ELSEVIER

Journal of Chromatography A, 979 (2002) 123–129

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Effect of feed zone width on product purity in preparative-scale, continuous free-flow isoelectric focusing separation of enantiomers

Ivan Spanik, Gyula Vigh*

Chemistry Department, Texas A&M University, College Station, TX 77842-3012, USA

Abstract

The effects of the increased width of the sample feed stream upon the purity of the collected fractions were examined in the continuous free-flow isoelectric focusing separation of the enantiomers of dansyl-tryptophan. Compared to the reference separation obtained with a narrow feed stream introduced through the central sample feed port of the continuous free-flow isoelectric focusing separation unit, the final pH gradient, the position of the enantiomer band centroids and the values of the cumulative product recoveries and cumulative product purities remained essentially identical as the width of the feed band of the racemic sample dissolved in the carrier ampholyte was increased up to the full width of the separation chamber suggesting that the current, limiting practice of narrow, central feed bands can be safely abandoned and dilute feedstock solutions can be utilized in preparative-scale isoelectric focusing enantiomer separations.

Keywords: Preparative electrophoresis; Isoelectric focusing; Enantiomer separation; Amino acids; Tryptophan

1. Introduction

Following the first description of the isoelectric focusing (IEF) separation of enantiomers with β-cyclodextrin in a carrier ampholyte-bearing polyacrylamide slab gel [1], enantiomer separations were also achieved by capillary IEF (cIEF) using both carrier ampholytes and Bier's buffers [2]. These cIEF enantiomer separations were quantitatively described with an equilibrium model that predicted the magnitude of the dynamically generated isoelectric point (pI) difference (ΔpI) between the centroids of the bands of the two enantiomers [2]:

$$\Delta pI_{,R,S} = \frac{1}{2}\log\left(\frac{1+K_{CDS^-}[CD]}{1+K_{CDR^-}[CD]} \cdot \frac{1+K_{CDRH_2^+}[CD]}{1+K_{CDSH_2^+}[CD]}\right) \quad (1)$$

where K_{CDR-}, K_{CDS-}, K_{CDRH2+}, and K_{CDSH2+} are the equilibrium constants for the formation of the anionic complexes (CDR^- and CDS^-) and the cationic complexes ($CDRH_2^+$ and $CDSH_2^+$) of the R and S enantiomers, and [CD] is the equilibrium concentration of the free, neutral cyclodextrin. Rizzi and Kremser [3,4] determined the equilibrium constants K_{CDR-}, K_{CDS-}, K_{CDRH2+}, and K_{CDSH2+} in Eq. (1) for a number of enantiomers by capillary electrophoresis. Recently, our group has used both a pressure-mediated capillary electrophoretic (Pre-MCE) method [5] and a full-column imaging capil-

*Corresponding author.
E-mail address: vigh@mail.chem.tamu.edu (G. Vigh).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01497-8

lary IEF method [6] to rapidly find the experimental conditions that lead to maximum ΔpI values.

In addition to its analytical utility, IEF is also attractive for the preparative-scale separation of enantiomers, because the isoelectric focusing mechanism can partially offset some of the instrumentation-related and flow-related band broadening effects (for a recent review see, e.g., Ref. [7]). Successful preparative-scale IEF enantiomer separations were achieved in a continuous free-flow electrophoretic system [2,6] and in an isoelectric membrane-based multicompartmental electrolyzer [8]. (For recent reviews of the continuous free-flow electrophoretic system and the isoelectric membrane-based multicompartmental electrolyzer see, e.g., Refs. [9,10], respectively.)

Based on the practice common in continuous free-flow electrophoretic separations, ampholytic enantiomers have been injected through the central sample feed port to obtain a narrow sample band, while the separation compartment was continuously supplied with either the same carrier ampholyte mixture through all of the background electrolyte inlet ports [6] or with different Bier's buffer compositions [11] fed into the adjacent background electrolyte inlet ports to preform the pH gradient [2]. When only dilute feedstock solutions are available, the use of a narrow injection band severely limits the production rates that can be achieved in continuous free-flow IEF enantiomer separations. The objective of the present paper was to investigate if sample injection through a single, central port could be replaced, without compromising product purity, with injection of the homogeneous analyte, chiral resolving agent and carrier ampholyte mixture across the entire width of the separation chamber.

2. Experimental

2.1. Chemicals

Chemicals for the preparation of the background electrolytes, both for the analytical and preparative-scale separations, were obtained from Sigma–Aldrich (Milwaukee, MI, USA) and included phosphoric acid, lithium hydroxide, triethanolamine, hydroxypropylmethylcellulose (HPMC, average molecular mass 86 000) and carrier ampholytes Pharmalyte, pH 2.5–5. The chiral resolving agent used for the preparative-scale separations, hydroxypropyl-β-CD (HP-β-CD) with a degree of substitution of 4.7, was purchased from Cerestar (Hammond, IN, USA), while the chiral resolving agent used for the analysis of the enantiomeric purity of the collected fractions, the sodium salt of heptakis(2-*O*-methyl-3,6-disulfo)-β-CD (HMediSuβCD), was synthesized and analytically characterized in our laboratory as described in Ref. [12]. The test analyte, the piperidinium salt of dansyl-tryptophan (Dns-Trp) was purchased from NBS Biological (Huntingdon, UK).

2.2. Background electrolytes

All solutions were freshly prepared using deionized water from a Milli-Q unit (Millipore, Milford, MA, USA). During the continuous free-flow IEF separations, 80 m*M* phosphoric acid was used as anolyte and 100 m*M* triethanolamine as catholyte.

Both solutions contained 0.2% HPMC. The background electrolyte for the preparative-scale IEF separations contained 4% Pharmalyte, p*I* 2.5–5 (carrier ampholyte), 60 m*M* HP-β-CD (chiral resolving agent) and 0.2% HPMC (electroosmotic flow controlling agent). The test sample, Dns-Trp, was dissolved in the background electrolyte. For the capillary electrophoretic (CE) analysis of the enantiomeric purity of the collected fractions, the background electrolyte was prepared by dissolving 8 m*M* HMediSu-CD in 25 m*M* H_3PO_4 that was titrated to pH 2.09 with LiOH.

2.3. Equipment

The continuous free-flow IEF separations were carried out with the Octopus unit (Dr. Weber, Kirchheim-Heimstetten, Germany). The instrument contained a pair of anolyte recirculating ports, a pair of catholyte recirculating ports, seven independently fed background electrolyte feed ports, a central sample feed port, and a counter-flow feed port [9]. Two independently operated membrane pumps circulated the anolyte and catholyte. Multichannel peristaltic pumps with adjustable delivery rates fed the background electrolyte, the sample solutions and the counterflow (deionized water). There were 96 sample

collection ports at the exit end of the separation chamber and provided a lateral resolution of about 1 mm/collection port. The separation chamber was 450-μm deep. The chamber was cooled through its bottom panel via a recirculated coolant that was thermostated at 10 °C.

The pH of the collected fractions was measured with a solid state microelectrode, pH16-SS, and a Model IQ240 pH meter (IQ, San Diego, CA, USA).

The volume of the collected fractions was measured by a gas-tight microsyringe (Hamilton, Reno, NV, USA). The collected samples were analyzed for enantiomeric purity by CE using a P/ACE 5510 unit (Beckman-Coulter, Fullerton, CA, USA) and an uncoated, fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 26 cm (injector-to-detector length 19 cm)×25 μm, and outer diameter of 150 μm. The applied potential was 10 kV, the cartridge coolant temperature was 25 °C, the UV detector was set at 214 nm. A five-point calibration curve was constructed for the quantitative analysis of the fractions using the time-normalized peak areas of the enantiomers.

3. Results and discussion

The separation conditions described in Ref. [6] for the continuous free-flow IEF separation of Dns-Trp enantiomers with a centrally injected, narrow sample band were used in this work as well. The residence time in the separation chamber was adjusted to 30 min, the applied potential was kept constant at 3 kV.

In order to establish a reference point, in the first experiment, the 1.5 m*M* Dns-Trp sample dissolved in the background electrolyte was fed through the central sample feed port as usual, and the seven background electrolyte feed ports were fed only with the background electrolyte. In the second to fifth experiments, the sample (dissolved in the background electrolyte) was fed through one or more of the background electrolyte feed ports. The concentration, width and position of the sample feed band was changed as indicated in Table 1. As the width of the feed band was increased, the concentration of Dns-Trp in the feed bands was lowered to provide comparable total sample loads. At these load levels, no precipitate formation was observed during any of the experiments.

Once the separation chamber was filled, the focusing potential was applied and the IEF separation was conducted for a period of 1 h to ascertain that steady state conditions had been achieved both in the separation chamber and in the fraction collection lines. Then, fractions were collected in a deep 96-well titer plate for half an hour. The pH of the collected solution in each well was measured immediately, followed by the measurement of the fraction volume which was used for the calculation of the material balance. (Adjacent fraction volumes varied due to variations in the resistance of the collecting lines. This effect causes ''spikes'' that can be observed in the bottom panels of Figs. 1–5.) Then, aliquots of the collected fractions were analyzed by CE to determine the enantiomeric purity in each fraction. Finally, using the calibration curves and the fraction volumes, the amounts of each enantiomer in each fraction were calculated, along with cumulative product recoveries and purities, as shown in Figs. 1–5. The cumulative values were calculated by pooling from the outward edges of the bands and proceeding toward the common boundary.

Fig. 1 indicates that in the reference run (central,

Table 1
Concentration of Dns-Trp (m*M*) in the background electrolyte fed into the respective background electrolyte feed ports of the Octopus unit

Expt.	Concentration of Dns-Trp (m*M*)						
	Port 1	Port 2	Port 3	Port 4	Port 5	Port 6	Port 7
1	0	0	0	0	0	0	0
2	0	0.5	0	0	0	0	0
3	0	0.25	0.25	0	0	0	0
4	0	0.125	0.125	0.125	0.125	0	0
5	0.0811	0.0811	0.0811	0.0811	0.0811	0.0811	0.0811

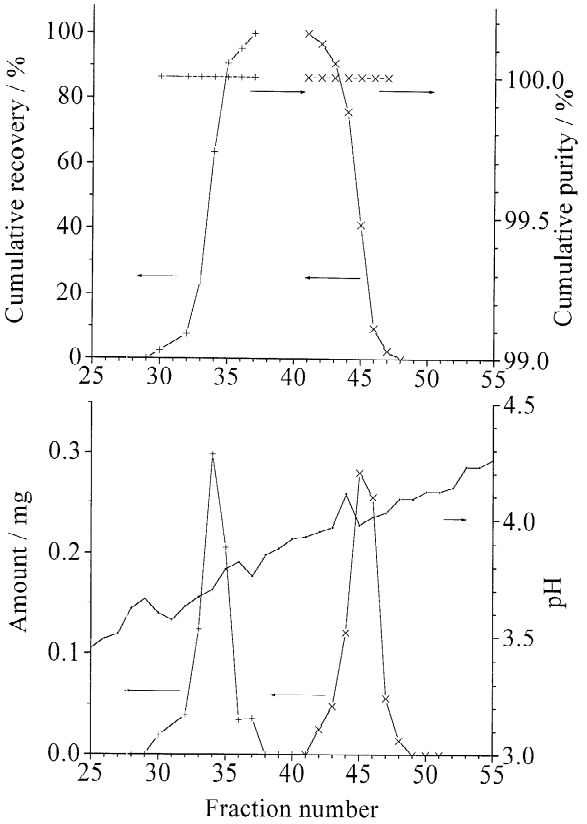

Fig. 1. Preparative-scale IEF separation of the enantiomers of Dns-Trp using a 4-mm wide injection band (Experiment 1). Bottom panel: left axis, amount of Dns-Trp enantiomers in the collected fractions; right axis, pH in the collected fractions. Top panel: left axis, calculated cumulative product recoveries; right axis, calculated cumulative product purities. Symbols: (+) lower p*I* enantiomer band; (×) higher p*I* enantiomer band. Background electrolyte: 0.2% HPMC, 60 m*M* HP-β-CD and 4% Pharmalyte, p*I* 2.5–5, carrier ampholyte. Residence time in the Octopus unit, 30 min; applied potential, 3000 V; sample injection, through the central sample feed port.

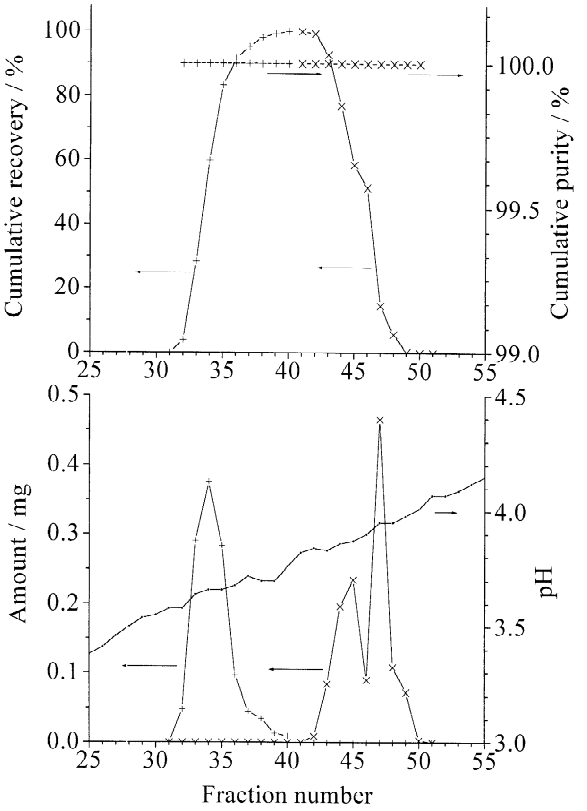

Fig. 2. Preparative-scale IEF separation of the enantiomers of Dns-Trp using a 13-mm wide injection band (Experiment 2). Bottom panel: left axis, amount of Dns-Trp enantiomers in the collected fractions; right axis, pH in the collected fractions. Top panel, left axis: calculated cumulative product recoveries. Top panel, right axis: calculated cumulative product purities. Symbols: (+) lower p*I* enantiomer band; (×) higher p*I* enantiomer band. Background electrolyte, 0.2% HPMC, 60 m*M* HP-β-CD and 4% Pharmalyte, p*I* 2.5–5, carrier ampholyte. Residence time in the Octopus unit, 30 min; applied potential, 3000 V; sample injection, through the second background electrolyte feed port.

narrow feed band, about 4-mm wide), the enantiomers of Dns-Trp are completely separated from each other.

The pH values of the fractions at the band centroids are about 3.7 and 4.0, corresponding to a ΔpI of 0.3, just as in Ref. [6]. The bands are separated by three fractions in which the concentration of both enantiomers is below the detection limits. Each enantiomer can be recovered at essentially 100% purity.

Fig. 2 shows the results for the second experiment wherein the sample was introduced through the second background electrolyte feed port as a 13-mm wide band. The pH values of the fractions at the band centroids are about 3.7 and 3.9, and the bands are separated by a single ''empty'' fraction in which the concentration of both enantiomers is below the detection limits. Each enantiomer can be recovered at essentially 100% purity.

Fig. 3 shows the results for the third experiment in which the sample was introduced through the second and third background electrolyte feed ports as an

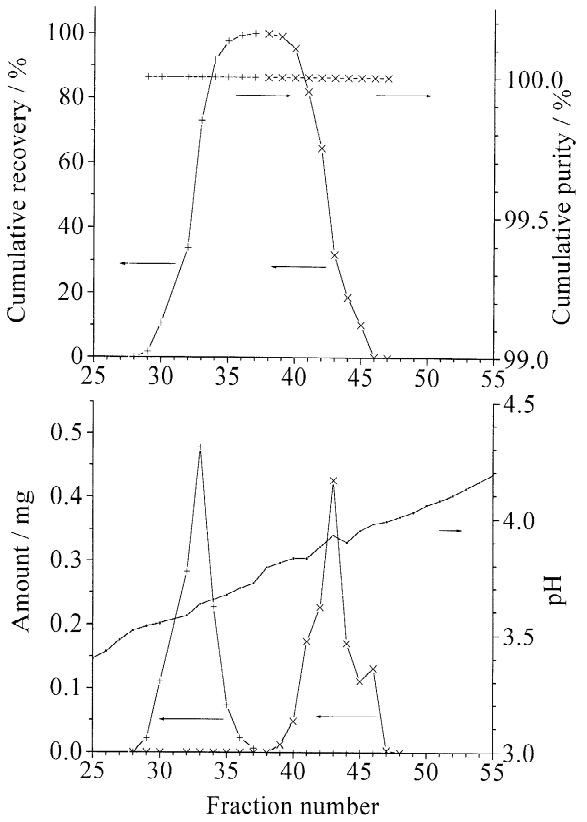

Fig. 3. Preparative-scale IEF separation of the enantiomers of Dns-Trp using a 25-mm wide injection band (Experiment 3). Bottom panel: left axis, amount of Dns-Trp enantiomers in the collected fractions; right axis, pH in the collected fractions. Top panel: left axis, calculated cumulative product recoveries; right axis, calculated cumulative product purities. Symbols: (+) lower p*I* enantiomer band; (×) higher p*I* enantiomer band. Background electrolyte: 0.2% HPMC, 60 m*M* HP-β-CD and 4% Pharmalyte, p*I* 2.5–5, carrier ampholyte. Residence time in the Octopus unit, 30 min; applied potential, 3000 V; sample injection, through the second and third background electrolyte feed ports.

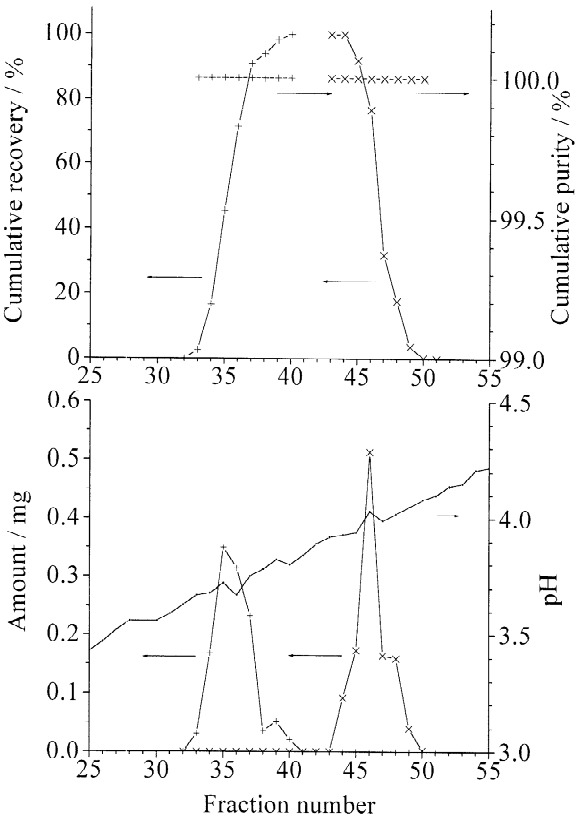

Fig. 4. Preparative-scale IEF separation of the enantiomers of Dns-Trp using a 58-mm wide injection band (Experiment 4). Bottom panel: left axis, amount of Dns-Trp enantiomers in the collected fractions; right axis, pH in the collected fractions. Top panel: left axis, calculated cumulative product recoveries; right axis, calculated cumulative product purities. Symbols: (+) lower p*I* enantiomer band; (×) higher p*I* enantiomer band. Background electrolyte: 0.2% HPMC, 60 m*M* HP-β-CD and 4% Pharmalyte, p*I* 2.5–5, carrier ampholyte. Residence time in the Octopus unit, 30 min; applied potential, 3000 V; sample injection, through the second, third, fourth and fifth background electrolyte feed ports.

approximately 25-mm wide band. The pH values of the fractions at the band centroids are about 3.6 and 3.9. There is still a single ''empty'' fraction between the bands: the concentration of both enantiomers is below the detection limits in this fraction. Thus, both enantiomers can still be recovered at essentially 100% purity.

Fig. 4 shows the results for the fourth experiment wherein the sample was introduced through the second, third, fourth and fifth background electrolyte feed ports as an approximately 58-mm wide band. (The chamber width covered by the adjacent background electrolyte inlet ports, as determined by the pre-separation injection of a red dye solution, varies slightly.) The pH values of the fractions at the band centroids are about 3.7 and 4.0. There are three fractions between the bands in which the concentration of both enantiomers is below the detection limits. Thus, despite the fact that the initial width of the sample stream is now half as wide as the entire separation chamber, both enantiomers can still be recovered at essentially 100% purity.

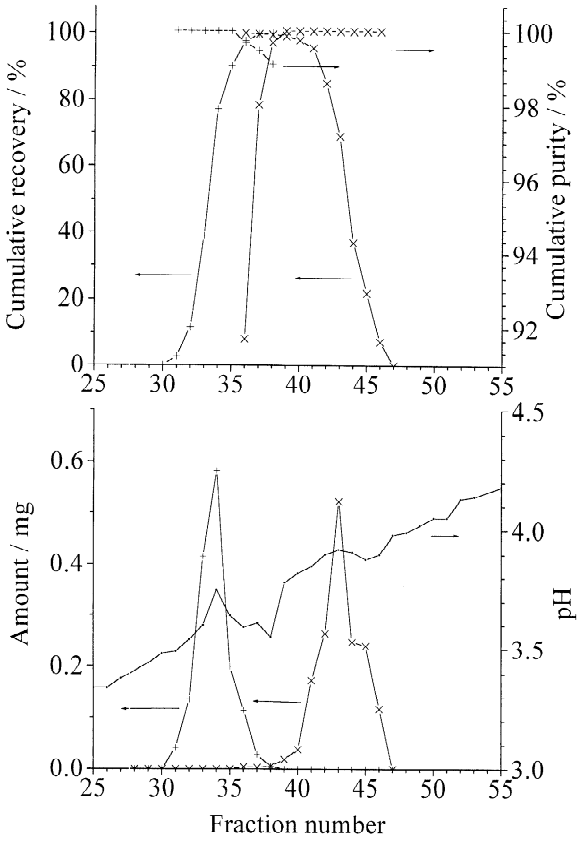

Fig. 5. Preparative-scale IEF separation of the enantiomers of Dns-Trp using a 100-mm wide injection band (Experiment 5). Bottom panel: left axis, amount of Dns-Trp enantiomers in the collected fractions; right axis, pH in the collected fractions. Top panel: left axis, calculated cumulative product recoveries; right axis, calculated cumulative product purities. Symbols: (+) lower p*I* enantiomer band; (×) higher p*I* enantiomer band. Background electrolyte: 0.2% HPMC, 60 m*M* HP-β-CD and 4% Pharmalyte, p*I* 2.5–5, carrier ampholyte. Residence time in the Octopus unit, 30 min; applied potential, 3000 V; sample injection, through the first, second, third, fourth, fifth, sixth and seventh background electrolyte feed ports.

Finally, Fig. 5 shows the results for the last experiment in which the sample was introduced through all seven background electrolyte feed ports, covering the entire width of the separation channel. The total sample load is about 10% higher than in the previous cases. The pH values of the fractions at the band centroids are once again about 3.7 and 3.9. However, the pH curve between the two enantiomer bands is not as smooth as in the previous cases indicating a minor experimental problem. Consequently, there are three fractions between the bands in which both enantiomers can be detected: this results in a 90 and 95% recovery for the more acidic and more basic enantiomer bands, respectively, at a purity of 100%.

4. Conclusions

Using Dns-Trp as a model substance, satisfactory preparative-scale IEF enantiomer separations have been achieved in the Octopus continuous free-flow electrophoretic unit, even when the width of the injected analyte band was increased from 4 to 100 mm, the full width of the separation compartment. These results show conclusively that the separation is brought about by a true isoelectric focusing mechanism, even though the p*I* difference of the enantiomer bands is generated dynamically by secondary chemical equilibria between the enantiomers and the chiral resolving agent, and focusing conditions for the chiral analyte are not as favorable as for proteins. The results should also dispel any lingering notion that the reported separation is brought about by a zone-electrophoretic separation mechanism that takes advantage of the narrow injection band width and the electrophoretic velocity difference of the two enantiomers (from a Discussion following the lecture by P. Glukhovskiy and Gy. Vigh, Analytical and Preparative-Scale Isoelectric Focusing Separations of Enantiomers, Frederick Symposium on Capillary Electrophoresis, Hood College, Frederick, MD, October 1999). Furthermore, with the ability to introduce the sample across the entire width of the separation channel, even dilute feedstock solutions can be processed at reasonable production rates and high enantiomeric purities.

Acknowledgements

The authors are indebted to Gradipore Pty (French's Forest, NSW, Australia) and the Texas A&M University Gradipore Chair in Separation Science Endowment for partial financial support of this project and to Beckman-Coulter (Fullerton, CA, USA) for the loan of the P/ACE unit.

References

[1] P.G. Righetti, C. Ettori, P. Chafey, J.P. Wahrmann, Electrophoresis 11 (1990) 1.
[2] P. Glukhovskiy, Gy. Vigh, Anal. Chem. 71 (1999) 3814.
[3] A.M. Rizzi, L. Kremser, Electrophoresis 20 (1999) 2715.
[4] A.M. Rizzi, L. Kremser, Electrophoresis 20 (1999) 3410.
[5] P. Glukhovskiy, Gy. Vigh, Electrophoresis 19 (1998) 3125.
[6] I. Spanik, P. Lim, Gy. Vigh, J. Chromatogr. A 960 (2002) 241.
[7] A. Rizzi, Electrophoresis 22 (2001) 3079.
[8] P. Glukhovskiy, T.A. Landers, Gy. Vigh, Electrophoresis 21 (2000) 762.
[9] G. Weber, J. Bauer, Electrophoresis 19 (1998) 1104.
[10] P.G. Righetti, A. Bossi, E. Wenisch, G. Orsini, J. Chromatogr. B 699 (1997) 105.
[11] M. Bier, J. Ostrem, R.B. Marques, Electrophoresis 14 (1993) 1011.
[12] D.K. Maynard, Gy. Vigh, Carbohydr. Res. 328 (2000) 277.