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Preparative capillary zone electrophoresis of synthetic peptides Conversion of an autosampler into a fraction collector

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Abstract

Preparative capillary zone electrophoresis of three synthetic peptides was performed either manually or automatically by simple manipulations of a commercial electropherograph that is equipped only with an autosampler without any built-in fraction collection capability. Manual fraction collection was achieved by replacing the outlet (cathode) beaker with a microcentrifuge tube, and automatic fraction collection was accomplished by converting the electropherograph's autosampler into a fraction collector. The latter was easily achieved mainly by the use of an extension wire, which completed the electrical circuit and facilitated fraction collection either at a specified time or within fixed time intervals.

1. Introduction

The publications of Jorgenson and Lukacs [1,2] in the early 1980s have popularized capillary zone electrophoresis (CZE) as an experimental technique for peptide separation and analysis [3–14]. High electrophoretic resolution can be achieved using high voltage (10–30 kV) because of the efficient heat dissipation that is achievable by using small internal diameter (I.D. 50–100 μm) thin-walled fused-silica or glass capillaries that have a large surface-to-volume ratio. Other advantages of modern CZE include a high level of detection sensitivity and selectivity, rapid analyses, on-line detection, interfacing to mass spectrometry (MS), long column life, low sample/reagent consumption and automation.

Fraction collection is also possible using this micro-column electrophoretic separation technique [15–30]. In most cases, electrical contact is maintained during the electrophoretic separation and collection, and voltage is turned off during the transfer of the capillary and electrode to a collection vial [15–24] that contains the electrolyte or to a moving membrane [25] that is submerged in the electrolyte. Alternatively, Huang and Zare [26,27] constructed an on-column frit to maintain the electrical contact and to collect the eluent on a moving surface. Furthermore, hydrodynamic elution using a syringe

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pump [28] after the voltage is turned off or using the available pressure [29] have also been reported. In all cases, automated fraction collection would facilitate repetitive and multiple collections.

Our laboratory has an electropherograph, which has an autosampler but not a fraction collector. Accordingly, in this present study, we report that such an autosampler can be easily converted to an automated fraction collector. That fraction collector was used to collect electrophoretically three synthetic peptides [Dynorphin A_{1-24} or A_{1-17} ($DynA_{1-24}$ or $DynA_{1-17}$), substance P (SP), and leucine enkephalin-lysine (LE-K)] separated in a single electrophoretic experiment. This conversion is significant, because automatic fraction collection is possible either at a specified time, or within a fixed time interval.

The applications of CZE in biomedical research have been reviewed recently [31–33]. For many years, this laboratory has been involved in analyzing neuropeptides from biological sources, including human tissues and fluids, by using multi-dimensional reversed-phase high-performance liquid chromatography (RP-HPLC) for sample preparation [34], and by using radioimmunoassay (RIA) [35], MS [36], and tandem MS (MS–MS) [37] for qualitative and quantitative analyses [38]. CZE may be used to substitute for, or to complement, RP-HPLC for sample preparation prior to RIA, MS, and/or MS–MS detection.

2. Experimental

2.1. Reagents and materials

$DynA_{1-17}$, SP, and LE-K were purchased from Sigma (St. Louis, MO, USA) and $DynA_{1-24}$ was obtained from Peninsula Labs. (Belmont, CA, USA). These synthetic peptides were used without any further purification. Ammonium formate (J.T. Baker, Phillipsburg, NJ, USA) and trifluoroacetic acid (TFA; Pierce, Rockford, IL, USA) were used to prepare the volatile CZE buffer. Fused-silica capillary with 50 or 100 μm I.D. and 360 μm O.D. was purchased from Polymicro Technologies, Phoenix, AZ, USA.

2.2. Instrumentation

An ISCO (Lincoln, NE, USA) Model 3140 electropherograph outfitted with an IBM (Armonk, NY, USA) Personal System/2 Model 30 286 computer was used. Operation of the instrument and data collection/analysis were controlled by the manufacturer's ICE 3.1.0 level software. According to a recent survey of capillary electrophoresis instrumentation [39], ISCO is listed as one of the major suppliers of the electropherographs that have been purchased. However, ISCO's electropherographs, including the Model 3140, are not equipped with any built-in fraction collection capability.

2.3. Methods

CZE

The fused-silica capillary used in the manual fraction collection experiment was a 98 cm × 50 μm I.D. capillary, with a 68 cm length from the inlet of the column to the detector; whereas a 100 cm × 100 μm I.D. capillary, with a 60 cm length from the inlet of the column to the detector, was used in the automatic fraction collection experiment. The 50 μm I.D. capillary column volume was 2 μl, and the 100 μm I.D. was 8 μl. Prior to daily use, the capillary was preconditioned with the following sequence of solvents by applying the electropherograph's "high vacuum" ($\Delta p = 28$ kPa) [40] from the outlet beaker for at least two column volumes for each solvent: water, 1 *M* NaOH, 0.1 *M* HCl, water and finally buffer (20 m*M* ammonium formate, titrated to pH 2.5 with TFA) for the experiment. This volatile buffer is similar to that used by Johansson et al. [41], and was chosen so that the fractions collected may be analyzed subsequently by MS. A 1 m*M* buffer was also used in some experiments.

The mixture of peptides contained 0.3 μg (75 to 439 pmol each) of each peptide (ca. 1 μg total for the three peptides) per μl of buffer (1 m*M*). Injection was performed by applying the instru-

ment's injection vacuum from the outlet beaker [40,42]. An injection volume of ca. 30 nl (corresponding to applied amounts of 2.4, 7.1, and 14.0 pmol for $DynA_{1-24}$, SP, and LE-K, respectively) was injected into the 50 μm I.D. capillary for the manual fraction collection. The injection volume for the 100 μm I.D. capillary in the automatic fraction collection was ca. 100 nl, corresponding to injected amounts of 15.1, 24.0, and 47.4 pmol of $DynA_{1-17}$, SP, and LE-K, respectively. Reinjections of those collected fractions were used to identify electrophoretically the collected peptide. The applied voltage was 27 or 13.5 ($\pm$ 1%) kV [43], and the temperature was regulated to 30 $\pm$ 0.5°C by the electropherograph's built-in air-circulating system.

Manual fraction collection

This fraction collection mode (cf. [16]) was performed by replacing the outlet buffer reservoir (beaker) with a microcentrifuge tube containing 10 μl of either 1 or 20 m*M* buffer. Because the electropherogram of the three synthetic peptides (e.g, Fig. 1A) provides only the electrophoretic migration time that it takes an analyte to reach the detector, the corresponding longer migration time that it takes an analyte to reach the capillary outlet must be calculated [18] (see below, *Calculation of migration time for fraction collection*). The analyte collected in the microcentrifuge tube was reinjected for electrophoretic confirmation. For example, the middle peak (SP) in Fig. 1A was collected and analyzed in this manner (See Fig. 1B and C).

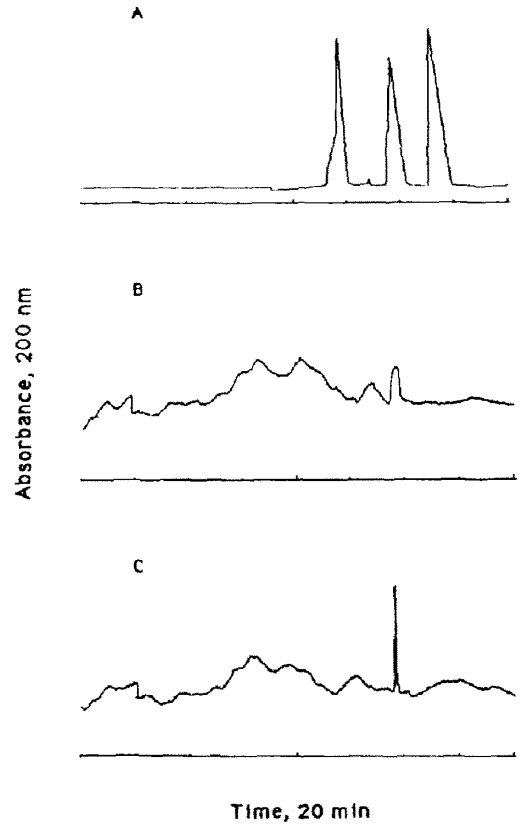

Fig. 1. Capillary zone electropherograms of synthetic peptides. (A) Electropherogram of three synthetic peptides; peaks from left to right are $DynA_{1-24}$, SP, and LE-K, respectively. (B and C) Electropherograms of the reinjected SP peak, collected in 20 m*M* CZE (B) or in 1 m*M* CZE buffer (C). AUFS values are 0.1, 0.002, and 0.002 for A–C, respectively.

Automatic fraction collection

The instrumental configuration for automating the fraction collection is shown in Fig. 2, where the autosampler is now labeled as a fraction collector. This scheme illustrates four basic different features between the original instrumental configuration and the modification required for automatic fraction collection. First, and most importantly, an extension wire is used to electrically connect the ground (−) outlet, positioned near the outlet beaker, with the platinum wire attached to the sampler arm. That platinum wire on the sampler arm was originally intended for electrokinetic injection using the autosampler. It now serves to complete the electrical circuit required for the electrophoretic migration of an analyte during fraction collection.

Second, the capillary inlet (segment A) and capillary outlet (segment B) are positioned opposite to the original configuration (i.e., on the opposing side of the detector), so that the capillary outlet end can now reach the fraction collector instead of only the outlet beaker.

Third, the sample vials (e.g., 300-μl microcentrifuge tube) originally used in the built-in sample carousel are now the vials used for the outlet buffer reservoir for the fraction collector.

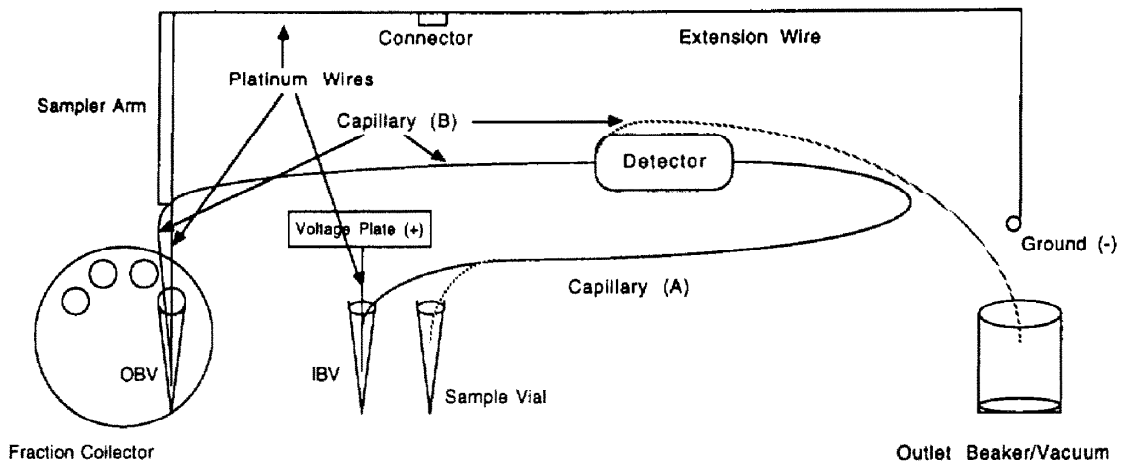

Fig. 2. Instrumental configuration for automatic fraction collection. IBV = Inlet buffer vial; OBV = outlet buffer vial. See text for details.

That same type of vial (or an inlet beaker) is used as the inlet buffer reservoir positioned beside the high voltage plate. Prior to the modification, the anode and cathode reservoirs both used the 10-ml inlet (not shown in Fig. 2) and outlet beakers, respectively, supplied by the company.

Finally, for vacuum injection, the capillary segment B (dashed line) is redirected back to the outlet beaker, and capillary segment A (dashed line) is inserted into a sample vial (positioned wherever convenient). Vacuum is applied as described [40]. After the sample injection, the capillary inlet is repositioned manually back to the inlet buffer vial (IBV), and the capillary outlet is also returned manually to the outlet buffer vial (OBV) via the sampler arm. Voltage can now be applied.

In the automatic fraction collection experiment, each peptide migrated into an OBV that contained 5 μl of the 20 m*M* CZE buffer. The applied voltage during the fraction collection was halved to achieve higher recovery [18]. To avoid peptide contamination from one collected fraction to the next, the ground electrode and the capillary outlet tip were rinsed by inserting them into a vial that contained a larger volume (e.g., 300 μl) of 20 m*M* buffer for a brief period (e.g., 15 s) without voltage prior to moving to the next OBV for the next fraction collection. At the end of each electrophoretic collection in an OBV, a programmed delay of 1 min maximized the diffusion of the eluted peptide from the column and electrode into the OBV solution. The collected samples were lyophilized and reconstituted in 2 μl water for reinjection analysis under "stacking" conditions [44].

Computer

The electropherograph is controlled via the computer, which has two operating modes: normal or extended. The normal mode gives the manufacturer's defined sequence of operations [43] for injection of sample from the autosampler and for electrophoresis in a continuous fashion. The extended mode allows one to instruct the electropherograph via the computer to perform every single step of the instrument operation, and to perform a group of distinct functions with a program specially written for that mode. The program is easily written by inputting the step-by-step commands selected from the available command selection [43]. Furthermore, several programs may be executed continuously in a "batch" mode.

For manual collection, one uses the normal mode or the programmable extended mode to perform an initial electrophoretic separation. However, after replacing the outlet beaker with a microcentrifuge tube, an extended mode pro-

gram must be used to restart the electrophoresis for fraction collection. In principle, the extended mode electrophoretic programs may be used to collect manually more than one fraction per separation.

For the automatic fraction collection, all operations are performed with the extended mode programs. First, an injection program is written to perform vacuum injection under the instrumental configuration shown in Fig. 2. Second, an extended mode program is written to instruct the electropherograph via the computer to perform electrophoresis. At the appropriate time within the program, voltage is turned off. Third, programs are written to move the sampler arm (and thus the platinum ground wire and the capillary outlet) to the next OBV in the carousel for fraction collection or to a rinsing vial between collections. Execution of the second and third programs in the "batch" mode is the basis of the automatic fraction collection. The electrophoretic migration times of the eluents and the time intervals required for fraction collection are input to the computer.

Calculation of migration time for fraction collection

It is necessary to calculate the window of migration time within which an analyte is collected. That window encompasses the leading and tailing edges of the UV absorbance. The following equation is used to calculate those migration times corresponding to those leading and tailing edges. The migration time of an analyte to the detector is converted to the corresponding migration time to the capillary outlet by the equation:

$$t_e = (L/l)t \quad (1)$$

where t_e = the electrophoretic migration time of the peak to the capillary outlet (min or s), L = total capillary length, l = capillary length from the inlet to the detector and t = migration time (min or s) to the UV detector.

For manual fraction collection, only the peptide (SP) that migrates in the middle of the three synthetic peptides was collected (see Fig. 1A). The detected leading and tailing portions of the SP peak are at 14.3 and 15.4 min, respectively. Thus, $t_e = 20.6$ and 22.2 min, respectively, because $L = 98$ cm and $l = 68$ cm. Therefore, a fraction collection beginning at 20 min, and lasting ca. 2 min was programmed for the collection of that SP peak. The safety margin of 0.6 min earlier than the expected time of collection was empirically determined.

For automatic fraction collection, three peptides were collected into three different fractions in a single electrophoretic separation. The electrophoretic time points (t_{1-4}) needed for the migration time calculations for the automatic fraction collection of the three migrating peaks are shown in the electropherogram in Fig. 3. Each of these time points gives the t (migration time as determined by UV detector) values (Table 1). Each t value is decreased by 15 s (due to the 30 s of the initial linear voltage ramping) to give t_c (migration time corrected for voltage ramping) value. These t_c values are converted to the respective t_e (migration time of analyte at the capillary outlet) values by multiplying by the factor (100/60) because $L = 100$ cm and $l = 60$ cm. Finally, wherever possible, a safety margin for time to compensate for any errors or irreproducibility was incorporated. In this case, 0.5 min was subtracted from t_{e1}, giving t_{fc1} (actual migration time used at time point 1 for fraction collection). Similarly, a safety margin of 0.5 min was added to t_{e4}, giving t_{fc4}. Therefore, the electrophoretic separation (at the UV window) ends at t_{fc1}. The duration of the electrophoretic collection of the first peak ($\Delta 1$) was calculated as follows,

$$\Delta 1 = [(t_{fc2} - t_{fc1} - 0.1 \text{ min}) \times 2] - 0.25 \text{ min} \quad (2)$$

where 0.1 min accounts for the voltage deramping time [18], the factor of 2 accounts for the fact the voltage was halved during the collection process, and 0.25 min accounts for the linear ramping time of 0.5 min used to ramp up the voltage up to a constant level (13.5 kV) during the electrophoretic fraction collection. The values of $\Delta 2$ and $\Delta 3$ are calculated similarly, except that for $\Delta 2$, t_{fc2} was subtracted from t_{fc3} and that for $\Delta 3$, t_{fc3} was subtracted from t_{fc4}. Accordingly,

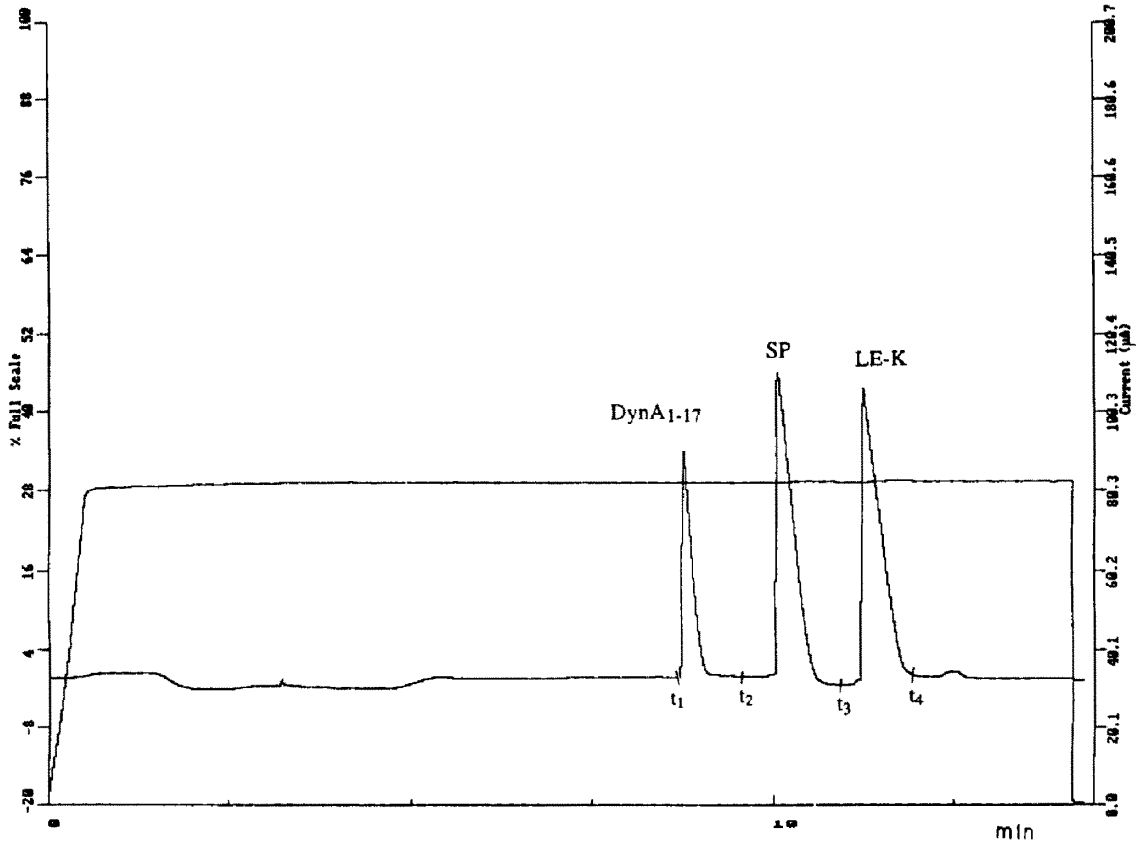

Fig. 3. Electropherogram for automatic fraction collection of three synthetic peptides. A 1 m × 100 μm I.D. capillary, with 60 cm to the detector, was used. The electrophoretic time points t_{1-4} were used for calculating collection windows and for programming the automatic fraction collection times. The horizontal line is the monitored current (ca. 83 μA). The ramping of current observed initially is due to the voltage ramping that occurs linearly in 30 s (see text). AUFS = 0.2.

Table 1
Conversion of the migration time at the detection window to its corresponding collection time

Time point	Migration time (s)			
	t	t_c	t_e	t_{fc}
1	521	506	843	813
2	574	559	932	932
3	655	640	1067	1067
4	713	698	1163	1193

t = Migration time as recorded on the electropherogram; t_c = migration time corrected for voltage ramping; t_e = migration time of electroelution at the capillary outlet; t_{fc} = the actual time used for programming the fraction collection.

Δ1–3 values equal to 211, 243, and 225 s, respectively, were put into the computer programs. These times were manually selected to allow for the collection of all three fractions into three different OBVs in a single electrophoretic separation.

3. Results and discussion

Fig. 1A shows the electropherogram from which the migration time of the middle peak (SP) is calculated, and used for the fraction collection. Fig. 1B shows the electropherogram of the reinjected SP that had been collected into

10 μl of the same CZE buffer (20 m*M*, pH 2.5) and demonstrates that a sufficient amount of SP was collected in a single electrophoresis for the reinjection to be detected by high sensitivity (0.002 AUFS) UV detection. Although the collected SP fraction was confirmed electrophoretically in Fig. 1B, reinjection of the sample collected in 1 m*M* of the CZE buffer gives a much narrower peak width (Fig. 1C) due to the improved electrophoretic "focusing" effect [44].

This fraction collection demonstrated by the data in Fig. 1 proves that manual fraction collection on this instrument is possible. However, manual fraction collection is tedious and involves a manual change of outlet beaker to collection vial, and a change of vials for multiple collections and for the capillary/electrode rinse between collections. The collection of more than one fraction becomes too time-consuming and impractical, especially when an experiment involves the fraction collection of a completely unknown sample and requires the collection of many different fractions for further analysis such as with MS to identify each analyte of interest. Furthermore, repetitive fraction collection is often necessary to accumulate a sufficient amount of material for further analysis. Therefore, the collection of more than one fraction in a single electrophoretic separation and repetitive collections would be facilitated by an automatic fraction collector.

Accordingly, the autosampler of our electropherograph was converted for use as an automatic fraction collector, although the instrument was not designed for such a function. Fig. 2 demonstrates this conversion. Basically, after sample injection (shown by the dotted lines), the capillary outlet was manually and easily positioned to the sampler arm that inserts both the capillary outlet and the ground electrode into the appropriate OBV in the 40-sample carousel. Through the extended mode computer program(s) that enables one to instruct the electropherograph to perform step-by-step operations, multiple fraction collection becomes possible using this modified configuration. Furthermore, repeated collection of the fractions is made more conveniently. The key to this successful conversion is the extension wire that electrically connects the ground (−) electrode to the autosampler (fraction collector), thus completing the electrical circuit required for electrophoresis.

Fig. 3 shows the four manually selected times used in Table 1. Table 1 summarizes the step-by-step transformation of those migration times into empirical times for fraction collection. Based on these calculations, $DynA_{1-17}$, SP, and LE-K were collected in a single electrophoretic separation. Fig. 4A is the electropherogram of the mixture used in Fig. 3, but now with a 50 μm I.D. capillary. In Fig. 4B–D the electropherograms of the three individual peptides that were reinjected from the three collected fractions are shown; they all agree well (>98%) with the migration times of the electropherogram of the mixture of the three peptides (Fig. 4A). Also, note the absence of any memory effect in the electropherograms in Fig. 4B–D. The recovery of peptide in the electropherograms shown in Fig. 4B–D, compared to Fig. 4A, is high (75%). That high recovery demonstrates that the calculation of each collection window is accurate.

We have observed that a combination of (a) a programmed delay of ca. 1 min after the termination of the applied voltage before moving on to the next fraction collection vial and (b) voltage ramping and a two-fold reduction of the voltage during the fraction collection increases the analyte recovery. The programmed delay allows the analyte that might have bound to the electrode and the capillary to diffuse into the solution in the collection vial. Voltage ramping at the onset of the electrophoretic collection process minimizes any rapid heating and thermal expansion of the buffer. Voltage reduction enhances peptide recovery, presumably because the interaction between the electrode and analyte decreases. Based on peak areas, we estimate that the recovery of each peptide was typically >65% under these experimental conditions. Biehler and Schwartz [18] also reported that, by reducing the voltage during fraction collection, recovery could be increased, and obtained a recovery of ca. 60%, also based on peak areas. Finally, to avoid any memory effect during the fraction collection, it is recommended that the

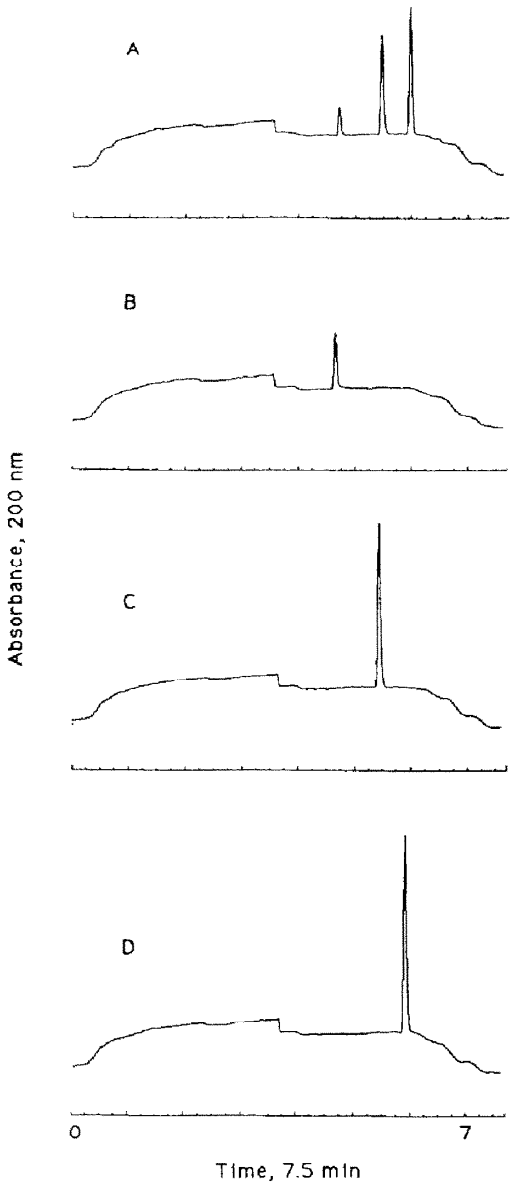

Fig. 4. Electropherograms of the mixture of three peptides, and of the three collected fractions reinjected under analytical conditions. A 63 cm × 50 μm I.D. capillary, with 41 cm length to the UV detector, was used. The injection volume was ca. 20 nl. (A) Electropherogram of the mixture of the three synthetic peptides used in Fig. 3 (in Fig. 3, an ca. 200-fold greater amount was injected); peaks from left to right represent $DynA_{1-17}$ ($t = 4.7$ min), SP ($t = 5.5$ min), and LE-K ($t = 6.0$ min), respectively. (B–D) Electropherograms of the individually collected $DynA_{1-17}$ ($t = 4.7$ min), SP (5.4 min), and LE-K (5.9 min) fractions, respectively. AUFS = 0.005 for A–D.

ground electrode and the capillary be rinsed in a buffer vial between collections.

4. Conclusions

Manual and automatic fraction collection are demonstrated for a commercial electropherograph using 50 and 100 μm I.D. capillaries. When important factors such as voltage ramping/deramping, electrode/capillary rinsing, and empirical safety margin are experimentally determined and regulated, precise single or multiple collection without any memory effect is possible under these experimental conditions. A combination of reduced voltage and a delay period after the voltage is turned off maximizes the peptide recovery. Subsequent manipulation and analysis of the analyte obtained from the preparative CZE are important steps in identifying a peptide such as with amino acid sequence determination, especially when studying samples from biological sources.

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