Arturo Araque
Jean Jaugey
Philippe Javet

Institute of Chemical Engineering, Swiss Federal Institute of Technology, Lausanne, Switzerland

Preparative isoelectric focusing and Joule effect: A purification cell that contains a heat exchanger

Reproducibility in protein purification by preparative isoelectric focusing depends greatly on temperature control during the separation process. A preparative apparatus is described, including a heat exchanger between compartments with isoelectric membranes. The selectivity of the isoelectric membranes was optimized as a function of isoelectric points of the separated proteins. At 2500 V and 60 W, 0.3 g of horse heart myoglobin from 0.2 g of whale skeletal muscle myoglobin could be separated in 1 h. At a total load of 2 g protein, 97% of bovine hemoglobin (2% initial concentration) was purified from bovine serum albumin (0.15%).

1 Introduction

Recent progress in the production of large quantities of biomolecules has revealed the importance of separation processes in biotechnology. Among them, preparative isoelectric focusing (PIEF), an isoelectric membrane separation process in our case, is a reliable technique for the purification of proteins, based on isoelectric points (p*I*). The method consists of focusing the biomolecules at their p*I* under conditions of a combined pH and potential gradient. Analytical IEF affords high resolution, attaining separations of proteins differing by 0.001 p*I* units [1]. Although the ultimate goal of PIEF is to obtain similar results, at present scaling up is limited by two factors: (i) the physicochemical properties of the membranes, and (ii) the difficulty in dissipating the heat produced by the Joule effect. The latter is detrimental for the separated proteins because of the risk of denaturation at temperatures higher than 40°C and other adverse phenomena such as oxidation or proteolysis, occurring already at temperatures as low as 4°C [2, 3]. In addition, Joule heating impairs the reproducibility of the method because temperature variations result in p*I* changes of the isoelectric membranes and proteins [1, 4].

Therefore, temperature control is essential for reproducible and safe purification with electrophoretical methods. Most PIEF devices, described in current literature, have an external cooling system for heat dissipations [5–10]. The PIEF cell described in this report encompasses an integrated heat exchanger inside the compartments. Jonsson and Rilbe [11] and Kyhse-Anderson [12] had suggested this type of approach before. Tarnopolsky *et al.* [13] addressed the problem differently, describing a combination of capillary and free flow electrophoresis.

Correspondence: Professor Philippe Javet, IGC-EPEL, CH-1015 Lausanne, Switzerland (Tel: +41-21-693-31-73; +41-21-693-36-71; Fax: +41-21-693-36-80)

Nonstandard abbreviations: PIEF, preparative isoelectric focusing; **pIa,** isoelectric point of acidic membrane; **pIb,** isoelectric point of basic membrane

Keywords: Electrophoresis / Preparative isoelectric focusing / Protein purification / Joule effect / Heat exchange

2 Materials and methods

2.1 The focusing cell

Our PIEF cell is a membrane module resembling a square filter press, composed of several plates made of Plexiglas. These hollow plates form 1.7 cm thick chambers, within which the separation occurs. Figure 1 shows the main elements of the module in a typical configuration. Each electrode consists of a metal plate inserted into a Plexiglas matrix with an electrical connection in the other side. The anode is a titanium plate with an iridium oxide coating (Ti/IrO_2), a so-called "dimensionally stable anode (DSA)." The cathode is a zirconium plate (Zr). The choice of these electrodes was based on their excellent properties: conductivity, high resistance to aggressive substances, ease of use and relatively low cost [14–16]. The electrodes have been omitted from Fig. 1 to simplify comprehension of the drawing; however, an indication of their position is given. Each chamber has four Plexiglas tubes (3 cm long, 5 mm ID, 7 mm OD, each) placed at a distance of 1 cm one over the other, constituting the internal heat exchanger of the cell. Cooling liquid flows through the tubes perpendicularly to both the electric field and the flow of solutions. The rectangular devices at the sides of the focusing module (detached from the module in Fig. 1) distribute the cooling liquid to the tubes, as indicated by the arrows. An added advantage of this cell is that the membranes are fastened without crushing, avoiding any possible leakage from the focusing compartments. Thus, the fragility of polyacrylamide gels, another limiting parameter for large-scale purification, does not represent a major problem. The surface area of the membranes corresponds to that of the electrodes, *i.e.*, 30 cm^2.

2.2 The whole device

Figure 2 shows the other elements of the PIEF device. A thermostatic bath (1) made of Plexiglas keeps the solutions at a low and constant temperature. It is composed of five Plexiglas cylindrical reservoirs (2), each with a capacity of 100 mL. The reservoirs have a bottom outlet connected to a Teflon tube (1 m long) of 3 mm ID (3). The thermostatic bath is constructed in such a way as to allow the solutions leaving the reservoirs to remain in the thermostatic bath while flowing through the Teflon tube. This procedure allows improved contact of the

0173-0835/96/0101-0168 $5.00+.25/0

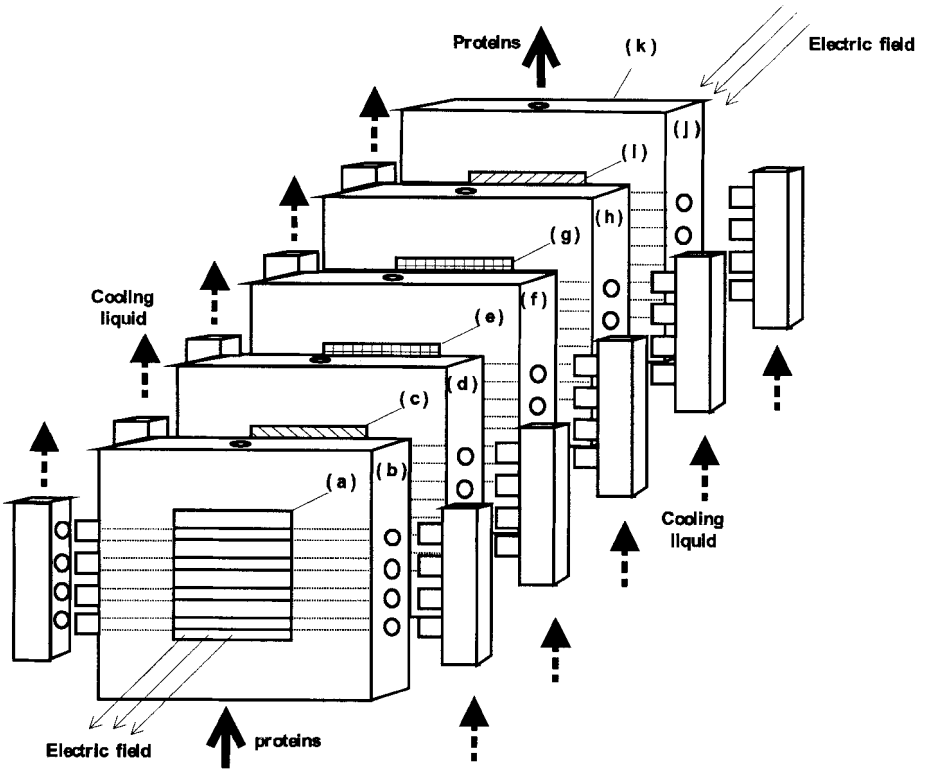

Figure 1. The PIEF cell with heat exchanger: (a) anode, (b) anolyte compartment, (c) anion exchange membrane, (d) acid compartment, (e) amphoteric membrane with acidic p*I*, (f) central compartment, (g) amphoteric membrane with basic p*I* (h) basic compartment, (i) cation exchange membrane, (j) catholyte compartment, and (k) cathode.

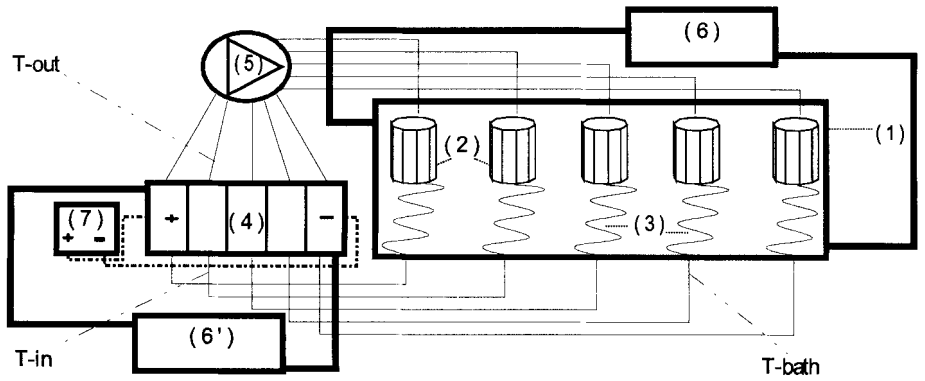

Figure 2. The whole PIEF device: (1) thermostatic bath, (2) Plexiglas reservoirs, (3) Teflon tubing, (4) the focusing cell, (5) peristaltic pump, (6 and 6′) cryogenic units, and (7) power supply.

solutions with a larger cold surface before they leave the bath on their way to the focusing cell (4). The peristaltic pump (5), placed between the focusing cell and the thermostatic bath, illustrates the clockwise flow direction of the solutions. Two cryogenic units furnish the cooling liquid. The first (6) supplies the thermostatic bath while the other one (6′) sends its cooling liquid directly to the focusing cell. A power supply (7) provides electric energy.

2.3 Membranes

The properties of isoelectric membranes have been improved with respect to such problems as electroendosmosis, swelling, selectivity and precision [10]. Poly-*N*-acryloylaminoethoxyethanol, a new polymer matrix grafted with Immobilines (Pharmacia Biotech, Uppsala, Sweden), offering a number of advantages, has recently been suggested by Chiari *et al.* [17]. The isoelectric membranes used for our experiments were 6% T and 4% C polyacrylamide matrices grafted with Immobilines. Righetti [18] has thoroughly characterized these membranes. An anion exchange membrane (QZR386) prevents the acid protein solution from mixing with the anolyte. A cation exchange membrane (CZR386) prevents any contact of the basic protein solution with the catholyte. Besides eliminating the risk of contamination with the electrolytes, these membranes (distributed by Ionics, Inc., Watertown, MA, USA) impede any possible reaction of the proteins at the electrodes [19].

2.4 Choice of a test system

The rapidity of separation at a given voltage depends mainly on the p*I* difference between the protein and the isoelectric membrane it has to cross [20]. Nevertheless, other parameters such as ionic mobility, molecular weight or pH play an important role. We chose a mixture of bovine hemoglobin (p*I* 6.8 at 25°C) and bovine serum albumin (p*I* 4.8 at 25°C) as a test system based on the facility of visual detection and overall knowledge of these two proteins [21]. Another test system consists of two myoglobins: horse heart (p*I* 6.87 and 7.33 at 25°C)

and whale skeletal muscle (p*I* 8.09 and 7.72 at 25°C). One should expect an increase of the protein's p*I* at the stationary run temperature (6°C) [22]. This has to be taken into acount prior to deciding which concentrations of Immobilines in the isoelectric membranes should be chosen (see Table 1 for details).

Table 1. Immobiline concentrations in the isoelectric membranes used for these experiments[a)]

Separation experiment	Immobiline p*K*	Concentration	Immobiline p*K*	Concentration	Membrane p*I*
BSA / hemoglobin	3.6	6.67 mM	7.0	7.90 mM	6.30, pIa
	3.6	6.67 mM	7.0	9.60 mM	6.70, pIb
Horse/whale myoglobins	4.6	6.67 mM	9.3	6.67 mM	6.95, pIa
	4.6	6.67 mM	9.3	7.33 mM	8.30, pIb

a) Membrane's p*I* estimated from p*K* values given by Pharmacia Biotech

2.5 Operation

Two isoelectric membranes were prepared for each experiment and placed according to increasing p*I* from the anode to the cathode. The protein or group of proteins of interest focus in the chamber where their p*I* corresponds to the pH range delimited by the isoelectric membranes enclosing that compartment. For the separation of bovine hemoglobin from bovine serum albumin (BSA), hemoglobin focused in the cathodic compartment, while BSA migrated toward the anodic compartment, both migrating from the central compartment. In the case of the two myoglobin separation, horse heart myoglobin focused in the central compartment, while whale skeletal muscle myoglobin was recovered in the cathodic compartment. All solutions are loaded in the reservoirs of the thermostatic bath: the electrolytes are at both extremes and the protein mixture is in one of the other three compartments, according to the purification strategy. The solutions leave the bath from the bottom and travel beneath the cell (Fig. 2). They climb through the PIEF module with a flow perpendicular to the electric field, proceeding back to the top of the reservoirs of the thermostatic bath after a short passage through the peristaltic pump.

2.6 Analysis

The temperature was measured by means of thermocouples placed as indicated in Fig. 2: at the entrance of each chamber (T-in), at the exit of each chamber (T-out) and at the exit of the thermostatic bath (T-bath). The p*I*s of proteins were determined with the Pharmacia Phast-System. Protein concentrations were measured by their absorbance at 280 nm and 405 nm using a Shimadzu UV-160 spectrophetometer.

3 Results

3.1 Efficiency of the heat exchanger

Several heat transfer phenomena occur during the heat dissipation process. Heat is produced when the electric current finds physical barriers in its way from the cathode to the anode (membranes and solutions). The heat passes from the bulk of the solutions to the Plexiglas walls by convection and is then transferred through the walls by thermal conduction until it reaches the internal wall of the Plexiglas tube where it is dissipated by convection into cooling liquid. The Joule effect P (watt) is described by:

$$P = \frac{1}{\chi}\frac{d}{S}I^2 \qquad (1)$$

where χ (ω^{-1} m^{-1}) is the conductivity of the solution, d (m) the compartment thickness, S (m^2) the compartment section, and I (A) the electric current.

Figure 3 is a plot of the average temperature of solutions leaving the cell (previously identified as T-out) from the different compartments. This figure facilitates the comparison of cell operation with and without circulation of cooling liquid through the tubes. The thermostatic bath was run during these experiments and constant operating conditions were maintained. Other measurements (not shown in Fig. 3) show that at a thermally stationary state, the solutions exhibit a temperature increase (from T-out to T-in) of approximately 15°C when no cooling liquid flows through the heat exchanger. With the circulation of cooling liquid a temperatures decrease of 1°C is observed and the thermal stationary state is attained more rapidly. The maximum temperature attained during the first minutes of focusing (Fig. 3) results from high current values.

3.2 Effect of voltage

The application of high voltages has a dual effect on electrophoresis: on the one hand separation time diminishes, which is desirable, but on the other hand temperature increases, which is undesirable. Figure 4 compares the behavior of the cell at two different voltages. At 1600 V, the stationary state is attained more rapidly than at 2000 V, with no temperature difference in the stationary state. In both experiments a 0.2% bovine hemoglobin solution was loaded in the anodic compartment and recovered in the cathodic compartment. The calculated p*I*

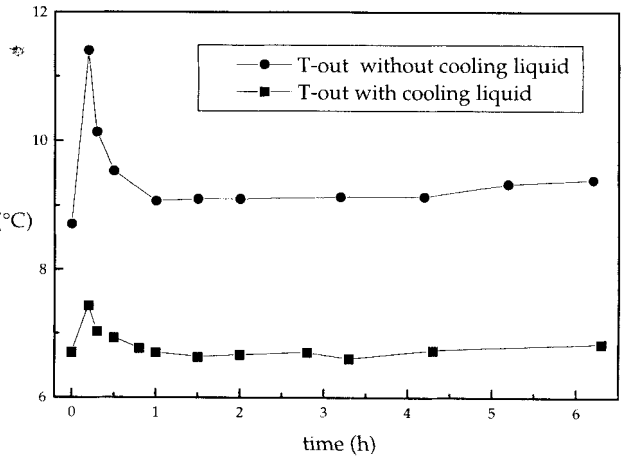

Figure 3. Average temperature of solutions leaving the cell (T-out). Bovine hemoglobin was loaded in the anodic compartment and focused in the cathodic compartment. Amphoteric isoelectric membranes were pIa 6.3 and pIb 6.7; voltage: 1600 V; power: 12 W.

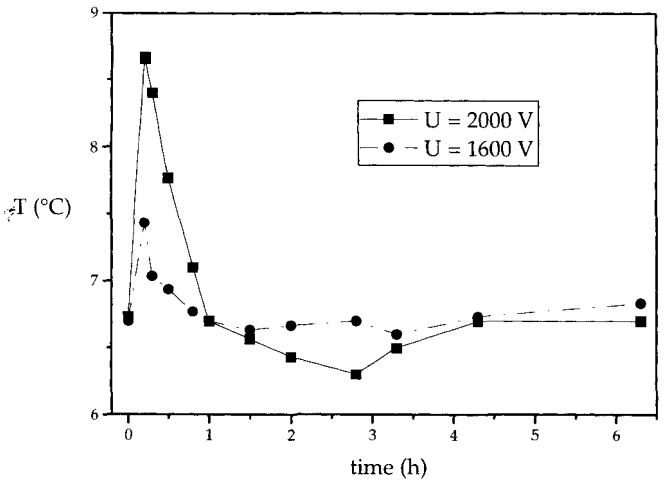

Figure 4. Temperature changes at 1600 V and 2000 V. Other details as in Fig. 3.

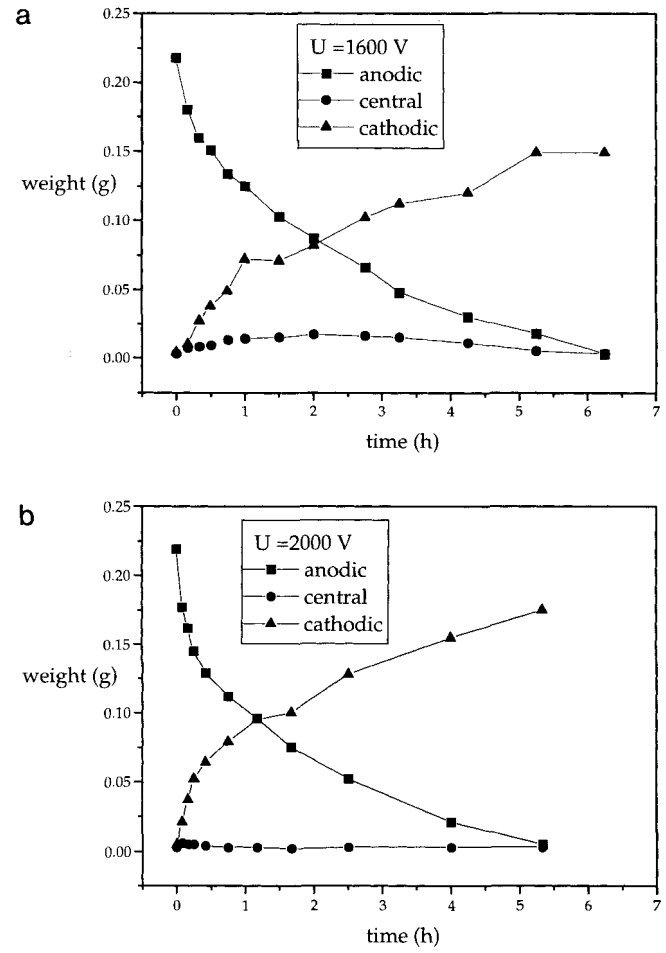

Figure 5. Total weight (g) of bovine hemoglobin as a function of time (h) in each compartment for: (a) 1600 V, and (b) 2000 V. Other details as in Fig. 3.

of the acid isoelectric membrane (pIa) was 6.3, whereas it was 6.7 for the basic isoelectric membrane (pIb). Separation was complete after 5 h at 2000 V, while it took more than 6 h at 1600 V (Fig. 5). Less hemoglobin accumulated in the central compartment at a higher voltage.

3.3 Resolution

The efficiency of any large-scale separation process is evaluated in terms of recovery ratio. In our case, it is the ratio of the protein of interest collected in the compartment where it is to focus, to that of the same protein present in the original mixture. In the case of the myoglobins (0.3 g of horse heart and 0.2 g of whale skeletal muscle), the separation required 1 h at 2500 V and 60 W. Analytical refocusing in a 3–9 IPG, followed by silver staining, revealed that after 60 min (lane 7) whale myoglobin had almost totally moved to the cathodic compartment (Fig. 6). Lane 8 corresponds to the sample of the cathodic compartment after 1 h. The recovery ratio was 80%. For the purification of 1.87 g of bovine hemoglobin from 0.13 g of BSA, we obtained a recovery ratio of 97%. Table 2 shows the evolution of protein concentration in the three compartments. BSA required 22 h at 1600 V and 12 W for focusing in the acid compartment, while bovine hemoglobin needed 4 h more to focus totally in the basic compartment.

4 Discussion

The efficiency of a heat exchanger is usually indicated in terms of the global heat transfer coefficient. A value of 1246 W/m^2 K was obtained for a cooling liquid flow of 0.45 m^3/h, fed at 2°C since the beginning. The configuration of the cell for this particular measurement contained no membranes; it was totally filled with deionized water without stirring or recycling. The increased thickness of the chambers does not affect the rapidity of separations. The larger distance that every biomolecule has to overcome is compensated by the possibility of applying more voltage. This observation is more evident in the case of hemoglobin crossing the two isoelectric membranes: the concentration in the central compartment is practically zero during the whole run at a high voltage.

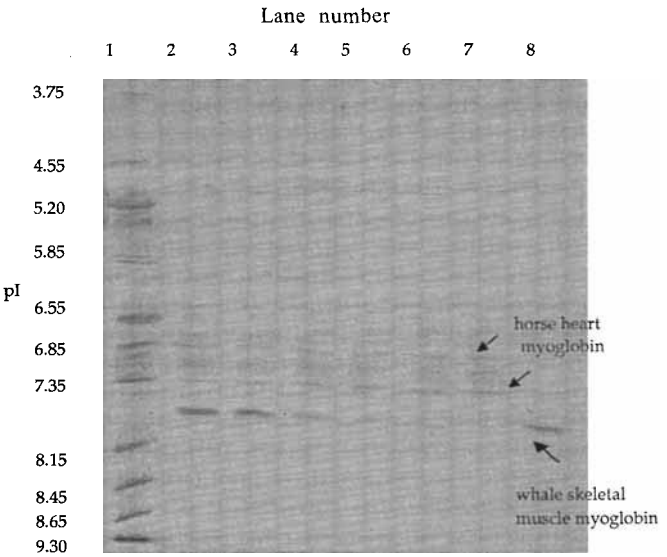

Figure 6. Analytical refocusing in a 3–10 IPG, followed by silver staining. Lane (1) p*I* marker proteins, (2)–(7) proteins in central compartment at time 0, 10, 20, 30, 40 and 60 min, respectively, (8) whale myoglobin in the cathodic compartment after 60 min.

Table 2. Protein amount in each compartment during purification[a)]

Time (h)	Total protein amount (g) Acidic compartment	Central compartment	Basic compartment	% Recovery
0	2.000	0.000	0.000	0.0
2	1.719	0.194	0.162	8.6
4	1.390	0.361	0.339	18.1
18	0.194	0.158	1.575	84.2
22	0.135	0.081	1.678	89.8
26	0.135	0.069	1.819	97.3

a) 1.87 g of bovine hemoglobin (p*I* 6.8 at 25°C) from 0.15 g of BSA (p*I* 4.8 at 25°C). Isoelectric membranes: pIa = 6.3, pIb = 6.7; U = 1600 V; P = 12 W

The resolution of the method depends on the selectivity of isoelectric membranes; this is improved due to constant low temperatures during the whole run. Small temperature variations may affect the selectivity of these membranes due to changes in their p*I*. The separation speed is mainly a function of the voltage applied. However, a greater p*I* difference between the protein and the isoelectric membrane accelerates the process considerably. The goal of PIEF, the purification of large quantities of biomolecules in the shortest possible time, can be attained if the Joule heat is immediately dissipated from the focusing apparatus. For industrial applications of PIEF, larger membrane surfaces than those in our focusing cell will be required. For this purpose, single isoelectric membranes with increased surface appear unsuitable due to the fragility of the polyacrylamide gels. Larger surfaces could be obtained by using multiple modules, either in series or in parallel as needed.

The authors wish to express their gratitude to Mr. Gerard Bovard for his significant advice on the construction of the focusing cell and the thermostatic bath. Thanks are also expressed to Mr. Dave Jemielity for the corrections made in this paper. This work was sponsored by the Swiss National Fund for Scientific Research.

Received March 27, 1995

5 References

[1] Bjellqvist, B., Ek, K., Righetti, P. G., Giannazza, E., Görg, A., Postel, W., Westermeier, R., *J. Biochem. Biophys Methods*, 1982, *6*, 317–339.

[2] Scopes, R. K., *Protein Purification: Principles and Practice*, Springer-Verlag, New York 1987, pp. 246–252.

[3] Creighton, T. E., *Proteins, Structures and Molecular Properties*, W. H. Freeman, New York 1983, pp. 133–158.

[4] Righetti, P. G., Bello, M. S., *Electrophoresis* 1992, *13*, 275–279.

[5] Martin, A. J. P., Hampson, F., *J. Chromatogr.* 1978, *159*, 101–110.

[6] Faupel, M., Barzaghi, B., Gelfi, C., Righetti, P. G., *J. Biochem. Biophys. Methods* 1987, *15*, 147–162.

[7] Bier, M., Twitty, G. E., Sloan, J. E., *J. Chromatogr.* 1989, *470*, 369–376.

[8] Wenger, P., Javet, P., *J. Biochem. Biophys. Methods* 1986, *13*, 275–287.

[9] Righetti, P. G., Wenisch, E., Faupel, M., *J. Chromatogr.* 1989, *475*, 293–309.

[10] Cherkaoui, S., Zell, P., Javet, P., *J. Biochem. Biophys. Methods* 1991, *25*, 61–73.

[11] Jonsson, J., Rilbe, H., *Electrophoresis* 1980, *1*, 3–14.

[12] Kyhse-Anderson, J., *Electrophoresis* 1989, *10*, 6–10.

[13] Tarnopolsky, Y., Roman, M., Phyllis, R. B., *Separ. Scie. Tech.* 1993, *28*, 719–731.

[14] Comninellis, C., Vercesi, G. P., *J. Appl. Electrochem.* 1991, *21*, 136–142.

[15] Comninellis, C., Vercesi, G. P., *J. Appl. Electrochem.* 1991, *21*, 335–345.

[16] Ettori, C., Righetti, P. G., Comninellis, C., *Electrophoresis* 1992, *13*, 55–58.

[17] Chiari, M., Nesi, M., Roncada, P., Righetti, P. G., *Electrophoresis* 1994, *15*, 9535–959.

[18] Righetti, P. G., *Immobilized pH Gradients: Theory and Methodology*, Elsevier, Amsterdam 1990.

[19] Wenger, P., *Développement d'une cellule de séparation d'acides aminés par focalisation isoélectrique, Ph. D. Thesis No. 604*, Swiss Federal Institute of Technology, Lausanne 1985.

[20] Cherkaoui, S., *Développement d'un appareil de séparation préparative de protéines par focalisation entre membranes isoélectriques, Ph. D. Thesis No. 958*, Swiss Federal Institute of Technology, Lausanne 1991.

[21] Yukawa, H., Hoshino, T., Kasokoshi, T., Hakoda, M., *J. Chem. Eng. Jap.* 1987, *21*, 262–266.

[22] Fredriksson, S. M., *J. Chromatogr.* 1978, *151*, 347–355.