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Two-dimensional protein separation by microcolumn size-exclusion chromatography–capillary zone electrophoresis

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ABSTRACT

A two-dimensional separation system for the study of proteins consisting of size-exclusion chromatography (SEC) and capillary zone electrophoresis (CZE) is presented. The SEC separation was carried out in a 1 m × 250 μm I.D. microcolumn. The effluent from this SEC microcolumn filled a sample loop on a computer controlled 6-port valve. The contents of this loop were swept past the grounded end of the CZE capillary for electromigration injection. Detection was by on-line UV detection at 214 nm. The system was initially tested with protein test mixtures, then was used to analyze human, horse and bovine sera. Data are presented as three-dimensional chromatoelectropherograms and grayscale images.

INTRODUCTION

The realization that in many cases no single separation technique is capable of completely resolving all the components in a complex mixture has spawned considerable interest in the area of two-dimensional (2D) separation techniques. Although the general theory underlying these 2D systems has been fully explained [1–4] and is widely accepted, the reduction to practice of this theory into viable 2D separation systems has been relatively slow. Bushey and Jorgenson [5] realized that capillary zone electrophoresis (CZE) was well suited as a second dimension separation due to its potential for high speed while maintaining high separation efficiency. They introduced the first automated "comprehensive" 2D system that coupled column liquid chromatography (LC) with CZE [5]. The focus of this work was the study of peptides by reversed-phase HPLC–CZE. Previous approaches to 2D coupled column techniques involved analysis of only certain regions of interest from the first dimension by the second dimension of separation. In the comprehensive 2D approach of Bushey and Jorgenson, all sample components from the first dimension are analyzed by the second dimension (although the entire volume is not re-injected). Since the inception of this 2D system, our research group has become increasingly interested in expanding the scope of 2D separations. Part of our interest lies in constructing a 2D system for separating proteins.

One of the major tasks facing bioanalytical chemistry is the separation of complex mixtures of biopolymers. Novotny [6] has outlined the recent advances in biomacromolecular separations and forecasts some of the new technology required to further the advancement in this area. He points out that HPLC, particularly when employing microcolumn separations, along with CZE, holds considerable promise for separation and microisolation of protein mixtures due to the high selectivity and efficiency these techniques provide. To address this point, we have constructed an automated 2D separation system for proteins based on microcolumn size-exclusion chromatography (SEC) and CZE.

As a first-dimension separation, SEC is particularly well suited for protein separation because it provides easily interpretable qualitative informa-

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tion: the molecular weight distribution of a mixture. In addition, SEC is run under non-denaturing conditions. This allows any biological activity that a protein might possess to be maintained. The relative merits of using microcolumns rather than conventional size columns has been previously discussed [7]. The major advantage —increased separation efficiency— is particularly important in SEC. In SEC, separation is based primarily on size, and as a result, is not noticably enhanced by the addition of mobile phase modifiers. Resolution in SEC is therefore essentially a sole function of separation efficiency. CZE, also run under non denaturing conditions, provides information on overall molecular charge. When operating in tandem in a 2D system, SEC–CZE can provide an estimate of overall charge within a certain molecular mass range of a mixture. This type of information may prove to be valuable in studies of biopolymers.

EXPERIMENTAL

Reagents

Protein standards, sera samples and buffer reagents were purchased from Sigma (St. Louis, MO, USA). Formamide was obtained from Fisher Scientific (Raleigh, NC, USA). All chemicals were used as received.

The buffer used for both separations was 10 m*M* tricine, 25 m*M* Na_2SO_4, 0.005% (w/v) sodium azide adjusted to pH 8.23 with NaOH. Buffer solutions were made with deionized water purified with a Barnstead Nanopure system (Boston, MA, USA) and were filtered with 0.2-μm nylon membrane filters from Alltech (Deerfield, IL, USA).

Sample preparation

A protein standard mixture was made from thyroglobulin (THYRO), bovine serum albumin (BSA), chicken egg albumin (OVA), and horse heart myoglobin (MYO) each present at 2% (w/v). This mixture also contained 5% (w/v) formamide (FA). Human serum (lyophilized powder) and bovine serum (lyophilized powder) were reconstituted in 500 μl of buffer containing 8% (w/v) formamide (1 ml was the original volume according to Sigma). Horse serum (lyophilized powder) was reconstituted in 1000 μl of buffer containing 7.5% (w/v) formamide (2 ml was the original volume). The protein standard mixture and the sera samples were subjected to centrifugation at 16 000 *g* for 2 min. After centrifugation, the supernatants were drawn off and stored at 4°C. Centrifugation removes any undissolved particulate matter from the samples. This approach was used rather than filtering due to the small volumes being used.

Column packing procedure

The size exclusion column used was a 250 μm inner diameter (I.D.) fused-silica capillary with length 105 cm (Polymicro Technologies, Phoenix, AZ, USA). The capillary was slurry packed in our laboratory with 6-μm, spherical, Zorbax GF450 particles (Rockland Technologies, Newport, DE, USA). The packing procedure has been described previously [7] and was followed with slight modification to the fritting process. A frit is constructed by tapping one end of the fused-silica capillary into a vial containing 100 μm diameter borosilicate glass beads (Sigma). Once a band of glass beads approximately 200 μm in length has been formed in the capillary, the beads are sintered by an arcing device built in the laboratory [8]. Prior to packing, frits are tested by placing the inlet end of the column in a reservoir of methanol and applying 3 bar (50 p.s.i.) of helium pressure for 30 s. Any unstable frits will dislodge during this process. The inlet end is next placed in a high pressure slurry reservoir containing a slurry of 1:10 (w/v) packing material to methanol. Methanol is forced into the reservoir at 200 bar (3000 p.s.i.) by an Altex model 110A pump. Columns are typically packed in 8–10 h and allowed to settle overnight under pressure. The pump is then turned off and the pressure allowed to bleed out through the column for 4 h. The column is then rinsed with deionized water for 4 h, followed by the running buffer which is allowed to rinse overnight.

Instrumentation

Fig. 1 is a schematic of the entire instrumental setup.

Chromatographic system. The chromatographic injection system has been modified from a static split injection system which had been developed for open-tubular LC in our laboratory [9]. Rather than performing a static split injection which requires several hundred microliters of sample per injection, injections were made from a pressure reservoir con-

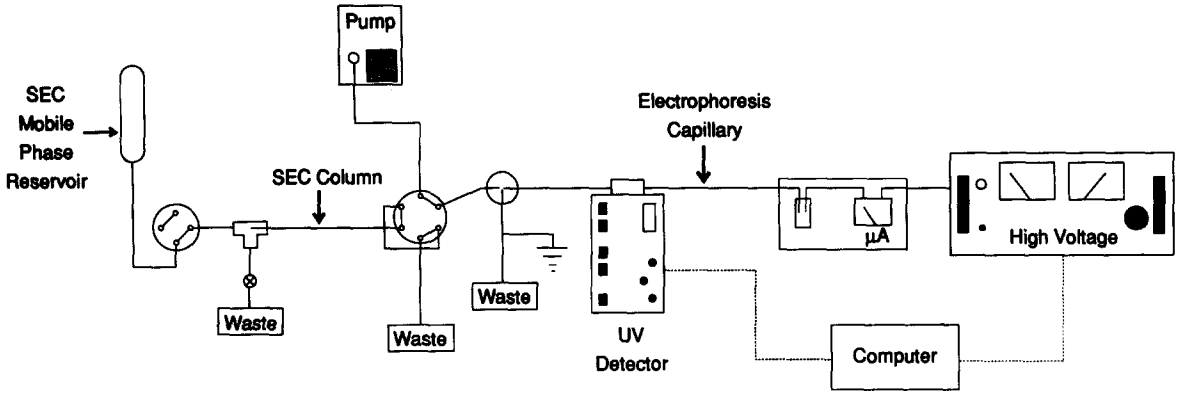

Fig. 1. Schematic of instrumental setup for 2D SEC–CZE.

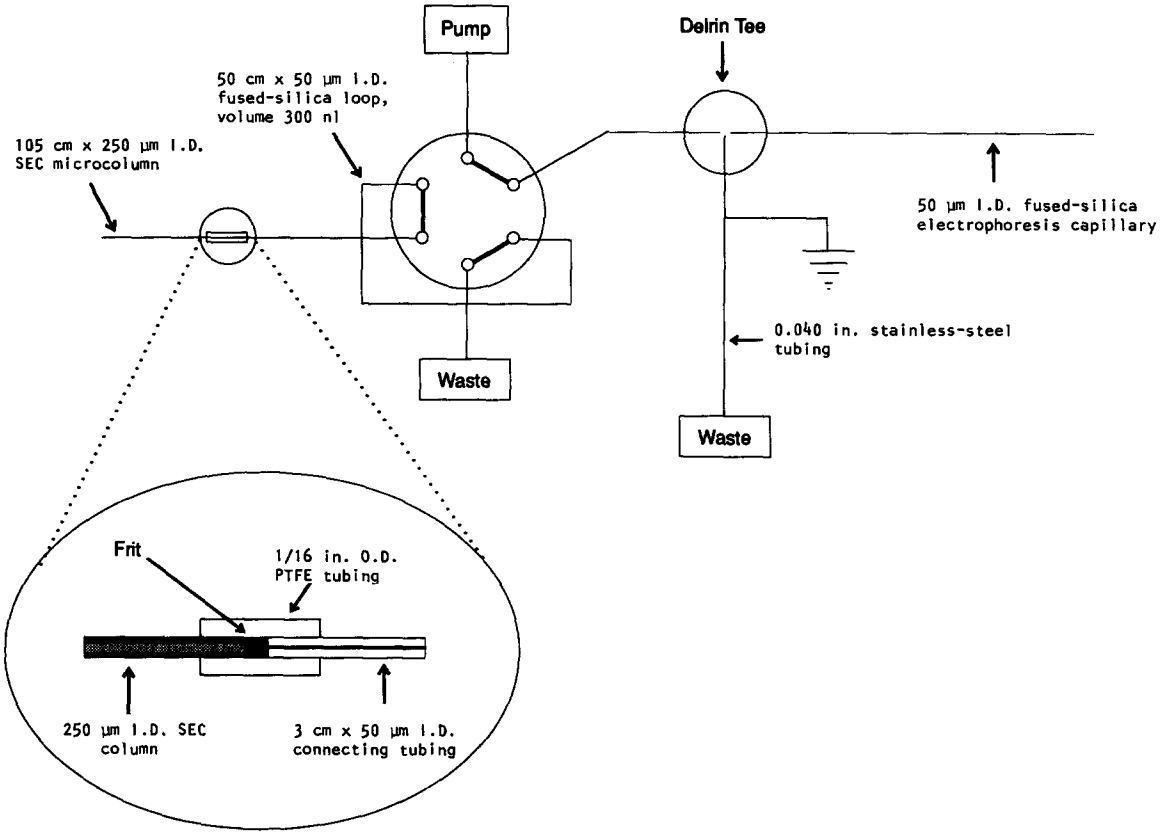

Fig. 2. Expanded view of the 6-port valve used to interface the SEC microcolumn with the CZE capillary. The inset shows the union between the SEC microcolumn and the fused-silica connecting tubing used to interface the microcolumn to the 6-port valve. Details are given in the text.

taining a small vial of sample. The microcolumn was removed from the static split injection tee and inserted into the pressure reservoir where head pressure of 7 bar (100 p.s.i.) was applied for a predetermined time to inject the desired volume of sample. For the protein standards sample, a 10-min injection was performed. A shorter injection time of 6 min was used for the sera samples. After the injection was complete the microcolumn was returned to the static split injection tee where 55 bar (800 p.s.i.) of head pressure was applied to begin chromatography. This head pressure generates an SEC flow-rate of approximately 360 nl/min.

Electrophoresis system. Capillary electrophoresis was performed in untreated fused-silica capillary with inner diameter of 50 μm. Capillary lengths and distance to the detection window varied and are reported in the figure captions. A ± 30 kV high-voltage power supply (Spellman High Voltage Electronics Corp., Plainview, NY, USA) was used in the negative high-voltage mode. Electromigration injections were done at −3 kV for 5 s. Typical run conditions were 4 min at −8 kV and 28 μA. Any changes in CZE operating conditions are provided in figure captions.

SEC–CZE Interface. The SEC microcolumn was interfaced to the CZE system through a 6 port electrically actuated valve. Fig. 2 shows a schematic of this interface. The 6-port valve (Valco, Houston, TX, USA) was fitted with a 300-nl collection loop fabricated in-house from a 15 cm long piece of 50 μm I.D. × 350 μm O.D. fused-silica capillary. Each end of the fused-silica loop was held in place in the valve by sleeving through polyether ether ketone (PEEK) tubing "liners". The liners were made from 2-cm lengths of 0.020 in. I.D. × 1/16 in. O.D. (1 in. = 2.54 cm) PEEK tubing (Upchurch Scientific, Oak Harbor, WA, USA). Each of these liners has a "Light-Touch" removable ferrule system (Upchurch Scientific) and a standard 1/16-in. stainless-steel nut for fittings.

Rather than directly exposing the relatively fragile fritted end of the microcolumn to the fittings necessary to hold the capillary in place within the valve, the microcolumn was coupled to a 3 cm long section of 50 μm I.D. × 350 μm O.D. fused-silica capillary. The union between these capillaries was made by sleeving the capillaries with a 1 cm piece of 0.007 in. I.D. × 1/16 in. O.D. PTFE tubing. When heated, the PTFE tubing expands and allows insertion of the capillaries. Upon cooling, a snug fit is obtained between the capillaries and the tubing. The 3-cm fused-silica connecting tube is also held in place within the valve by a PEEK tubing liner. Protecting the delicate frit in this manner increases column lifetime and allows easy insertion and removal of the SEC microcolumn from the valve.

The electrophoresis capillary was coupled to the valve through a Delrin tee (Alltech) which was connected to the valve by a 5-cm section of 0.005 in. I.D. × 1/16 in. O.D. PEEK tubing (Upchurch Scientific). The capillary extended in to the tee and was held in place near the center of the tee with a PEEK tubing liner. A 20-cm section of 0.040 in. I.D. stainless-steel tubing was also inserted into the Delrin tee. This tubing serves as both a waste line and as the ground electrode (anode) for the CZE system. A microammeter was placed between the tubing and the connection to ground to allow for current monitoring. The height of this stainless-steel waste line was kept level with the cathodic buffer reservoir to minimize hydrodynamic flow within the CZE capillary. The general operation and timing sequence of the valve have been previously described [5]. A flush rate of 100 μl/min was provided by a Hewlett-Packard 1050 pump (Hewlett-Packard, Palo Alto, CA, USA).

Detection. A Linear model 200 variable-wavelength UV–VIS detector outfitted with an on-column capillary flowcell was used for UV detection (Linear Instruments, Reno, NV, USA). Detection was done at 214 nm with a sensitivity of 0.05 absorbance units full scale (AUFS) and a rise time of 0.1 s.

Instrument control. A Hewlett-Packard Vectra 386/25 computer with 25 MHz clock speed and math coprocessor was used to control the 6-port valve, high-voltage power supply and data collection system. The computer was outfitted with a Labmaster multifunction data acquisition board (Scientific Solutions, Solon, OH, USA). Details concerning data collection with this interface board were previously described [5]. The data collection rate for all experiments was 2 points per second.

Data analysis and display. Laboratory-written software with QuickBasic 4.5 (Microsoft Corp, Redmond, WA, USA) provided control over experimental parameters and allowed for data processing

and analysis. The primary means of data display are provided by two commercial software packages. Three-dimensional (3D) chromatoelectropherograms are provided by Surfer 4.0 (Golden Software, Golden, CO, USA). This software allows for viewing 3D data plots from any angle of observation or rotation. These plots are useful for showing peak profile characteristics such as peak symmetry and extent of tailing. Spyglass Transform and Spyglass Format (Spyglass, Champaign, IL, USA), imaging software for the Apple Macintosh, provide grayscale images. There are built in mathematical functions within Spyglass that allow data manipulation (*e.g.* logarithms) and interpolation to be performed.

System preparation. Prior to a 2D separation, samples are run by CZE alone in order to gauge the performance of the electrophoretic system. This ensures that any change in electroosmotic flow can be compensated for by adjusting CZE voltage. This testing is done by manually injecting sample into the collection loop. This is accomplished by inserting a syringe adaptor into the valve port where the microcolumn normally resides. Injections are made with a 25-μl injection syringe. After manual injections are complete, fresh buffer is aspirated into the capillary prior to 2D operation.

RESULTS

Fig. 3A is a Surfer-generated 3D chromatoelectropherogram of the separation of the protein standards mixture. The 360 nl/min SEC flow-rate yields a 2-h run time for the entire 2D separation. Fig. 3B shows a Spyglass-generated grayscale image of the same separation. Unlike the 3D view which requires several observation angles for total viewing, the grayscale image provides easy observation of all separated sample components. The peak assignments indicated were confirmed by independently running each protein with formamide by CZE. Formamide plays two important roles in the 2D separation. Being a small molecule it serves as a totally included marker for SEC. As such, it indicates when the SEC separation is complete. This is useful for initial testing of the system because its elution time sets the upper limit on run time for any given set of conditions. Any peak elution after formamide indicates that a mechanism in addition to size exclusion is occuring. Also, because formamide is a neutral species, it serves as an electroosmotic flow marker in the CZE separation. Any variation in migration time for formamide can be attributed to changing electroosmotic flow. More importantly, neutral species do not tend to undergo adsorptive interactions with the fused-silica capillary surface and therefore usually yield symmetric peaks. Evidence of peak tailing in CZE for formamide is an

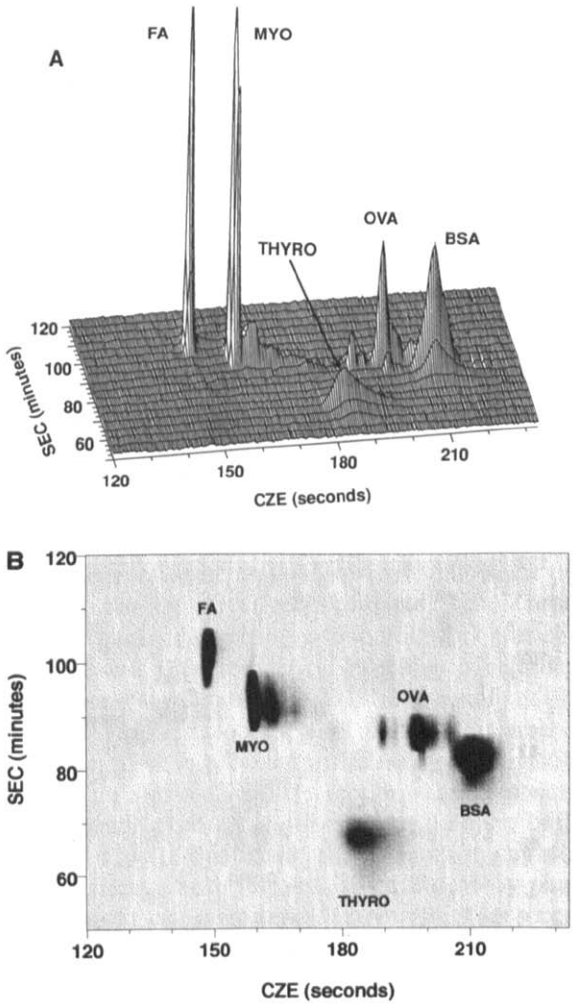

Fig. 3. Separation of protein standards by 2D SEC–CZE. SEC injection was 10 min at 7 bar (100 p.s.i.). Head pressure of 55 bar (800 p.s.i.) was applied to generate a flow-rate of 360 nl/min for the chromatographic separation. The electrophoresis capillary was 38 cm long, 20 cm to the detection window. CZE conditions are 5-s electromigration injection at −3 kV and 4-min runs at −8 kV. Data collection was 2 points/s. Shown here: (A) Surfer-generated 3D chromatoelectropherogram and (B) Spyglass-generated grayscale image.

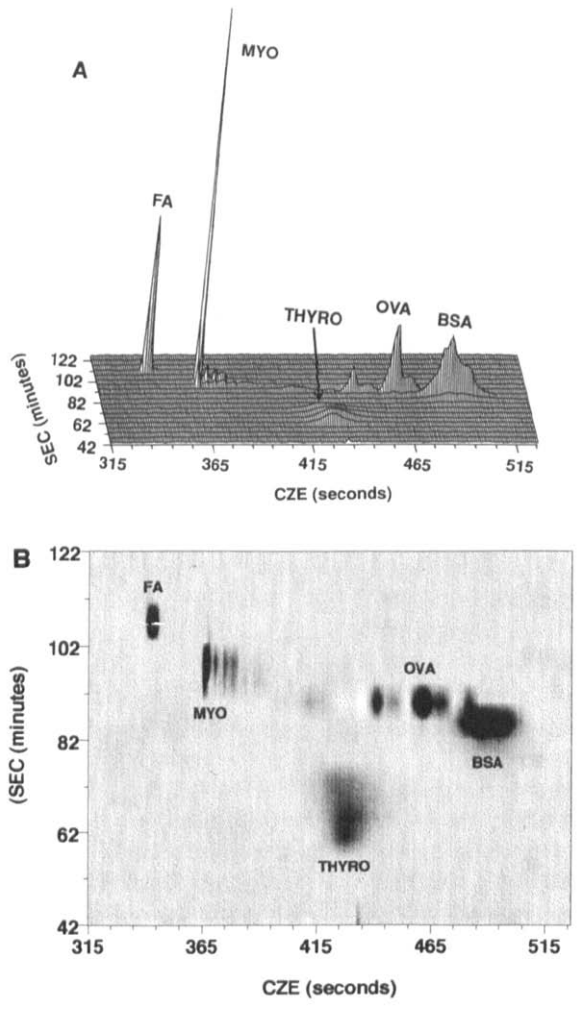

Fig. 4. Separation of protein standards by 2D SEC-CZE with overlapping CZE runs. SEC conditions as in Fig. 3. The electrophoresis capillary was 58 cm long, 40 cm to the detection window. CZE conditions are 5-s electromigration injection at −5 kV and 4.5-min runs at −11 kV. The actual required run time is 9 min. Data collection was 2 points/s. Shown here: (A) Surfer-generated 3D chromatoelectropherogram and (B) Spyglass-generated grayscale image. Data are in logarithm (base 10) scale.

indicator of an injection problem or poor interface washout characteristics.

In taking a closer look at Fig. 3B it becomes evident that the time span in which all the sample components migrate in the CZE dimension does not make full use of the entire separation time available. Although the CZE run time is 4 min long, all sample components migrate within a 1-min span. More importantly, detection of the first sample component does not begin until after half of the CZE run time has passed. This means that the entire first 2 min of the CZE run is analytically "wasted" time. One way to make use of this separation time is to overlap one CZE run onto the empty time of the next CZE run. In doing so, two samples reside in the capillary at any point in time prior to detection. Fig. 4 shows a separation of the proteins standards mixture obtained by overlapping the CZE runs. The capillary length was increased from 38 cm overall, 20 cm to the window, to 58 cm overall, 40 cm to the window. The run time now increased from 4 min to 9 min. The overlap was achieved by performing an injection every 4.5 min. There is a noticable increase in CZE resolution as a result of the added capillary length. The faint band after the main component of myoglobin in Fig. 3B has been clearly resolved into 3 distinct bands in Fig. 4B. Clearly, under separation conditions which allow for its use, overlapping CZE runs is a simple means of enhancing overall 2D resolution.

The combined resolving power of SEC and CZE is evident in separations involving complex sample mixtures. Fig. 5 contains grayscale images of 2D SEC–CZE separations of human serum (A), horse serum (B) and bovine serum (C). It is clearly evident that neither separation run independently could have resolved all the components that the combined use of 2D SEC–CZE was able to resolve. There are distinct pattern differences between the three sera samples shown in Fig. 5. Human serum seems to contain more higher-molecular-mass species than either the horse or bovine sera. All three samples show the presence of a high albumin constituent concentration.

DISCUSSION

We have presented what we believe is the first "comprehensive" 2D system to couple two capillary separation techniques that both use liquid mobile phases. The system provides greater resolving power than either dimension alone could running under the same conditions. The current system generates run times on the order of 2 h. If the SEC separation was being operated at an optimal mobile phase velocity for a mid-range molecular-mass protein (*e.g.*

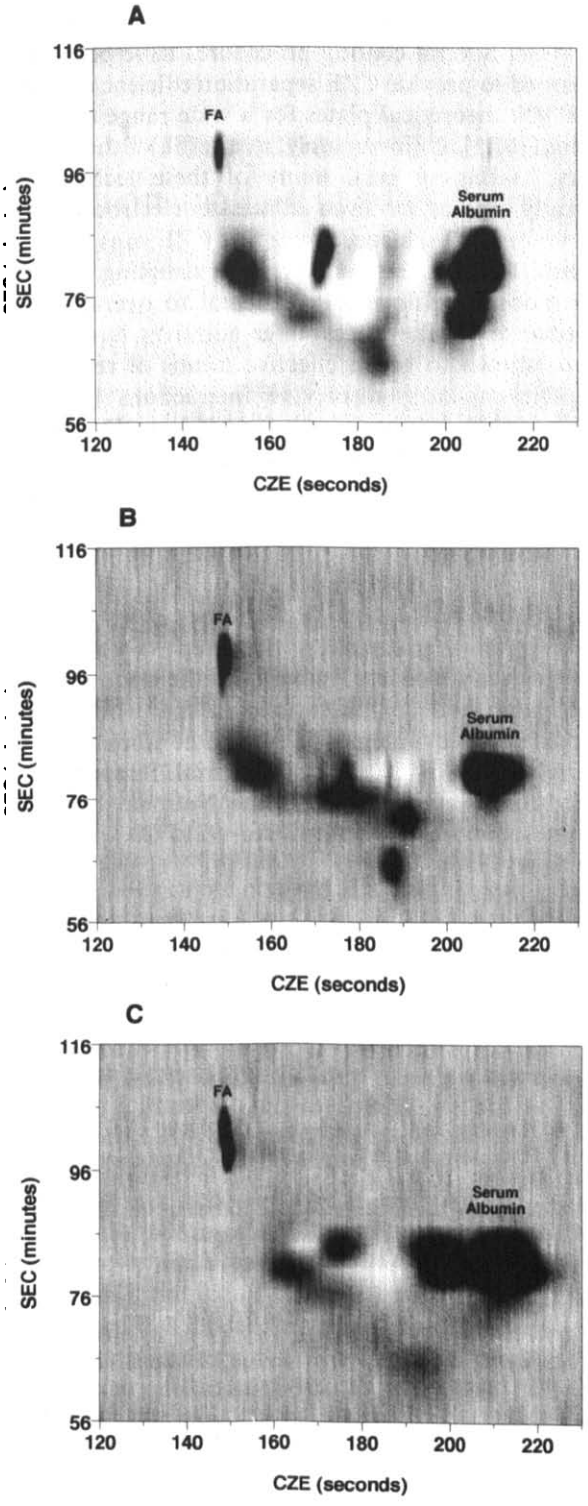

Fig. 5. Separation of sera samples by 2D SEC–CZE. Conditions as in Fig. 3. Spyglass-generated grayscale images are shown for : (A) human serum, (B) horse serum and (C) bovine serum. Data are in logarithm (base 10) scale.

ovalbumin), the run time would approach 12 h. Run times on the order of h are necessary in order to approach the maximum separation efficiency attainable from the chromatographic separation. This arises out of the slow diffusion rates associated with protein species. Although these run times might seem long, they are typical of many of the separation techniques used in protein analysis.

One area of major concern in constructing this 2D separation system was the compatibility of the operating conditions for each separation. Traditional silica-based SEC is performed under conditions of high salt concentration, often approaching 0.5 *M*. Salt concentrations of this magnitude are needed due to the excess residual surface charge associated with the silica. In marked contrast, CZE is typically carried out under salt concentrations around 50 m*M*. Clearly, a compromise in operating conditions must be made in order to allow the two systems to be effectively coupled. The Zorbax GF450 packing material used in this work was chosen because it has the desirable quality of relatively low excess surface charge. The end result of this lowering of surface charge is that we are able to perform SEC in a buffer with a salt concentration compatible with CZE. We are, however, restricted to separating proteins with a p*I* less than the buffer pH of 8.23. If this is not the case, strong protein adsorption to both the SEC silica stationary phase and the fused-silica capillary results. The second area of concern in the construction of the system was the means of achieving the interface between the SEC microcolumn and the CZE system.

The characteristics of the type of interface used to couple the SEC microcolumn to the CZE capillary ultimately determines the operating conditions of the entire system. In the work presented here this serves to set the minimum SEC column size and flow-rate able to be used. This limit arises primarily out of the extra (non-loop) volumes contained within the valve itself. For the 6-port valve used here, each port provides 160 nl of extra volume. The valve rotor, used to make the internal connections between ports, has three engravings that each provide an additional 220 nl of volume. These volumes are in addition to the volume of the sample loop. Regarding the sample loop itself, the minimum distance to connect any two ports (in the proper configuration) and allow for fittings is 15 cm. The

smallest I.D. tubing (of 15 cm length) that could be used without generating excessively high back pressure upon flushing was determined to be 50 μm. A 15 cm long section of 50 μm I.D. fused-silica capillary provides 300 nl of volume. With the extra volume of the valve taken into account, the valve/loop assembly shown in Fig. 2 requires approximately 900 nl of sample volume to enter the valve in order to fill the 300-nl loop with sample. This fixed volume of 900 nl must be generated within the time of one CZE run. This relationship imposes severely limiting restrictions on SEC and CZE operating conditions.

An ideal 2D SEC–CZE system would allow SEC operation near the optimum flow-rate with a CZE injection frequency great enough to prevent recombination of components separated by SEC. To meet these ideal conditions with the system presented here would require an SEC flow-rate near 50 nl/min and CZE runs 2–3 min long. Under these conditions, only 100–150 nl of sample would be generated in the time for one CZE run. This is nearly six times less volume than the 900 nl required to fill the valve/loop assembly. Collection of 900 nl would take 18 min at an SEC flow-rate of 50 nl/min. Although an 18-min CZE run time would provide the ability for very high CZE resolution, gross undersampling of the SEC dimension would result. The operating conditions chosen for the work presented here provided an acceptable trade-off between the factors of resolution (in both SEC and CZE) and sampling frequency. A new interface design is required if lower SEC flow-rates are to be used, or if a smaller I.D. microcolumn is desired. The combination of lower SEC flow-rates, approaching optimum conditions, and higher performance smaller I.D. microcolumns is an important consideration for maximizing the overall performance of 2D SEC–CZE. It has been shown that a 1 m × 50 μm I.D. SEC microcolumn run under optimum conditions for a protein sample can generate nearly 100 000 theoretical plates [7]. A column with this separation efficiency, coupled with the separation efficiency of CZE, would certainly be a powerful system for protein separations. In addition to a new type of interface, improvements in CZE performance will be necessary to further develop the potential of 2D SEC–CZE.

We did not investigate using any type of capillary coating technique to improve the CZE protein separation. Several coating procedures have been developed to provide CZE separation efficiencies near 100 000 theoretical plates for a wide range of proteins [10,11]. Unfortunately, as a result of the capillary coating process, many of these techniques greatly reduce or even eliminate electroosmotic flow. In a 2D scheme, where fast CZE runs are required for frequent first dimension sampling, loss of electroosmotic flow is detrimental to overall performance. Zwitterionic buffer additives have also been shown to be an effective means of reducing protein–capillary adsorptive interactions [12,13]. This approach is promising for use in a 2D system because electroosmotic flow is maintained. New interfacial schemes and methods of improving CZE performance are currently under investigation in our laboratory.

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REFERENCES

1 J. C. Giddings, *Anal. Chem.*, 56 (1984) 1258A.
2 J. M. Davis and J. C. Giddings, *Anal. Chem.*, 57 (1985) 2168.
3 J. M. Davis and J. C. Giddings, *Anal. Chem.*, 57 (1985) 2178.
4 J. C. Giddings, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 319.
5 M. M. Bushey and J. W. Jorgenson, *Anal. Chem.*, 62 (1990) 978.
6 M. Novotny, *J. Microcolumn Sep.*, 2 (1990) 7.
7 R. T. Kennedy and J. W. Jorgenson, *J. Microcolumn Sep.*, 2 (1990) 120.
8 A. M. Hoyt Jr., S. C. Beale, J. P Larmann, Jr. and J. W. Jorgenson, *J. Microcolumn Sep.*, in press.
9 E. J. Guthrie and J. W. Jorgenson, *J. Chromatogr.*, 255 (1983) 335.
10 R. M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
11 J. K. Towns and F. E. Regnier, *Anal. Chem.*, 63 (1991) 1126.
12 M. M Bushey and J. W. Jorgenson, *J. Chromatogr.*, 480 (1989) 301.
13 M. Merion, B. Bell-Alden, E. Grover, U. Neue and J. Peterson, presented at the *3rd International Symposium on High Performance Capillary Electrophoresis, San Diego, CA, February 4–6, 1991*, Poster PT-69.