sample solution whereas no improvement is achieved through introduction of the capillary conditioner to the anolyte. The catholyte (NaOH) is shown to be the major contributor to the electroosmotic flow. Variations of its concentration and that of the carrier ampholytes affect the resolution and migration time. Higher NaOH and/or lower carrier ampholyte concentrations increase the residence time and resolution. With the sample loading time (initial sample zone length) the steepness of the pH gradient can be manipulated, this having an effect on the resolution, efficiency of separations and the speed of analysis. From the instrumental side, the applied voltage and capillary length are the two parameters with which resolution and retention times are influenced. Increased voltage/capillary length are shown to result in shorter/longer retention times, configurations which are obtained at the expense/benefit of resolution, respectively. Finally, the rinsing procedure between runs is shown to have a strong effect on reproducibility. Etching of the capillary surface (with 1 *M* NaOH) followed by a short renewal of the dynamic coating is shown to provide data of the highest reproducibility.

The proteins used in this study are characterized by large differences in p*I*. Under the investigated conditions, they are generally well separated. The resolution of isoforms of proteins, however, is shown to require favourable conditions, which appear to be protein specific. Further work is needed to define the limit of resolution of components with very close p*I* values. Other topics of interest include improved control of electroosmosis (*e.g.*, with external electric fields [20,21]), the description of the complete separation and transport dynamics of CIEF and a careful comparison between the method described here and that of Mazzeo and Krull [17,18].

ACKNOWLEDGEMENTS

The authors acknowledge stimulating discussions with J. Chmelík. This work was partly sponsored by the Research Foundation of the University of Berne and the Swiss National Science Foundation.

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