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Journal of Chromatography B, 736 (1999) 273–287

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

# Characterization of a solid-phase extraction device for discontinuous on-line preconcentration in capillary electrophoresis-based peptide mapping

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Received 23 June 1999; received in revised form 11 October 1999; accepted 12 October 1999

**Abstract**

Peptide mapping by capillary electrophoresis (CE) with UV detection is problematic for the characterization of proteins that can only be obtained at low micromolar concentrations. Dilution of peptide fragments during digestion of the protein can further reduce the detection sensitivity in peptide mapping to the point where analysis at sub-micromolar concentrations is not possible. A remedy to this problem is preconcentration (sample enrichment) of the proteolytic digest by solid-phase extraction (SPE). To minimize non-specific adsorptive losses during sample handling, on-line SPE–CE is preferred. However, packed-inlet SPE–CE is not always feasible due to either instrument or sample limitations. We describe here a simple method of preconcentration by discontinuous on-line SPE–CE, specifically applied to peptide mapping in low-pH separation buffer after protein digestion in a solid-phase enzyme microreactor. The SPE–CE system does not require application of a low pressure during electrophoretic separation to overcome reversed electroosmotic flow because the preconcentrator device is disconnected from the separation capillary before the electric field is applied. Up to a 500-fold preconcentration factor can be achieved with this device, which can be reused for many samples. Parameters such as the volume of desorption solution, the adsorption/desorption (chromatographic) process, reproducibility of packing the SPE preconcentrator and effects of sample concentration on the peptide map are investigated. 

*Keywords:* Preconcentration; Peptide mapping

## 1. Introduction

Over the past 17 years capillary electrophoresis (CE) has proven very effective for peptide and protein analysis amongst many other applications. CE has particularly established its capability in the area of peptide mapping [1–4]. This comparative technique is useful for confirming the identity of a protein which, for example, can intervene in diseases like Hepatitis C [5] and gastric cancer [6]. Peptide mapping essentially consists of site-specific cleavage of the protein by an enzyme, followed by separation and detection of the ensuing peptide fragments. As a consequence, the post-translational modification of a protein can be detected by a shift in migration time of the peptide bearing that alteration. The limitation of this technique is that biological samples often yield low concentrations of proteins in relatively

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0378-4347/99/$ – see front matter 
PII: S0378-4347(99)00472-7

small volumes after extraction and purification, making the peptide fragments created by proteolysis difficult to detect.

The advantages associated with CE arise from one important characteristic of the capillary – its micrometer dimensions, which allow for efficient dissipation of Joule heating. Therefore, separations can be performed at high electric field strengths and thus are often faster than by high-performance liquid chromatography (HPLC). However, injected sample volumes in CE must be extremely small (1–10 nl) to avoid column overloading. This is advantageous when only very small volumes of analytes are available for analysis. In this respect, CE is convenient for peptide mapping of small volume samples. However, low concentrations of analyte rather than very small sample volumes are more commonly encountered. Compounding this problem, on-column UV absorption detection of only a few nanoliters over an optical path length of 50–100 μm severely handicaps visualization of the protein digestion fragments. To overcome this detection limitation, various chemical and instrumental techniques have been developed. For example, injection techniques to enhance concentration include analyte stacking [7–10] and field amplification [11]. In these cases, analyte zones are stacked or focused due to differing ion mobilities in various field strengths or chemical environments. Instrumental methods to improve the sensitivity of detectors involve increasing the optical pathlength by means of using a bubble cell, Z-cell and other devices [12–14]. Chemical methods that produce extraordinary detection enhancement for CE can be achieved by laser-induced fluorescence. Unfortunately, the limitation of this technique is the relative inefficiency of the reaction between the fluorescent derivatization reagent and many analytes [15].

Another approach to improve detectability in CE is to undertake extensive off-line sample pretreatment and concentration. However, substantial losses of analytes to exposed surfaces can occur for most organic molecules and especially biopolymers like proteins and peptides. Also, extensive handling of concentrated solutions of proteins or peptides can lead to denaturation, aggregation and eventually precipitation and sample loss. Traditional preconcentration (sample enrichment) methods often suffer from a lack of convenience or unsuitability with either the CE system employed or the type of analyte under consideration. As a result, many researchers have recently focused on the development and use of solid-phase extraction (SPE) – based preconcentration methods coupled on-line with CE [16–31]. For example, Guzman et al. [16] constructed a concentrator device that consisted of a bed of an immunoaffinity resin and was coupled to a CE capillary for analysis of specific analytes. Debets et al. [17] described a micro pre-column that could be switched on-line and off-line with the separation capillary by means of a valve that contained the solid-phase material. A packed-inlet capillary developed by Waters Company (Accusep C/PRP capillary), which contained $C_{18}$ (octadecylsilane) reversed-phase HPLC packing scintered in place, was used to improve the sensitivity of CE analysis of pharmaceuticals [20]. Landers and co-workers [25,26], as well as that of Tomlinson et al. [23] and Beattie et al. [21], designed a detachable preconcentrator cartridge containing $C_{18}$ beads.

While SPE–CE is efficient at concentrating hydrophobic compounds, this method suffers from several drawbacks: reversal of the electroosmotic flow (EOF) at low pH [32], memory effects [33] and peak broadening [34]. In the past five years, Naylor and co-workers have developed and implemented a membrane-based preconcentrator for use on-line with CE (mPC-CE) [22–24,35–45]. The philosophy behind the development of mPC-CE was to decrease or remove potential problems associated with SPE–CE. Using a membrane, for example polymeric styrene–divinyl benzene copolymer (SDB), it is possible to minimize the bed volume at the capillary inlet as well as avoid the need for frits. As a result, a smaller volume of desorption solution is used in comparison to that needed with preconcentration using a packed bed of $C_{18}$ beads. The smaller desorption volume should greatly increase peak efficiency in the electropherogram [36]. Li et al. [30] made a comparison of the two sorbents, $C_{18}$ beads and SDB membrane, for the preconcentration of hydrophobic peptides. The most significant difference noted was a slight increase in sensitivity with the SDB membrane for most of the peptides. The only drawback of the SDB membrane was the lower coefficient of linearity observed over the analyte concentration range ex-

amined. Non-linearity at higher concentrations was attributed to the membrane having reached its maximum adsorption capacity.

Our previous experience in developing a capillary-sized protein microsequencer [46] in which both adsorptive membranes and porous silica bead cartridges were studied showed that the small, packed bed of silica beads had a higher adsorptive capacity for polypeptides than the membrane. In addition, the packed bed was better equipped to trap larger sample volumes. Therefore, for preconcentration of protein digests, we have chosen to investigate the use of a $C_{18}$-bead packed bed for SPE–CE, similar to that used by Landers and co-workers [25,26,32,47] and Beattie et al. [21]. Most papers describing the development of SPE–CE techniques use well-behaved samples to demonstrate the utility of the device. In this paper, we describe the preconcentration of a more complex mixture of polar and non-polar peptides (e.g., a tryptic digest), highlighting the effect of the preconcentrator fabrication (i.e., packing), the volume of desorption solution, the peptide concentrations and the adsorption/desorption (chromatographic) process on the peptide map. The tryptic peptides are obtained by digestion of protein sample in an immobilized-enzyme microreactor described elsewhere [48].

## 2. Experimental

### 2.1. Materials

Acetonitrile (MeCN), methanol (MeOH), controlled pore glass–diisothiocyanate–trypsin (CPG–DITC–trypsin) and bovine β-casein, dephosphorylated, were purchased from Sigma (St. Louis, MO, USA). Monobasic sodium phosphate and ammonium acetate were purchased from Anachemia (Montreal, Canada). $C_{18}$ beads (40 μm diameter) were purchased from Aldrich (Milwaukee, MI, USA). A 5 p.s.i.g. gauge and valve were purchased from Labcor (Anjou, Canada) (1 p.s.i.=6894.76 Pa). Vials (20 ml) were purchased from Fischer Scientific (Nepean, Canada). Swagelock connectors, a reducing union, 0.2 μm pore nylon syringe filters and PTFE tubing were purchased from Chromatographic Specialties (Brockville, Canada). Fused-silica (f.s.) capillary tubing (75 μm I.D.×350 μm O.D.) and f.s. Innerlok capillary connectors were obtained from Polymicro Technologies (Phoenix, AZ, USA). Polyethylene tubing (0.38 mm I.D., Intramedic No. 7405 Clay Adams) and syringes (30 ml and 1 ml) were purchased from VWR-Canlab (Mississauga, Canada). Laboratory-distilled water was purified with a multicartridge Millipore water filtration/deionization system before use.

### 2.2. Buffer and sample preparation

A 50 m*M* sodium phosphate, pH 2.5, buffer prepared from Millipore-purified water and filtered (0.2 μm pore membrane) was used for all CE separations and manipulations involving the SPE preconcentrator. Elution of peptides from the preconcentrator was achieved using a solution of acetonitrile–50 m*M* sodium phosphate at pH 2.5 (90:10, v/v). The peptide sample was obtained from tryptic digestion of β-casein using an immobilized-enzyme microreactor, described in detail elsewhere [48]. Briefly, a 40 μl solution of β-casein was perfused at 0.15 μl/min through a microreactor (28 cm×530 mm I.D.) packed with CPG–DITC–trypsin beads. Collection of the peptide fragments (~36 μl) into a 500-μl vial was made after approximately 2.5 h.

### 2.3. CE instrumentation

Separations were carried out on a SpectroPHORESIS 100 (Thermo Separation Products, Mississauga, Canada) equipped with a variable-wavelength UV detector. In this work, detection of peptides was carried out at $\lambda$=200 nm. Separations were performed in a 75 μm I.D. uncoated f.s. capillary with an effective length of 45 cm. The capillary was rinsed with 0.1 *M* NaOH for 2 min followed by a buffer rinse for 2 min prior to sample injection. Analog output from the detector was either recorded on an Chromjet Reporting Integrator (Thermo Separation Products) or digitally converted and acquired on an IBM-PC computer using Maxima 820 software (Dynamic Solutions, Division of Millipore). Stored electropherograms were imported as data files from the Maxima software into a Microcal

Origin (Northampton, MA, USA) spreadsheet for plotting.

### 2.4. SPE preconcentrator fabrication and operation

Construction of the SPE preconcentration device was based primarily on that described by Landers and co-workers [25,26] and Beattie et al. [21]. Essentially, the preconcentrator consists of a short (1 cm) piece of polyethylene tubing which contains a 50–225 nl bed (i.e., 0.25 to 1.5 mm length) of $C_{18}$ beads held in place by f.s. capillary tubing at both ends. To make this device, a 6 cm piece of 75 μm I.D. f.s. capillary was first inserted into one end of the polyethylene tube. By gently tapping the free end of the polyethylene tubing into an Eppendorf tube containing the $C_{18}$ beads, several beads can be trapped inside the tubing and held in place by inserting the second short piece (3 cm) of f.s. capillary tubing. The $C_{18}$ beads were conditioned by exposure to methanol vapors for 2 min prior to packing to eliminate electrostatically induced clumping. Care was taken to keep the packing loose in order to avoid a high back-pressure during sample loading and elution, even though the $C_{18}$ bed length was $\leq$1.5 mm. The exact length of the bed was measured using a microscope. No frits were needed as bed supports because the $C_{18}$ beads stayed firmly inside the cartridge once the f.s. tubing was inserted. Finally, the SPE preconcentrator cartridge was rinsed with approximately 10 volumes of methanol by using a 1-ml disposable Tuberculin syringe that had been adapted with a f.s. Innerlok connector to fit the 75 μm I.D. f.s. capillary tubing. In the above-mentioned literature references, the SPE preconcentrator was directly attached to the separation capillary and remained in place throughout sample loading, desorption and CE separation. For reasons discussed in Section 3, this was not feasible and we thus had to resort to the procedure that follows. A summary of the times required for preconcentrator-cartridge fabrication and the loading/desorption steps is given in Table 1.

#### 2.4.1. Sample adsorption

Loading of the tryptic digest was achieved by flushing the peptide solution (40 or 100 μl) through the SPE preconcentrator cartridge manually at a rate of approximately 8 μl/min using the same type of 1-ml syringe/Innerlok system described above in the rinsing step.

#### 2.4.2. Discontinuous on-line desorption

Elution of the tryptic digest was achieved using a laboratory-built liquid delivery system based on air pressurized by a manually operated 30-ml syringe (Fig. 1). CE separation buffer and desorption solvent were delivered from a 20-ml glass vial (Kimble Glass, Vineland, NJ, USA) having a screw cap through which a 1.3 cm diameter hole had been drilled out. A PTFE (Teflon) disc, 1.5 cm in diameter, 0.2 cm thick, was tapped with two threaded holes: one for a 3/8 in. connector and the other for a 1/16 to 1/32 in. reducing union (1 in.=2.54 cm). This disc fit snugly between the glass vial and screw cap, sealed by an o-ring to make the device gas tight. The 3/8 in. connector was linked by 60 cm of 0.04 in. I.D. PTFE tubing to a 30 kPa gauge (Cole-Parmer) and a 30-ml syringe. The reducing union

Table 1
Times required for fabrication and manipulation in discontinuous on-line preconcentration CE

| Procedure | Time |
|---|---|
| Precondition $C_{18}$ beads with methanol | 2 min |
| Preconcentrator fabrication and packing followed by 20 s methanol wash | 2 min |
| Sample loading (40 μl tryptic digest) by syringe | 5 min |
| Connect preconcentrator inlet to liquid delivery system; 20 s buffer rinse | 40 s |
| Connect preconcentrator outlet to CE capillary inlet; change delivery vial to acetonitrile–50 m*M* phosphate (90:10, v/v); 5 s desorption | 20 s |
| Change delivery vial to buffer and transfer elution plug into CE capillary | 50 s |
| Disconnect CE capillary inlet from preconcentrator and immerse into buffer reservoir | 20 s |
| CE separation | 25–35 min |

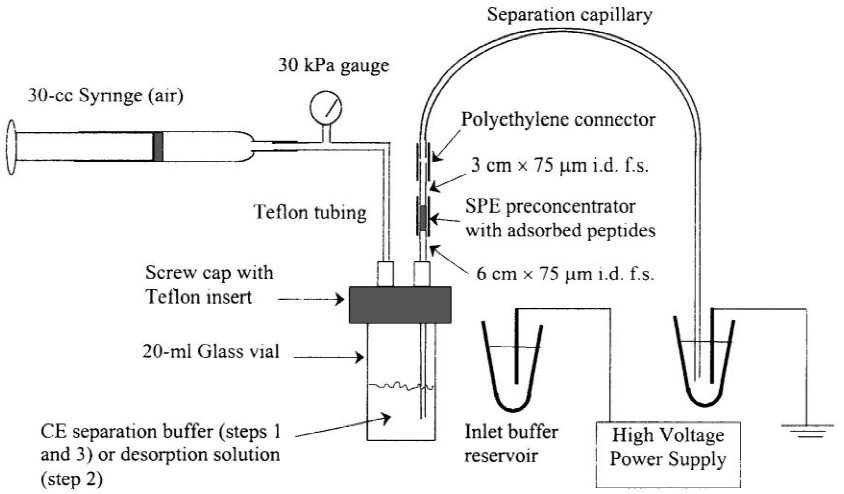


Fig. 1. Schematic representation of the SPE preconcentrator–CE system used for discontinuous on-line desorption. Details of its operation can be found in Section 2.4. Briefly, once sample has been loaded onto the SPE preconcentrator cartridge, desorption takes place as follows: (1) the preconcentrator inlet (6 cm f.s. tube) is connected to the liquid delivery system and the device is rinsed with separation buffer for 20 s, (2) the separation capillary inlet is attached to the preconcentrator outlet (3 cm f.s. tube) and tryptic peptides are eluted from the preconcentrator in 65±15 nl desorption solution by pressurization for 5 s, (3) separation buffer is pressurized for 45 s to push the elution plug well into the separation capillary, (4) the separation capillary is disconnected from the polyethylene connector and dipped into the inlet buffer reservoir to commence separation.

was connected to the 6 cm end (inlet) of the SPE preconcentrator cartridge, such that the f.s. capillary could be threaded through to the bottom of the glass vial, which contained either 10 ml of pH 2.5 CE separation buffer or 10 ml of desorption solution (acetonitrile–50 m*M* phosphate at pH 2.5, 90:10, v/v). The other end (outlet) of the SPE preconcentrator was either left open (first step, below) or connected to the separation capillary (second step) via a sleeve of polyethylene tubing.

In the first step, the SPE preconcentrator cartridge (containing adsorbed peptide) was flushed with separation buffer by applying a pressure of 25 kPa for 20 s inside the glass vial by manually activating the piston of the 30-ml syringe. In the second step, the separation capillary inlet was attached to the outlet of the preconcentrator and the glass vial containing separation buffer was replaced with one containing desorption solution, which was perfused through the preconcentrator by applying a pressure of 25 kPa for 5 s (to deliver 65±15 nl). In the third step, the glass vials were quickly switched back so that buffer solution could be used to push the *entire* desorption plug completely into the separation capillary inlet by applying 25 kPa for 45 s. Finally, the separation capillary was disconnected from the SPE preconcentrator cartridge and dipped into the inlet buffer reservoir of the CE system and the high voltage applied. Although the steps involved in discontinuous on-line desorption required several manual manipulations, they could be carried out rapidly (see Table 1) and easily.

### *2.4.3. Off-line desorption*

Elution of the tryptic digest was achieved using, for the most part, the same system as above except that the 30-ml syringe and 30 kPa gauge were replaced by an argon tank with a two-stage pressure regulator. The SPE preconcentrator was not connected to the separation capillary. Instead, after adsorption and rinsing with separation buffer, the outlet of the SPE preconcentrator was inserted into a 0.5-ml polypropylene microcentrifuge tube. By applying a pressure of 35 kPa argon in the 20-ml glass vial, the desorption solution was flushed through the SPE preconcentrator and the first 5 μl of this solution was retrieved at the cartridge outlet, in the microcentrifuge tube. This tube was then placed in

the CE system carousel and approximately 15 nl was injected for separation.

## 3. Results and discussion

Critical in peptide mapping is the reproducibility of maps (electropherograms) for a given protein. In order to achieve this, we constructed an immobilized trypsin-based microreactor for protein digestion [48]. Trypsin is a highly specific enzyme that cleaves proteins at the C-terminal side of lysine and arginine residues, resulting in a relatively simple series of peptide fragments from the parent protein sample. The protein used in this study was dephosphorylated bovine β-casein, which has a relatively open conformation (i.e., no disulfide bridges) to facilitate digestion by trypsin. One of the major advantages of digestion in the solid-phase microreactor compared to solution phase proteolysis is the lack of trypsin autoproteolysis products, thereby simplifying the peptide map and improving its reproducibility [48–50]. We are therefore working under conditions that should result in highly reproducible maps. To demonstrate this, Fig. 2 shows the tryptic peptide maps, without preconcentration, of three aliquots of β-casein (80 μ*M*) digested in three different microreactors.

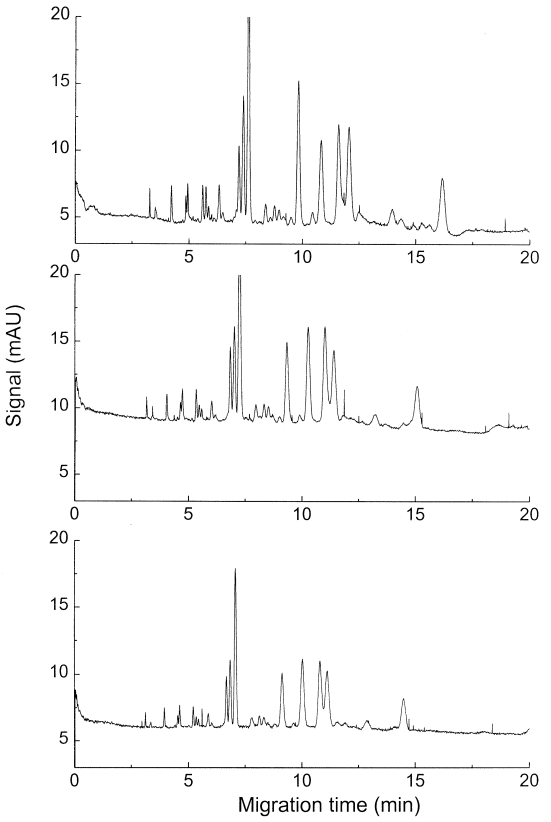


Fig. 2. Electropherograms showing the reproducibility of peptide mapping without preconcentration. Each panel represents the tryptic map of a different aliquot of 80 μ*M* of dephosphorylated β-casein in 100 m*M* ammonium carbonate, pH 8.1. Digestion was carried out by perfusion of 40 μl protein solution at 0.1 μl/min through an enzyme microreactor. Separations were carried out at 25 kV in 50 m*M* sodium phosphate buffer, pH 2.5. Sample was injected by vacuum at the capillary outlet for 0.35 s (15 nl injected).

As seen in Fig. 2, the reproducibility of the digests is very good in terms of relative peak height and relative migration time. Our previous study on the trypsin microreactor [48] showed that absolute peak heights and areas varied up to 47% when comparing three peptide maps. Nonetheless, absolute migration times displayed <3% variation in that study, which is sufficient for peptide mapping applications. It should be noted that the peak pattern in Fig. 2 differs from that seen in Ref. [48] because an old batch of immobilized trypsin and the phosphorylated form of β-casein were used for the previous study.

The goal of this work was to generate reproducible peptide maps from dilute protein solutions by means of SPE–CE with a $C_{18}$-based cartridge after first digesting the protein in our enzyme microreactor. To this end, several parameters were investigated such as the influence of chromatographic processes on the peptide map (i.e., adsorption), the volume of desorption solution, the volume of the solid-phase bed (i.e., the length of the SPE preconcentrator cartridge), and the sample concentration. It is important to point out here that unlike previous SPE preconcentrator designs (e.g., Refs. [21,25,26]), frits were not used to retain the solid-phase material in the cartridge. It has been suggested that bed supports (i.e., frits) can cause excessive dead volume, irreversible adsorption and loss of efficiency during preconcentration [51]. The only solid-phase material that we investigated was 40 μm diameter $C_{18}$ beads, primarily to avoid high backpressures and the need for frits.

### 3.1. Influence of the chromatographic process on peptide mapping

In our first attempt to investigate the effect of chromatographic processes arising from the SPE preconcentrator, we left the device attached to the capillary inlet during separation, similar to that described by Landers and co-workers [25,26] and Beattie et al. [21]. We wanted to compare the peptide map of an 80 μ*M* β-casein sample to one in which the same digest was diluted 100 times, then loaded (40 μl) on the preconcentrator cartridge. A 65±15 nl plug of the desorption solution (acetonitrile–phosphate buffer, 90:10, v/v) was injected and pushed past the preconcentrator to elute the peptides from the $C_{18}$ sorbent. Upon application of high voltage across the SPE-CE system, no peptides were detected. This phenomenon has also been reported by Strausbauch et al. [32] who postulate that peptide eluting from the $C_{18}$ sorbent is carried into the separation capillary and then re-adsorbed shortly after application of the electric field due to reversal of EOF in the capillary. Essentially, the peptide begins to electrophoretically migrate at the cathodic interface of the desorption and buffer solutions. However, the field strength is not constant throughout the capillary due to the presence of the desorption plug so a strong reversed EOF is generated in the region of the SPE preconcentrator. Strausbauch et al. [32] believe that unrecovered peptide bound to the solid-phase material creates a cationic charge on its surface, leading to the localized reversal of the EOF. They were unable to restore the cathodic EOF, even after rigorous washing of the SPE device. The same group observed highly suppressed EOF (>85 min) for preconcentration of peptides using the C/PRP capillary from Waters. Their solution was to apply a small pressure of 3.5 kPa concomitant with application of high voltage during the CE separation.

Inversion of the EOF in our studies only occurred at low pH. While CE separation at higher pH where the cathodic EOF is stronger might overcome flow reversal, this is not the optimum pH for peptide mapping. In addition, Chien and Burgi [11] reported that a mismatch of local EOF velocities in such a discontinuous pH buffer system can create mixing and a laminar back-flow at the interface of the buffers, seriously degrading resolution and peak shape. Therefore, we chose to retain the low-pH separation buffer (pH 2.5) to match the desorption solution pH. In off-line preconcentration studies (data not shown), the desorption solution was found to give better overall peptide maps when it contained 10% sodium phosphate buffer (pH 2.5) rather than 10% dilute HCl, which was used in other SPE–CE studies [32]. We found that HCl in the desorption solution caused unstable and irreproducible baselines at separation voltages over 15 kV.

Application of a small pressure to ensure a positive EOF as described by Strausbauch et al. [32] is not feasible with all CE instruments, can lead to additional peak broadening and would have to be optimized depending on the sample, thus making it a potentially time-consuming step. Therefore, to circumvent the reversed EOF problem, we decided to disconnect the SPE preconcentrator cartridge from the capillary inlet just prior to separation (Fig. 1). By performing this ''discontinuous on-line desorption'' process, where 100% of the volume desorbed is transferred into the separation capillary, we could reach a higher preconcentrator factor than with traditional off-line SPE preconcentration. Fig. 3 compares three peptide maps: (1) without any sample enrichment (80 μ*M* digest, Fig. 3A), (2) with off-line preconcentration (80 μ*M* digest diluted 10-fold, Fig. 3B), and (3) with discontinuous on-line preconcentration (80 μ*M* digest diluted 100-fold, Fig. 3C). These three electropherograms have been plotted on the same scale to demonstrate relative peak heights. Off-line preconcentration (Fig. 3B) was achieved by loading 100 μl of the digest (diluted to 8 μ*M*) onto the solid-phase followed by elution in 5 μl of the desorption solution (i.e., a 20-fold predicted preconcentration factor), from which 15 nl was injected for CE separation. Discontinuous on-line preconcentration (Fig. 3C) was achieved by loading 40 μl of 800 n*M* digest onto the SPE device followed by elution in 65±15 nl desorption solution, directly into the separation capillary (i.e., an approximately 500-fold predicted preconcentration factor).

In comparing Fig. 3A and B (without and with preconcentration, respectively), some variations in the relative peak heights can be seen. Obviously, the peak height of each peptide depends strongly on its hydrophobicity. The more hydrophobic the peptide, the more it is retained on the sorbent and, hence,

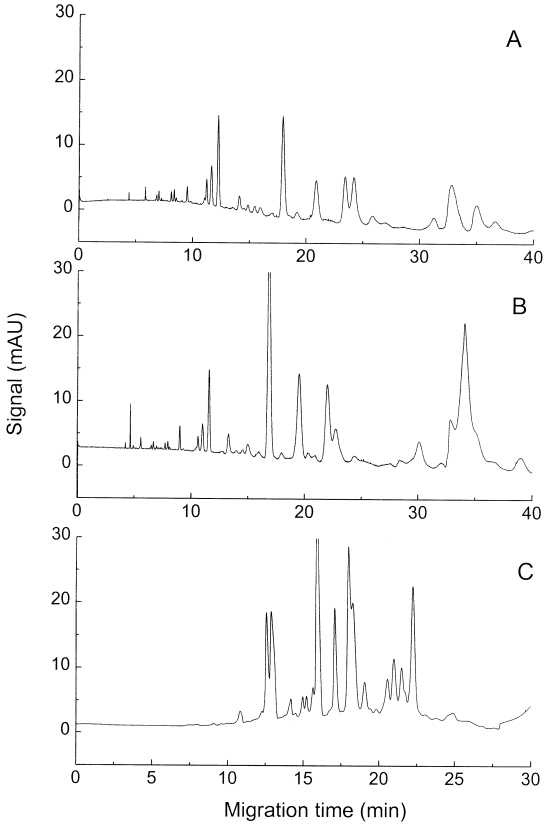


Fig. 3. Electropherograms showing the effect of preconcentration on the peptide map. (A) 80 μ*M* β-casein solution without preconcentration; (B) off-line preconcentration of a 10-fold dilution of the same 80 μ*M* β-casein solution, with 100 μl loaded and elution in 5 μl desorption solution; (C) discontinuous on-line preconcentration of a 100-fold dilution of the same 80 μ*M* β-casein solution, with 40 μl loaded and elution in 65±15 nl desorption solution (MeCN–50 m*M* phosphate buffer at pH 2.5, 90:10). A 1.5 mm SPE preconcentrator cartridge was used in (B) and (C). In all three cases, separations were carried out at 18 kV in 50 m*M* sodium phosphate buffer at pH 2.5. Injection times were 0.35 s by vacuum for (A) and (B).

preconcentrated to a higher degree. While the predicted equivalent concentration in Fig. 3B is 160 μ*M* (i.e., 80 μ*M*÷10-fold dilution×20-fold preconcentration), several peaks were not 2-times higher than the 80 μ*M* sample (Fig. 3A). Recovery from the SPE preconcentrator, like enrichment, varies greatly with the hydrophobicity of each peptide. Our early studies of off-line desorption using leu-enkephalin as a model peptide showed 79% recovery [52]. This value agrees with that found by Bateman et al. [33]. These authors also reported a preconcentrator memory effect where subsequent application of elution solvent provided variable amounts of peptide ranging from 20 to 40% of the first elution plug, although this effect was more severe for 5 μm than 40 μm $C_{18}$ particles.

In the case of discontinuous on-line preconcentration (Fig. 3C), the peptide map of the same tryptic digest is completely different. The hydrophobicity of each peptide has an influence on the map not only in terms of the quantity of peptide retained on the sorbent and its absolute recovery from the sorbent but also in terms of its desorption rate. A highly hydrophobic peptide has a lower desorption rate than a less hydrophobic peptide, leading to a modification of the overall migration time. The SPE elution plug, which is also the CE injection plug, will not be uniform in composition across the length of the plug. In essence, a reversed-phase chromatographic process becomes superimposed on the CE separation. Such a large chromatographic effect was not seen by Strausbauch et al. [32], who simply noted a slight increase in resolution between two highly hydrophobic model peptides after preconcentration.

The presence of such a large quantity of elution solvent (65±15 nl) in the separation capillary should bring about an additional stacking effect on top of the theoretical preconcentration factor. The predicted equivalent concentration in Fig. 3C should be ca. 310 μ*M* taking into account an average recovery of 79% but not considering any transient stacking effects. While we do not have sufficient information (i.e., peptide standards or diode array data) to match up the peaks from Fig. 3A to C to determine the true preconcentration factor, there is clearly a large increase in the areas of several peaks in Fig. 3C. To determine if we could increase the elution window observed in Fig. 3C and improve sensitivity, we investigated the effect of increasing the desorption volume for the discontinuous on-line preconcentration system.

### *3.2. Influence of the volume of desorption solution on peptide mapping*

Fig. 4 shows the influence of tripling the desorption solution volume for discontinuous on-line preconcentration peptide mapping. The same tryptic

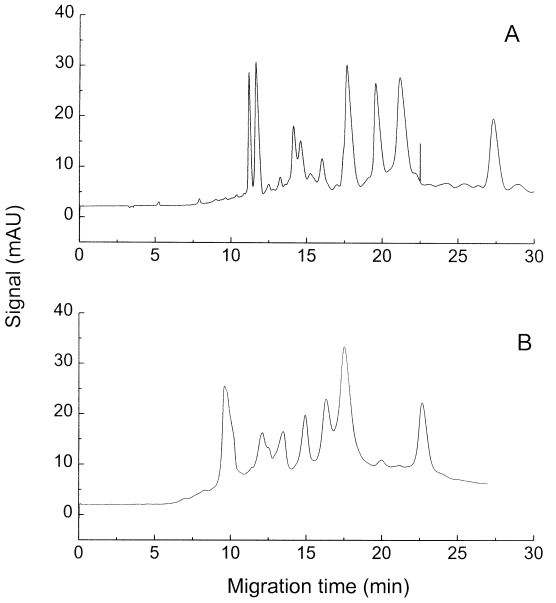


Fig. 4. Electropherograms showing the effect of tripling the volume of desorption solution for the same 800 n*M* tryptic digest (100-fold dilution of 80 μ*M* β-casein digest) in which 40 μl have been loaded on a 1.2 mm SPE cartridge for discontinuous on-line preconcentration. (A) Elution in 65±15 nl desorption solution; (B) elution in 175±20 nl desorption solution. Separations were carried out at 18 kV in 50 m*M* phosphate, pH 2.5.

digest (80 μ*M*, diluted 100-fold) and loaded sample volume (40 μl) were used in Fig. 4A and B, whereas the desorption solution volumes were 65±15 nl and 175±20 nl, respectively. Clearly, the volume of the elution plug has a strong influence on the peptide map in terms of peak shape and resolution. Within the large elution plug, the electric field is reduced due to the presence of organic solvent [53]. Therefore, the separation, which depends on differential electrophoretic mobility, is affected. Moreover, the diffusion process is much greater when the injection plug is so large. These two processes are commonly responsible for the lack of good peak efficiency and resolution in peptide mapping, highlighting the fact that elution volume optimization is necessary. For an SPE cartridge 0.3 mm in length, Strausbauch et al. [32] found that the optimum volume of desorption solution was 130 nl, about 2.5-times greater than the volume of $C_{18}$ sorbent. In their work, peak sensitivity was compromised for smaller volumes than this. Interestingly, these authors also reported that no reversed EOF was observed when the volume of desorption solution was <130 nl. In our case, the volume of desorption solution was 3.5 times *smaller* than the $C_{18}$ bed (225 nl), yet we still observed reversed EOF when the SPE preconcentrator was on-line (i.e., as a packed-inlet) with CE. The results in Fig. 4 show that increasing the desorption solution volume to 175±20 nl severely degrades resolution and does not significantly enhance peak heights or areas as hoped.

### *3.3. Influence of the preconcentrator cartridge length on peptide mapping*

Fig. 5 shows the influence of SPE preconcentrator length (i.e., quantity of solid-phase material) on the peptide map for the same tryptic digest of β-casein. Aliquots of 40 μl from an 80 μ*M* digest diluted to 800 n*M* were loaded onto a series of SPE preconcentrators of lengths ranging from 0.5 mm to 1.5 mm. With a length of 0.5 mm, only five small peaks could be seen (Fig. 5A). Essentially, there was insufficient sorbent for retention of the tryptic peptides, with only a small fraction of the most hydrophobic peptides being adsorbed. Increasing the preconcentrator cartridge length to 1 mm (Fig. 5B) led to increased peak heights, but not a significant increase in the number of peaks. While these two factors depend on the volume of solid-phase in the preconcentrator cartridge, they also depend on the relative analyte hydrophobicity [29]. Further increasing the preconcentrator length to 1.5 mm (Fig. 5C) produced more peptide peaks with, for the most part, increased heights. The peaks at 12 to 13 min in Fig. 5B and C showed no change in height when increasing the preconcentrator length from 1 to 1.5 mm. These are likely the most hydrophobic peptides, retained just as well on the shorter as on the longer preconcentrator cartridge. As can be seen in Fig. 5, the overall length of the preconcentrator affects the pattern of peaks in the peptide map because the chromatographic process during desorption is a function of the length of $C_{18}$ stationary phase. On the other hand, it was expected that peptide maps obtained using the shorter preconcentrator would more closely resemble those without preconcentration. This was not the case. Therefore, a length of 1.5

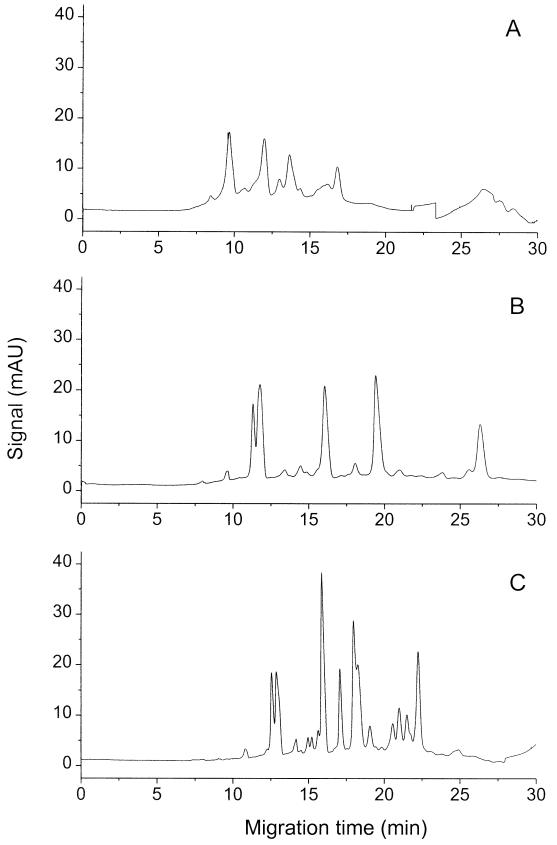


Fig. 5. Electropherograms showing the effect of SPE preconcentrator cartridge length on the peptide map of 800 n*M* β-casein tryptic digest (80 μ*M* solution diluted 100-fold). (A) 0.5 mm long preconcentrator; (B) 1 mm long preconcentrator; (C) 1.5 mm long preconcentrator. In each case, 40 μl tryptic digest was loaded onto the SPE preconcentrator and peptides were eluted in 65±15 nl desorption solution. Separations were carried out at 18 kV in 50 m*M* sodium phosphate buffer, pH 2.5.

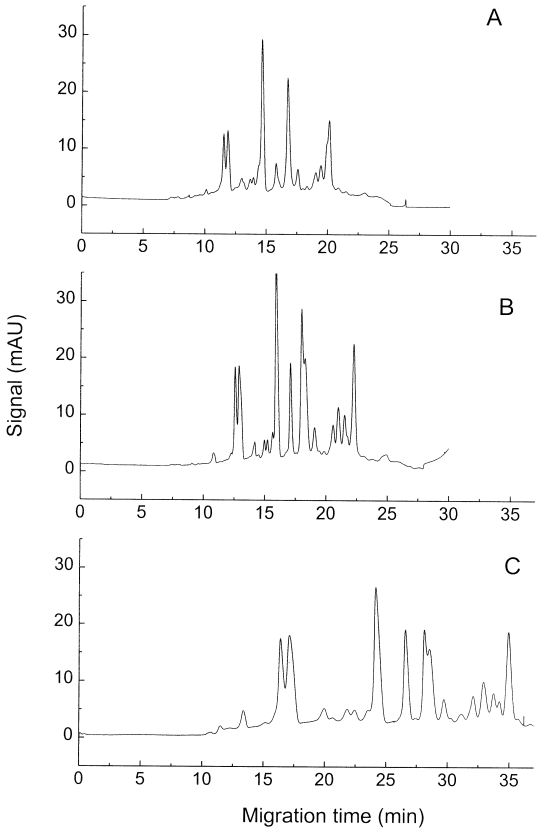


Fig. 6. Electropherograms showing the reproducibility of preconcentrator packing on peptide maps of 800 n*M* β-casein tryptic digest (80 μ*M* solution diluted 100-fold) for three different SPE preconcentrator cartridges (panels A, B and C), each 1.5 mm in length. Loading, elution and separation conditions were the same as in Fig. 5.

mm was determined to be the best amount of sorbent in the SPE preconcentrator cartridge.

### *3.4. Packing reproducibility*

To evaluate the reproducibility of packing the SPE preconcentrator, which is essentially a measure of the efficiency of fabricating the device, we compared the cartridge-to-cartridge variation for three preconcentrators. Fig. 6 shows three peptide maps in which 40-μl aliquots of the same digest (80 μ*M*, diluted 100-fold) were loaded onto three different preconcentrators. Although the preconcentrator cartridge length as well as the desorption and separation conditions were identical in each of the three cases, large variations in the peak migration times from Fig. 6A–C can be seen. While relative peak heights are similar for the three peptide maps, resolution is vastly different and absolute migration times vary from 18% for the first peak to 31% for the last peak. The differences seen in Fig. 6 arise from the packing procedure. For example, a tighter packing induces a higher backpressure, which is problematic during the desorption step and when pushing the elution plug into the separation capillary. In both these operations, a fixed pressure is applied for a specified time

interval to deliver a certain volume. In the case of desorption, a 50- to 80-nl variation in elution solvent volume, which is also the injection volume, arises leading to variable resolution of peaks. During transfer of this volume into the separation capillary, the elution plug does not travel as far into the capillary when backpressure from the SPE cartridge is high making the effective length of separation longer. Therefore, the migration times appear longer. This is the case for the electropherogram in Fig. 6C. On the other hand, if the packing is too loose, then a 1.5 mm SPE cartridge will have less overall sorbent material than a cartridge of the same length packed tightly. As a result, the chromatographic process will be different. In addition, the volume of desorption solution will not be compatible, which probably accounts for the poor resolution seen in the peptide map in Fig. 6A. While several discrepancies exist between peptide maps made with different SPE preconcentrators, this is much less of a problem when the same preconcentrator is reused as described in the next section.

### *3.5. Reusability of a preconcentrator cartridge for peptide mapping*

The reproducibility of peptide maps made using the same SPE preconcentrator (i.e., reusability) is shown in Fig. 7. In each case, 40-μl aliquots of the same digest (80 μ*M* diluted to 800 n*M*) were loaded onto the preconcentrator. The three electropherograms in Fig. 7 have been presented with offset time scales to show their similarities in terms of peak height and peak pattern. Differences in absolute migration times are manifested somewhat as a compression or expansion of the elution window, but

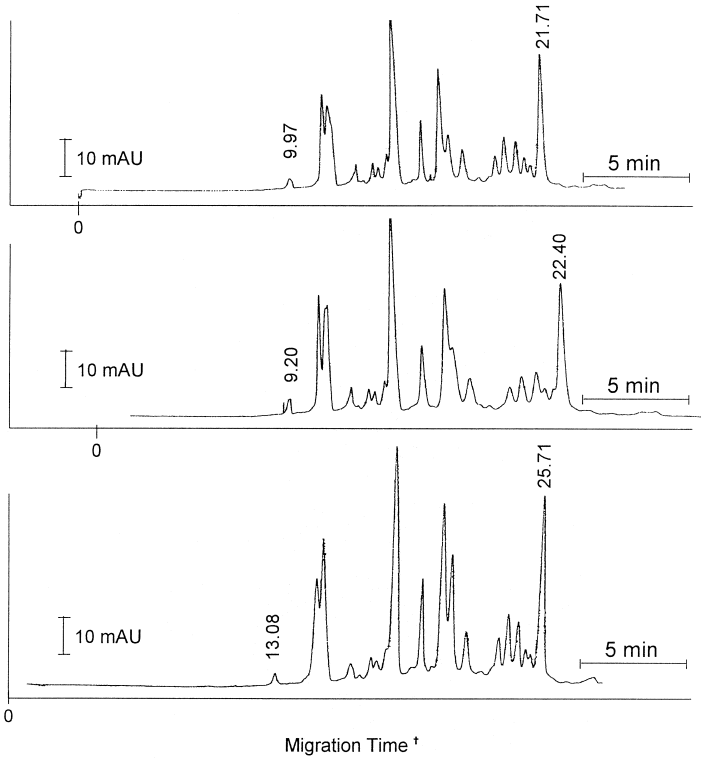


Fig. 7. Electropherograms showing the reusability of the same SPE preconcentrator (1.5 mm long) for peptide mapping of an 800 n*M* β-casein tryptic digest (80 μ*M* solution diluted 100-fold). Loading, elution and separation conditions were the same as in Fig. 5. †Data were originally recorded on an integrator and were thus scanned to offset the images for comparison of the peptide maps. Displacement of the elution windows with respect to each other is discussed in the text.

mostly as a displacement of the elution window. These differences arise from the method by which the SPE–CE system is pressurized. A slight change in the solid-phase packing organization after repeated sample loadings can lead to a tighter packing, thus increased backpressure. As mentioned in Section 3.4, the elution plug is not pushed as far into the capillary when backpressure is higher. The overall result is longer absolute migration times because the effective length of the capillary, from injection plug to detector, is longer. Even though the same desorption and separation conditions were used in each case, the large deviation in desorption solution volume ($65\pm15$ nl) accounts for the differences in resolution seen amongst the three peptide maps in Fig. 7. On the other hand, relative migration times are very reproducible. Table 2 presents the mean migration times and the RSD in migration time for the three maps in Fig. 7 using a two-peak normalization method [54]. The first and last peak were used for migration time correction, leading to migration time RSDs of less than 2.5%, which is sufficient for detection of protein modifications by comparison of two peptide maps. One SPE preconcentrator device was used for up to 30 runs (data not shown), demonstrating its robustness.

### *3.6. Effect of sample concentration on preconcentrator performance*

The effect of initial peptide sample concentration on the SPE preconcentrator performance was investigated. The SPE–CE peptide maps for concentrations ranging from 800 to 100 n*M* of the same tryptic digest of β-casein (initial concentration of 80 μ*M*) are shown in Fig. 8. The same preconcentrator cartridge was used in the 4 experiments, starting with the lowest concentration sample. Clearly, a peptide-specific correlation between sample concentration and detectability exists. There is a bias towards larger peptides, which are (1) typically more hydrophobic than small peptides, thus better retained by the SPE preconcentrator, and (2) often have a higher molar absorptivity that small peptides, so signal intensity is higher. From the electropherogram in Fig. 8D, loading 100 n*M* appears to be the minimum concentration of β-casein digest that can be mapped with our discontinuous on-line SPE preconcentrator design.

Strausbauch et al. [32] performed a similar study, although they increased the injected volumes of peptide standards as concentrations decreased so that mass loadings were equivalent. They found that

Table 2
Relative migration time precision for three peptide maps obtained using the same SPE preconcentrator

| Peak No.[a] | Mean migration time (min)[b] | Migration time RSD (%)[b] |
|---|---|---|
| 1 | 9.2 | 0 |
| 2 | 10.7 | 1.1 |
| 3 | 11.0 | 0.47 |
| 4 | 12.3 | 0.87 |
| 5 | 13.0 | 2.4 |
| 6 | 13.3 | 1.7 |
| 7 | 13.6 | 2.3 |
| 8 | 14.2 | 0.52 |
| 9 | 15.7 | 0.20 |
| 10 | 16.7 | 0.29 |
| 11 | 17.2 | 0.17 |
| 12 | 18.0 | 0.27 |
| 13 | 19.7 | 1.9 |
| 14 | 20.2 | 1.7 |
| 15 | 20.9 | 2.0 |
| 16 | 21.4 | 1.5 |
| 17 | 21.8 | 1.6 |
| 18 | 22.4 | 0 |

[a] Numbers correspond to sequential peaks in Fig. 7.
[b] Migration times were corrected using a two-peak normalization method [54].

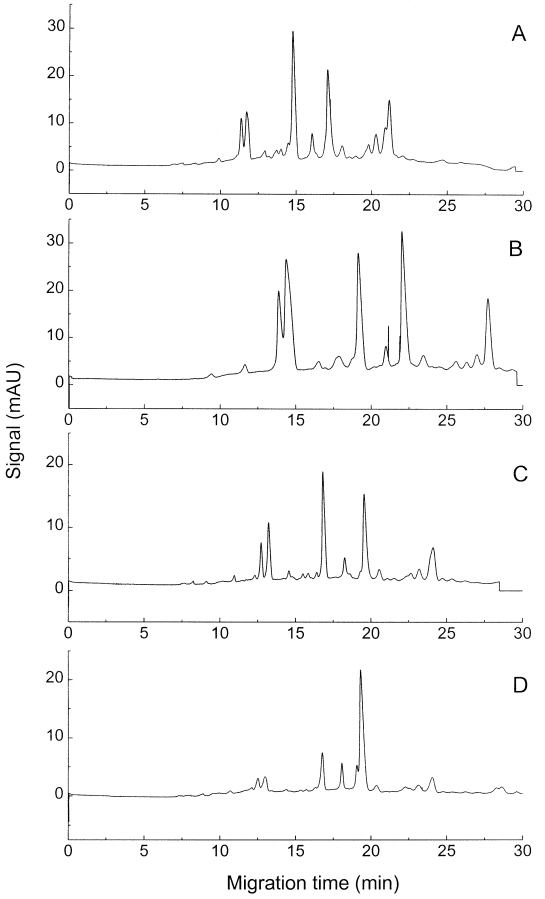


Fig. 8. Electropherograms showing the effect of sample concentration on SPE–CE for peptide maps of various dilutions of an 80 μ*M* β-casein tryptic digest. (A) 800 n*M* (100-fold dilution); (B) 400 n*M* (200-fold dilution); (C) 200 n*M* (400-fold dilution); (D) 100 n*M* (800-fold dilution). Loading, elution and separation conditions were the same as in Fig. 5. The same preconcentrator cartridge, 1.5 mm long, was used in all four experiments.

SPE–CE is concentration dependent, with loss of sensitivity at low concentrations (~400 n*M*) likely being caused by adsorptive losses during sample manipulation. Our results concur with theirs, although we did not adjust injected volumes as concentration changed. Instead, we used a constant injected volume of 40 μl because this is the volume of digest exiting the enzyme microreactor. On one hand, Strausbauch et al. [32] reported that the retentive capacity of their SPE cartridge (0.3–0.7 mm long) was not exceeded when loading up to 288 μl peptide (780 ng). This implies that we might improve peptide mapping sensitivity if the volume of protein sample loaded into the microreactor (currently only 40 μl) prior to SPE–CE was increased. On the other hand, attaining good peptide maps for lower protein concentrations may not be achievable due to the concentration dependence of SPE–CE.

For example, we examined the difference between the SPE–CE peptide maps of a 400 n*M* sample of β-casein (Fig. 9A) versus an 80 μ*M* sample of β-casein for which the peptide fragments were diluted to 400 n*M* (i.e., 200-fold) before SPE–CE (Fig. 9B). The same SPE cartridge and desorption and separation conditions were used to obtain the peptide maps shown in Fig. 9. While the relative migration window is the same for the two electropherograms, that in Fig. 9B is shifted towards zero due to variability in the distance the elution plug is pushed into the separation capillary as the $C_{18}$ packing tightens. Significant differences in relative peak height for the two peptide maps are seen in Fig. 9. The smaller peaks obtained for the digest of 400 n*M* protein (Fig. 9A) are likely due to sample losses in either the enzyme microreactor or the handling

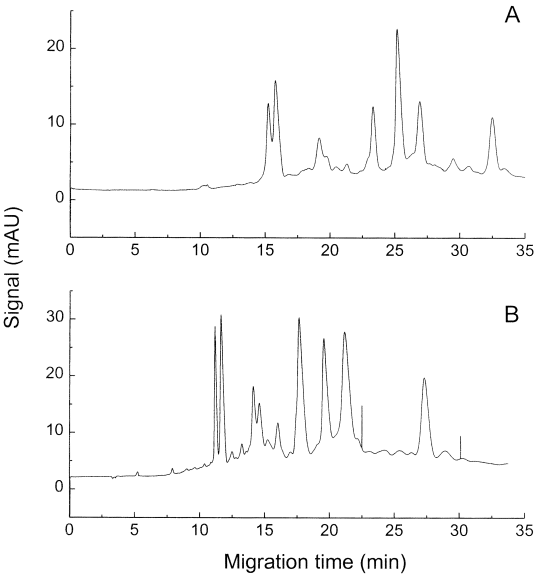


Fig. 9. Electropherograms comparing peptide maps for dilution before and after protein digestion followed by SPE–CE. (A) Tryptic digest of 400 n*M* β-casein and (B) a 200-fold dilution of a tryptic digest of 80 μ*M* β-casein (i.e., 400 n*M* sample). Loading, elution and separation conditions were the same as in Fig. 5. The preconcentrator cartridge was 1.2 mm long.

between digestion and preconcentration. At such low concentrations and small volumes (i.e., 40 μl loaded), any loss of peptide inside the microreactor due to adsorption on the CPG–trypsin beads or on the capillary wall will dramatically reduce the recovery of peptides. Further studies are underway to estimate recovery from the microreactor.

The peptide maps in Fig. 9 differ from that in Fig. 8B (also 400 n*M*) for the following proposed reasons. First, the SPE cartridge length was 0.3 mm shorter for the maps obtained in Fig. 9 than in Fig. 8, which implies a slight variation in the chromatographic process. Secondly, we used a different batch of dephosphorylated β-casein, which may contain impurities in the form of β-casein isoforms. For example, in studies where the protein was denatured with urea and electrophoresed in a bare f.s. capillary, we found that β-casein had several isoforms (data not shown). Presumably there are batch-to-batch differences in the quantity of the major isoform with respect to the total amount of protein. Finally, according to the label, the protein sample was approximately 80% dephosphorylated, which meant that variability in the quantity of phosphorylated versus dephosphorylated β-casein can probably occur between batches. Unfortunately, we were not equipped to further purify the protein before enzymatic digestion, which is highly desirable for high precision mapping. Otherwise, the electropherogram represents superimposed peptide maps of each unique protein in the sample, provided their concentrations are sufficient for detection. The fact that we have observed several differences, mostly in relative peak height, for peptide maps of different batches of β-casein digested using the same microreactor (data not shown) strongly suggests that our protein sample does not have a unique composition.

## 4. Conclusions

We have presented here a rigorous study of several operational aspects of a discontinuous on-line desorption SPE–CE system for mapping the peptides of dephosphorylated β-casein. This protein presented some complications with respect to evaluating peptide map reproducibility because it was found to have isoforms. However, the choice of using a peptide map for evaluating the performance of the SPE–CE device was deliberate, in that the complex mixture of hydrophobic and hydrophilic peptides provided a more realistic sample than the typically used peptide analogs or drugs. In particular, our results are the first to point out the severe effect of the chromatographic process from SPE–CE, which is superimposed on the electrophoretic separation.

While the quality of peptide maps was good for a 1.5 mm long bed of $C_{18}$ beads and a 65±15 nl volume of desorption solution, our results clearly show that a more precise method for metering the desorption solution, independent of the SPE cartridge backpressure, is necessary. We are currently testing an on-line SPE–CE design using micro valves to insure accurate and precise delivery of desorption solution. On the other hand, the current design of our discontinuous on-line desorption system (Fig. 1) could easily be adapted for delivery of buffer and desorption solution from 2-ml sample vials that fit into the CE instrument sample carousel to create a more compact system.

## Acknowledgements

Funding for this work was provided by the Natural Sciences and Engineering Research Council of Canada. E.B. gratefully acknowledges a graduate fellowship from the Université de Montréal. Part of this work was first presented at the 9th Annual Frederick Conference on Capillary Electrophoresis, Frederick, MD, 19–21 October 1998.

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