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# Membrane fraction collection for capillary electrophoresis

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ABSTRACT

A simple instrument system combining high-performance capillary electrophoresis (CE) and membrane technology is described. CE fraction collection is successfully implemented using a membrane assembly at the exit end of a capillary to complete the electrical circuit for electrophoretic separation. This membrane assembly consists of a poly(vinylidene difluoride) membrane, a buffer reservoir (two layers of 3 MM Chrom filter-paper) and a stainless-steel plate as the ground electrode. Two model proteins are separated and collected on the membrane. Direct protein sequencing is demonstrated from this membrane after CE fraction collection.

## INTRODUCTION

Membrane technology has found application in electrophoretic separations for some time, principally as a blotting medium on to which separated analytes (proteins or DNA) can be transferred after denaturing or non-denaturing one- or two-dimensional gel electrophoresis [1–4]. Following blotting, a wide variety of staining, immunoassay and chemical analysis techniques can be applied. With the rapid growth of powerful bioanalytical methods such as protein sequencing, membrane transfer media have found increased importance as substrates in such systems. Membrane materials such as poly(vinylidene difluoride) (PVDF) have been developed and their physical structures engineered to produce membranes with properties optimized for these kinds of applications [4,5]. Beck [6] demonstrated the direct blotting of analytes from a slab gel on to a moving membrane belt submerged in buffer solution.

In this paper, we demonstrate the feasibility of membrane fraction collection for capillary electrophoresis (CE). CE has developed into a powerful tool for the separation of ions and also neutral analytes because of its remarkable separation efficiency [7–11]. In CE, separation occurs in a capillary tube filled with an electrolyte and with the ends immersed in electrolyte reservoirs. Typically, the elution of the analytes is detected near one end of the capillary, and the separated species are discharged into the outlet reservoir. Previous attempts at fraction collection for CE have used small vials for sequential capture of eluted species. A high degree of dilution results. In addition, it is difficult to preserve the spatial resolution of closely spaced sample bands even when the capillary outlet is moved from one fraction vial to another using a programmable timer [12,13]. Hjertén and Zhu [14] used a variant of this technique in which analytes eluting from the electrophoresis capillary emerged into a flowing buffer stream and were carried to the test-tubes of a fraction collector.

Another approach for CE fraction collection, reported by Huang and Zare [15], utilizes a glass frit to create an electrical connection on-column. A porous frit is made near the exit end of a separation capillary. This frit is then immersed in a reservoir filled with electrolyte to complete the electric circuit for CE separation. This method reduces the dilution effect but it is difficult and time consuming to construct the fritted hole. In addition, a portion of the sample can be lost from the capillary through the frit and into the reservoir.

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We recently reported a simple and rugged means for recovering separated samples from a CE process without significant sample loss or dilution that still maintains good spatial resolution [16]. This paper describes further work with this simple instrument system combining CE separation and membrane technology. To demonstrate the utility of a membrane interface, direct protein sequencing of the recovered, separated proteins is described.

## EXPERIMENTAL

### *Construction of the CE membrane fraction collector*

The experimental configuration is illustrated in Fig. 1. A high-voltage d.c. power supply (Spellman High Voltage Electronics, Plainview, NY, USA) was used to provide a 20-kV potential for electrophoretic separation. The positive terminal of this power supply was applied to the sample or buffer vial and then across an untreated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) (70 cm × 75 μm I.D. × 365 μm O.D.). On-column detection was carried out at 58.5 cm from the injection end. UV absorbance was monitored at 210 nm (Model 206 PHD spectrophotometer; Linear Instruments, Reno, NV, USA). The ground terminal of the power supply was connected to the stainless-steel plate of the membrane assembly.

The membrane assembly consisted of an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA), an electrolyte reservoir consisting of two layers of Whatman 3MM Chrom filter-paper (Whatman, Maidstone, UK) and a stainless-steel ground electrode connected to the ground end of the power supply. This membrane assembly was rotated by a stepping motor during electrophoretic separation at *ca.* 2.2 revolutions per hour.

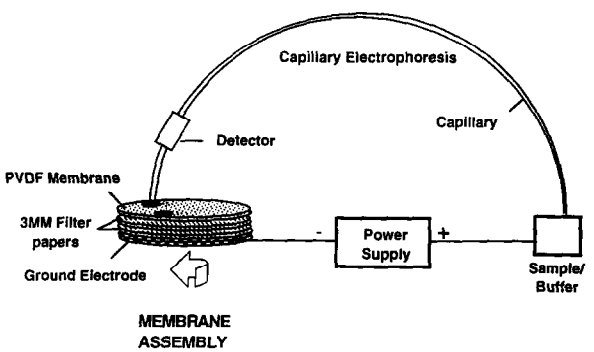


Fig. 1. Schematic diagram of membrane fraction collection for capillary electrophoresis.

### *Sample and reagents*

Myoglobin (horse heart, p*I* 6.76 and 7.16) and β-lactoglobulin (bovine milk, p*I* 5.13) were purchased from Sigma (St. Louis, MO, USA). A mixed myoglobin (8 mg/ml) and β-lactoglobulin (8 mg/ml) solution was prepared in 25 m*M* phosphate buffer (pH 7); 1 *M* zwitterion solution (AccuPure Z1-Methyl; Waters, Milford, MA, USA) was prepared in phosphate buffer (pH 7) to a final phosphate concentration of 20 m*M*.

Membrane staining reagent was prepared from 1.25 g of Brilliant Blue R250 (Coomassie Brilliant Blue R; Sigma) in 500 ml of a fixative solution (200 ml of methanol, 35 ml of glacial acetic acid and 265 ml of water). The destaining solution was methanol–water (90:10, v/v).

### *Procedure*

The procedure for membrane fraction collection for capillary electrophoresis is outlined as follows:

(1) An electrolyte reservoir consisting of two layers of 3MM Chrom filter-paper saturated with phosphate buffer was laid on top of a stainless-steel ground electrode.

(2) A PVDF membrane was prewetted with methanol for 5 s and then laid on top of the electrolyte reservoir (taking care to remove any air bubbles trapped in between).

(3) The membrane assembly was raised to provide good contact with the exit end of the capillary.

(4) The sample solution was injected via electromigration at 6 kV for 10 s. Electrophoretic separation was run at 20 kV using a phosphate buffer with or without added zwitterions.

(5) Rotation of the membrane assembly was started at the beginning of the run; the voltage was turned off after the 20-min CE separation and the membrane was removed from the assembly.

(6) The membrane was stained with Coomassie Brilliant Blue solution for *ca.* 10 min and destained three times.

### *Direct protein sequencing*

The protein bands were excised with a clean razor blade and then subjected to automated Edman deg-

radation in a Waters Model 6600 ProSequencer. The whole β-lactoglobulin band was subjected to sequencing, and only a portion of the myoglobin band was sequenced. No precycling of solvent or reagents was performed and degradations were done using the ADS100 (adsorptive) protocol with automatic on-line high-performance liquid chromatographic (HPLC) identification of PTH-amino acids. PTH-amino acids were measured at 269 nm, with dehydro derivatives of serine and threonine residues confirmed by detection at 313 nm.

The PTH-Maxima chromatography analysis software package (Waters) provides a data analysis in which successive HPLC traces are first optimally aligned and then subtracted to give difference traces for adjacent residues. Difference peak-area data were reduced to a semilog arithmic plot of log (PTH yield) *versus* cycle number and a line fitted by least-squares regression analysis. The average repetitive sequencing yield was derived from the slope of a least-squares regression linear fit to the data.

## RESULTS AND DISCUSSION

### *Membrane fraction collection*

The two-component mixture containing myoglobin and β-lactoglobulin in phosphate buffer (pH 7) was injected into the capillary of the system shown in Fig. 1. The grounding of the capillary was through the PVDF membrane laid on top of the filter-paper electrolyte reservoir containing sorbed buffer solution. The current during the separation is shown in the upper trace in Fig. 2. The current fluctuated between 100 and 125 μA. This fluctuation is due to Joule heating in the capillary and the fact that the capillary was operated in open air. It was also found that the current fluctuated between 100 and 125 μA for the next four consecutive electrophoretic separations which used the same applied voltage and buffer. We have previously reported that the presence of the membrane assembly does not significantly affect the current observed during the separation, indicating that the membrane does not introduce significant additional resistance to current flow [16].

The separation of these two model proteins is shown in the lower trace in Fig. 2. Peaks $A_1$ and $A_2$ are myoglobin (p*I* 7.16 and 6.76, respectively) and peak B is β-lactoglobulin (p*I* 5.13).

CE fraction collection onto a continuously rotating PVDF membrane is demonstrated in Fig. 3. This figure, representing a membrane after staining and destaining with Coomassie Brilliant Blue, illustates the resulting collection from five consecutive injections of a two-component mixture of the above sample solution. The membrane assembly was rotated to the same position prior to each run. Many blue spots are clearly visible in this picture and the reproducibility of the analysis is unacceptable. This phenomenon is due to migration time shifts during successive CE separations. The relative standard deviation (R.S.D.) was found to be 7.7% and 11.6% for the migration time of myoglobin (peak $A_1$ in Fig. 2) and β-lactoglobulin (peak B in Fig. 2), respectively. This observation suggests that the capillary inner surface was progressively modified by

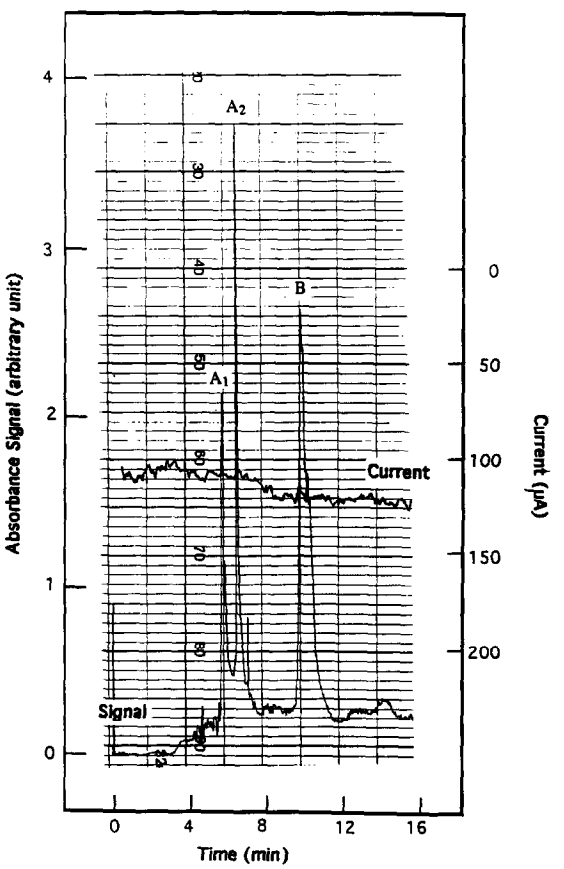


Fig. 2. Electropherogram (lower trace) and current (upper trace) for the separation of a mixture of myoglobin and β-lactoglobulin in a pure running buffer system.

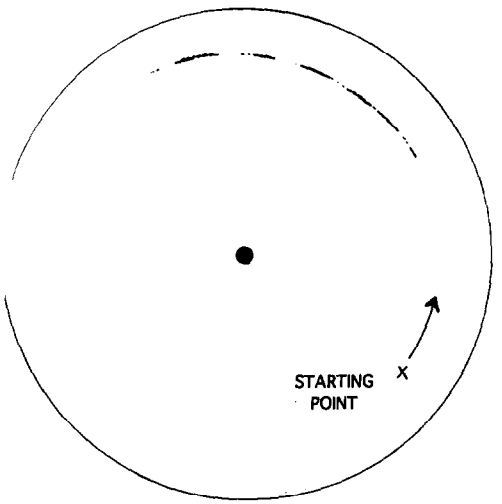


Fig. 3. Photocopy of membrane after staining and destaining with Coomassie Brillian Blue from five consecutive CE membrane fraction collections in a pure running buffer system.

irreversible adsorption of protein. Similar results have been observed in other protein separations [17–20].

*Effect of zwitterion solution of CE membrane fraction collection*

Some of the methods that have been used to counteract adsorption include electrophoresis in buffers with a pH higher than the isoelectric point of the sample proteins [17], electrophoresis in buffers with a very low pH [18] and capillary surface treatments [19,20]. To improve the migration time reproducibility in the present experiments, a zwitterionic compound (AccuPure Z1-Methyl) was added to the buffer solution. This particular compound has a high dipole moment, a zwitterionic character over a broad pH range, good solubility in aqueous buffers and low UV absorbance (0.2 absorbance for a 1-*M* aqueous solution at 214 nm, 1-cm path length).

The lower trace in Fig. 4 shows the electropherogram from one of the four consecutive injections of the previous two-component mixture containing myoglobin and β-lactoglobulin using 1 *M* zwitterion in phosphate buffer. The current fluctuated between 52 and 56 μA, as shown in the upper trace in Fig. 4. Running the experiment with the AccuPure Z1-Methyl additive greatly improved the reproducibility and peak shape, also yielding a better signal-to-noise ratio. The R.S.D. was found to be 0.6% and 1.1% for the migration time of myoglobin (peak $A_1$) and β-lactoglobulin (peak B), respectively. Average migration times and R.S.D.s for the separation of these two proteins with and without zwitterion modifier are given in Table I. A longer migration time was observed when the zwitterions was added to pure running buffer solution, owing to the increased viscosity. The resolution between peaks $A_1$ and $A_2$ in a pure running buffer system (as shown in the lower trace in Fig. 2) is better than in the 1-*M* zwitterion additive buffer system (as shown in the lower trace in Fig. 4). Perhaps the zwitterions interact with the protein analytes.

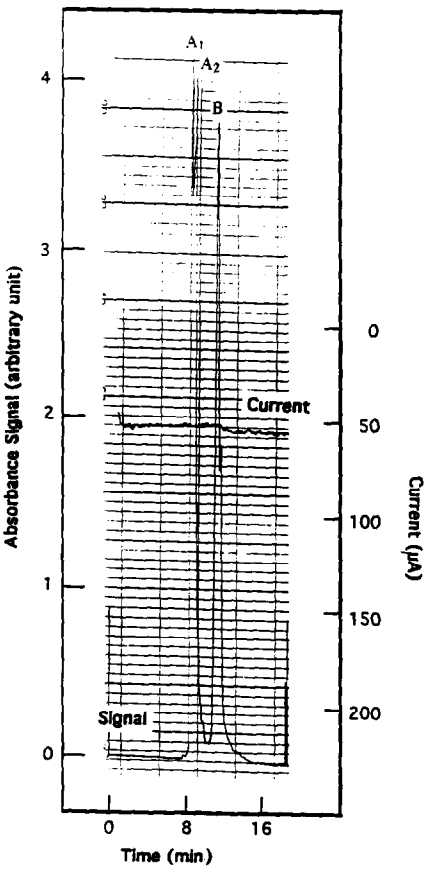


Fig. 4. Electropherogram (lower trace) and current (upper trace) for the separation of a mixture of myoglobin and β-lactoglobulin in a phosphate buffer–1 *M* zwitterion additive system.

The improved migration time reproducibility of

TABLE I

COMPARISON OF AVERAGE MIGRATION TIMES AND RELATIVE STANDARD DEVIATIONS FOR THE SEPARATION OF PROTEIN WITH AND WITHOUT ZWITTERION MODIFIER (1 *M*) IN THE RUNNING BUFFER

| Component | Migration time (min) | | | |
|---|---|---|---|---|
| | Without zwitterion | | With zwitterion | |
| | Mean ($n$ = 5) | R.S.D. (%) | Mean ($n$ = 4) | R.S.D. (%) |
| Myoglobin | 5.90 | 7.7 | 8.68 | 0.6 |
| β-Lactoglobulin | 9.12 | 11.6 | 10.90 | 1.1 |

CE separation when zwitterions were added to the running buffer solution generated better fraction collection onto a membrane. Fig. 5 shows, after staining and destaining with Coomassie Brilliant Blue, the resulting fraction collection from four consecutive fraction collections using 1 *M* zwitterion additive in the buffer. Only two elongated blue spots are clearly visible. Spot A is myoglobin and spot B is β-lactoglobulin. The amount of each solute introduced into the capillary is determined by the mobility of the solute. On this basis, we calculate the total amounts introduced into the capillary from a total four replicate injections to be 28 pmol of myoglobin and 10.8 pmol of β-lactoglobulin.

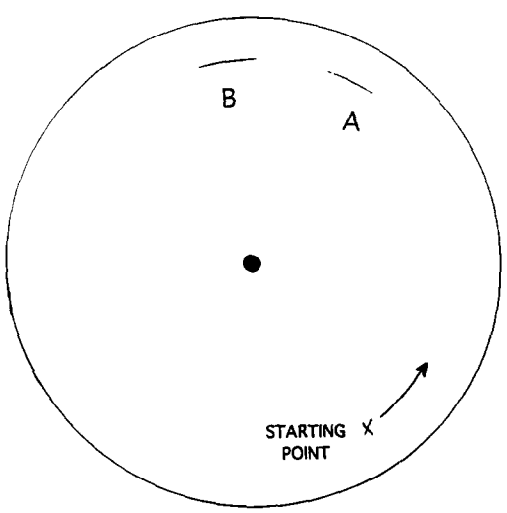


Fig. 5. Photocopy of membrane after staining and destaining with Coomassie Brilliant Blue from four consecutive CE membrane fraction collections in a phosphate buffer–1 *M* zwitterion additive system.

The spatial resolution of the CE separation is preserved in the pattern on the membrane. It is possible to reduce the length of the spots along the writing track and to increase the distance between separated species on the track, as one can adjust the speed of the motorized fraction collection carousel. From the distance between the on-line detector and the exit end of the capillary, the calculated mobility of each analyte and the width of each peak, the times at which the carousel speed should be reduced or increased can be calculated.

*Direct protein sequencing from the immobilon-P transfer membrane after CE fraction collection*

Coomassie Brilliant Blue-stained bands from the above four replicate CE membrane fraction collection were excised from PVDF membrane and subjected to sequence analysis. A representative sequence of the amino terminal fragment is shown in Figs. 6 and 7 for myoglobin and β-lactoglobulin, respectively.

The repetitive yield was 97.8% and 97.6% for myoglobin and β-lactoglobulin, as shown in Figs. 8 and 9, respectively. The initial yields were 1.9 and 6.8 pmol for myoglobin and β-lactoglobulin. Initial yield is defined as the amount of each protein band loaded on to the sequencer holder (not the total amount of each protein injected onto the capillary for CE separation) that gives a phenylthiohydantoin (PTH) signal in the first sequencing cycle; therefore, the recoverd yield for β-lactoglobulin is 63% (as calculated by 6.8/10.8 picomoles), as the entire β-lactoglobulin band was subjected to protein sequencing.

## CONCLUSIONS

The feasibility of membrane fraction collection for CE has been demonstrated. The system is simple, cost-effective, rugged and efficient. The advantage of the system is that it enables one to collect separated sample species from high performance capillary electrophoresis continuously while preserving the spatial resolution and preventing dilution. Sample species collected on a membrane after

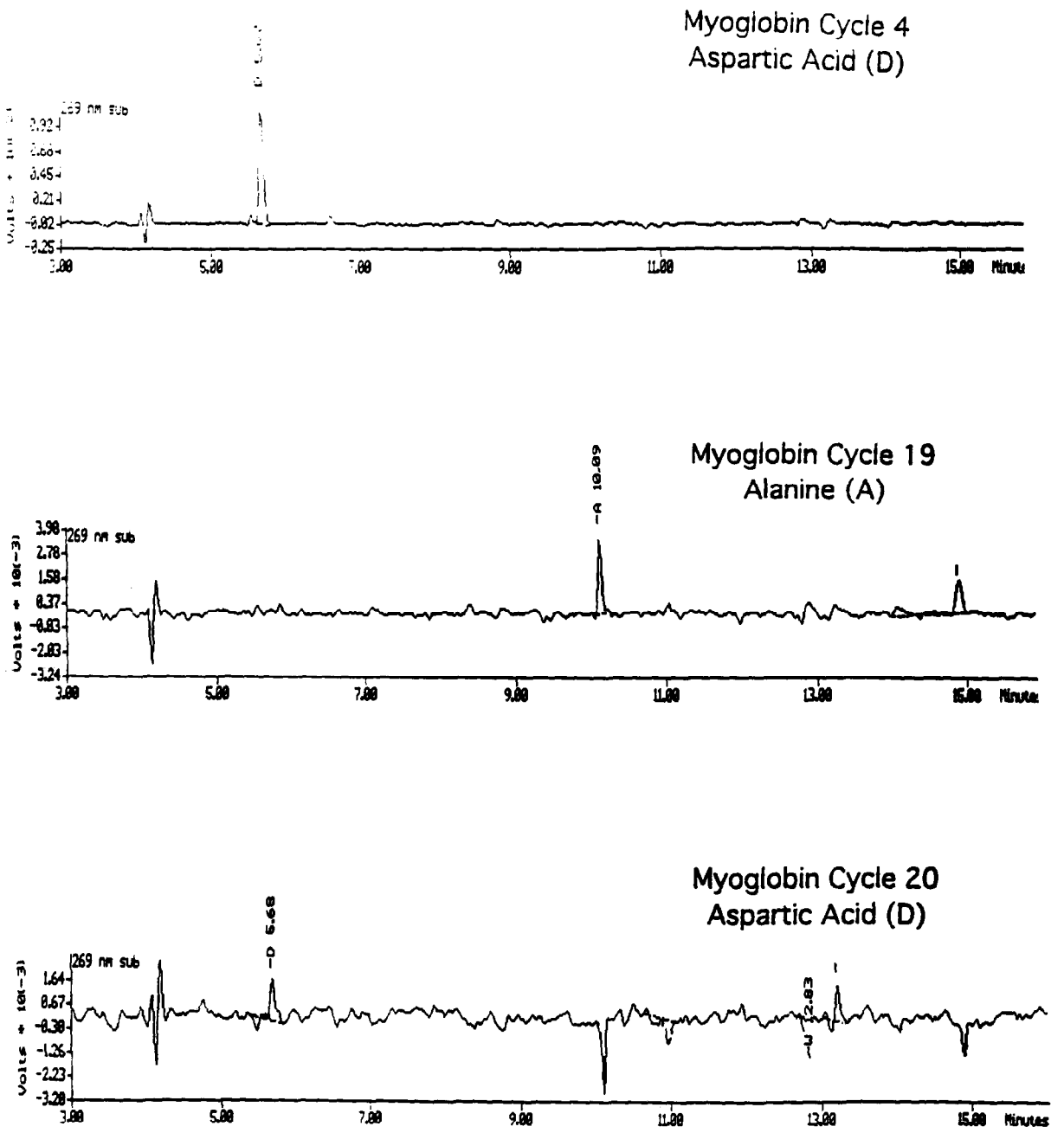


Fig. 6. Representative sequencer cycles from direct sequence analysis of myoglobin after CE membrane fraction collection on to an underivatized PVDF membrane.

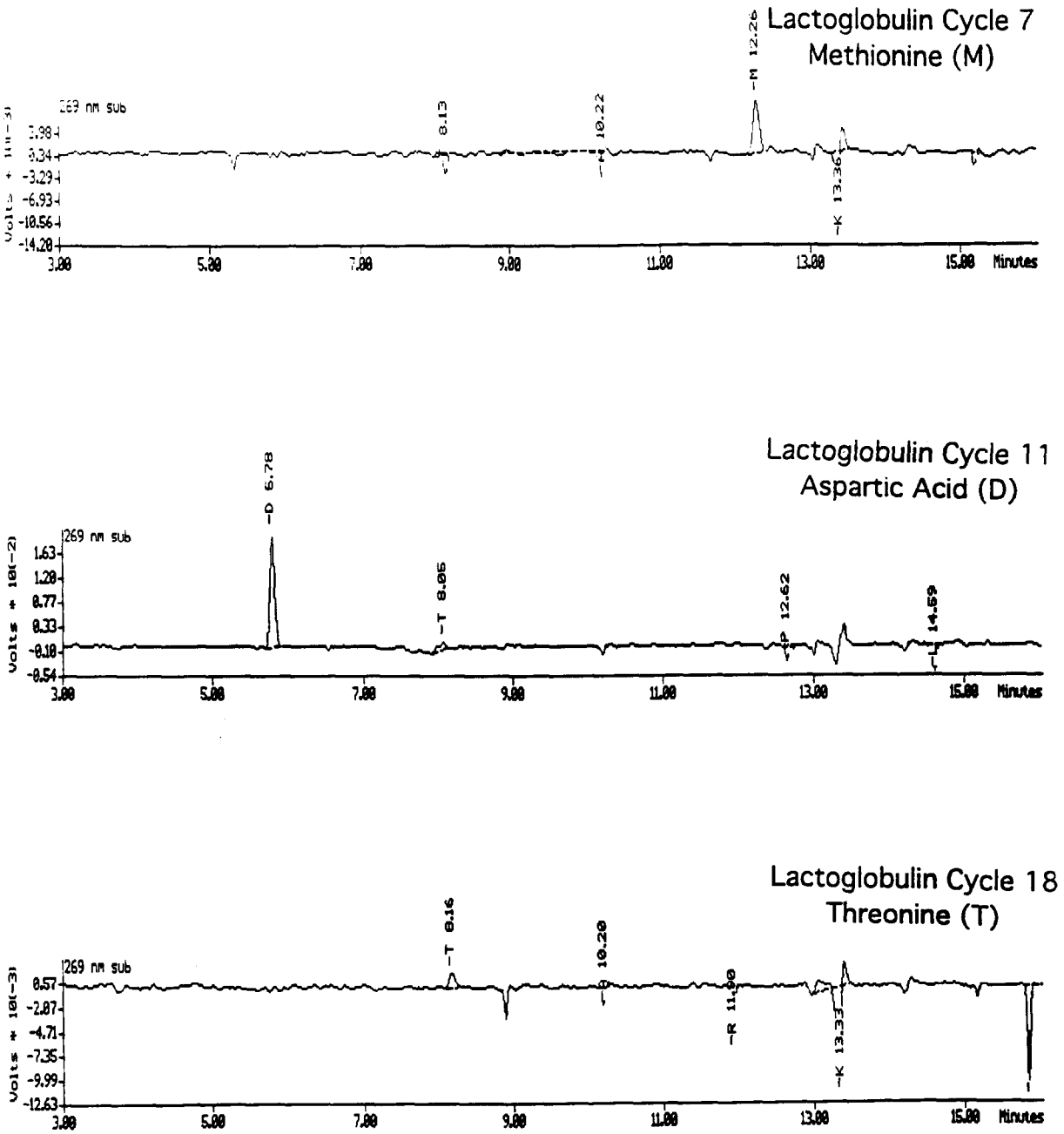


Fig. 7. Representative sequencer cycles from direct sequence analysis of $\beta$-lactoglobulin after CE membrane fraction collection on to an underivatized PVDF membrane.

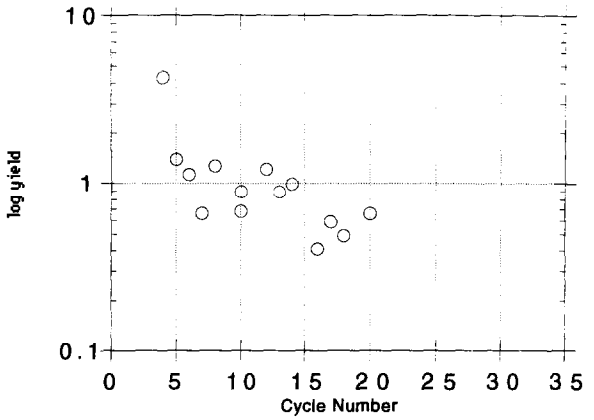


Fig. 8. Yield (○, pm) plot from the direct sequence analysis of myoglobin after CE membrane fraction collection.

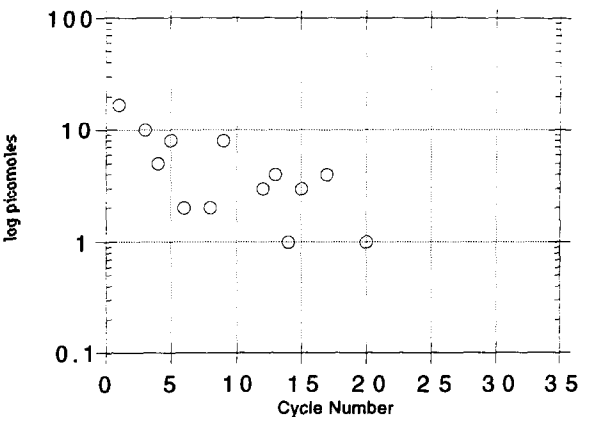


Fig. 9. Yield (○, pm) plot from the direct sequence analysis of β-lactoglobulin after CE membrane fraction collection.

CE separation have been successfully sequenced through direct protein sequence analysis. The combination of high-efficiency CE separation and membrane technology will provide a powerful tool for bioanalysis.

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