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# Serial displacement chromatofocusing and its applications in multidimensional chromatography and gel electrophoresis: II. Experimental results

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ABSTRACT

Part I of this study investigated the theory and basic characteristics of "serial displacement chromatofocusing" (SDC). In Part II of this study, SDC is applied to two prototype applications which have potential uses in proteomics and related areas involving the analysis of complex analyte mixtures. In the first application, SDC was used as a prefractionation method prior to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to separate a human prostate cancer cell lysate. It was observed that the resolution achieved in narrow-p*I*-range 2D-PAGE was improved when using SDC prefractionation, so that SDC may be useful as a low-cost, high-speed, and highly scalable alternative to electrophoretic prefractionation methods for 2D-PAGE. The second application involves the use of SDC as the first dimension, and reversed-phase chromatography as the second dimension, to produce a novel, fully automated, two-dimensional high-performance liquid chromatography technique. The method was shown to have performance advantages over one-dimensional reversed-phase chromatography for peptide separations.

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## 1. Introduction

Traditional chromatofocusing techniques employing a polyampholyte buffer have previously been used for sample prefractionation in a gel-based proteomics method [1], and as the first dimension in a two-dimensional high-performance liquid chromatography (2D-HPLC) proteomics method [2–6]. In these techniques, chromatofocusing achieves not only a high-resolution protein separation, but also a reasonably accurate isoelectric point (p*I*) based fractionation which facilitates its use in various multidimensional separation strategies. The usefulness of chromatofocusing can be increased if various shortcomings of the technique as traditionally practiced are eliminated. One shortcoming is the use of polyampholyte elution buffers, which often exhibit lot-to-lot variations in composition which may affect the repeatability of the method [7]. Polyampholytes also tend to form association complexes with proteins [8,9], which may complicate subsequent procedures, such as reversed-phase chromatography, mass spectrometry (MS) [10] or gel staining [1]. A second shortcoming is that a column packing with a uniform buffering capacity over a broad pH range has traditionally been used, in which case the choices for the packing are limited.

A variant of the chromatofocusing technique, termed "serial displacement chromatofocusing" (SDC), eliminates many of the shortcomings of the traditional chromatofocusing technique and involves the elution or displacement of proteins or peptides using a multistep, retained pH gradient formed using simple mixtures of buffering species. In Part I of this study, general aspects of SDC were investigated, including the use of computer-aided methods to design the gradient. In Part II of this study, the method will be used in multidimensional chromatography and gel electrophoresis and will be applied to prototype problems pertinent to the field of proteomics and related areas involving complex mixtures of analytes.

One particular application of SDC that will be investigated is its use as a sample prefractionation method prior to narrow-p*I*-range, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). When used this way, SDC provides an alternative to various chromatographic or electrophoretic sample prefractionation methods that have recently become popular in proteomics applications [1,10–39]. As shown in Fig. 1, when used as a prefractionation

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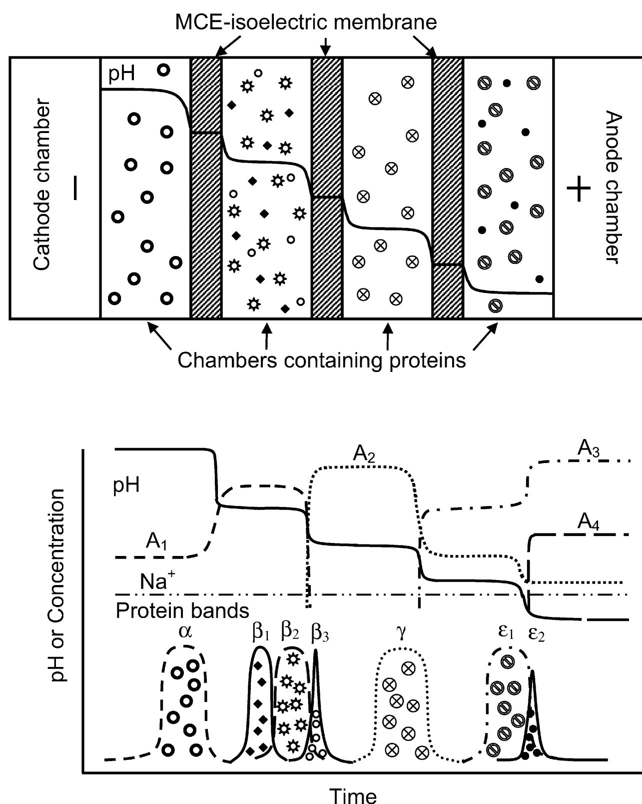


**Fig. 1.** The MCE-IM (top) and SDC (bottom). The SDC illustration is based on a strong-base ion-exchange column packing, in which case the buffer species $A_1$–$A_4$ would typically be tricine, MES, acetic acid, and formic acid, respectively, and the proteins $\alpha$, $\beta$, $\gamma$, $\varepsilon$ could be, for example, cytochrome *c* (p*I* ∼9–10.5), hemoglobin variants (p*I* ∼ 6.9–7.5), conalbumin (p*I* ∼ 5.9–6.6), and lactoglobulin variants (p*I* ∼ 5.0–5.5), respectively.

method, SDC can be considered to be the chromatographic analog of the multicompartment electrolyzer with isoelectric membranes (MCE-IM) [10,32,33,35–39], since the latter technique isolates proteins between membranes having a fixed pH similar to the way proteins are localized between pH plateaus in SDC. However, SDC has some potential advantages compared to the MCE-IM. For example, the pH at which a protein elutes during chromatofocusing, termed the apparent p*I*, differs somewhat from the true p*I* [40–42], so that proteins are slightly charged as they migrate down the column and therefore have an enhanced solubility in comparison to isoelectric focusing (IEF). At the same time, the true and apparent p*I* values are still sufficiently similar so that proteins generally elute in the order of their true p*I* values, in which case chromatofocusing is likely to yield fractions suitable for narrow-p*I*-range 2D PAGE. Nevertheless, by proper design of the method it may be possible to exploit small differences in the values of the p*I* and apparent isoelectric point ($pI_{app}$) to separate proteins not separable using exclusively a gel-based approach, since SDC may be able to direct proteins with nearly identical p*I* values to different narrow-p*I*-range 2D-PAGE gels by using their different $pI_{app}$ values.

A second application of SDC considered here is its use as the first dimension, coupled with reversed-phase chromatography as the second dimension, to form a 2D-HPLC method for the fractionation of peptide mixtures. This method may have applications in a non-gel-based "bottom-up" or "shotgun" proteomics approach where tryptic peptides obtained from a protein mixture digest are identified along with their protein source, either by the direct use of 2D-HPLC followed by tandem MS [43,44], or by using 2D-HPLC and tandem MS in combination with an affinity chromatography selection method or isotope labeling [45–47].

## 2. Experimental

### 2.1. Materials and columns

Trypsin Gold was obtained from Promega (Madison, WI, USA). Horse cytochrome *c*, bovine $\beta$-lactoglobulin A, horse myoglobin, and chicken lysozyme were obtained from Sigma (St. Louis, MO, USA). A protease inhibitor cocktail was obtained from Roche (Indianapolis, IN, USA). All chemicals were obtained from Sigma–Aldrich, except for formic acid, which was obtained from J.T. Baker (Philipsburg, NJ, USA). All buffer compositions are described in the figure captions, and the buffer solutions were prepared using deionized water which was vacuum filtered using a 47-mm diameter nylon membrane filter with 0.2-μm pores (Whatman, Clifton, NJ, USA).

A 7.5 cm × 0.75 cm I.D. TSK-GEL SP-5PW strong-acid ion-exchange column was obtained from Tosoh Bioscience (Montgomeryville, PA, USA). A 25 cm × 0.4 cm I.D. ProPac SAX-10 strong-base ion-exchange column was a gift from Dionex (Sunnyvale, CA, USA). A 0.75 cm × 0.21 cm I.D. P-HR trap column was obtained from Alltech (Deerfield, IL, USA), and a 5 cm × 0.21 cm I.D. ZORBAX 300 SB-C18 column was a gift from Agilent (Palo Alto, CA, USA).

### 2.2. Tryptic digest

Myoglobin, lysozyme, $\beta$-lactoglobulin A, and cytochrome *c*, each at 5 mg/mL, were dissolved individually in 6 M guanidine–HCl, 50 mM Trizma–HCl (pH 8), and 4 mM dithiotreitol (DTT). The protein solutions were heated to 95 °C for 20 min, cooled to room temperature, and then diluted 1:10 with 50 mM $NH_4HCO_3$ (pH 7.8). Trypsin Gold was then added to each individual protein solution to a final protease:protein ratio of 1:30 (w/w), and each solution was incubated at 37 °C for about 18 h. The protein digests were stored at −20 °C.

### 2.3. Cell lysate

Lymph node carcinoma of the prostate (LNCaP) cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in RPMI 1640 media (Gibco-BRL, Bethesda, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Biosource, Rockville, MD, USA) at 37 °C and 5% (v/v) $CO_2$, and harvested at 80% confluence. The cells were washed three times with phosphate-buffered saline (PBS) and centrifuged (6000 × g, 4 °C, 20 min), and then resuspended in lysis buffer containing 150 mM NaCl, 50 mM Trizma base, 0.25% (v/v) Nonidet P 40 (NP40), 1 mM ethylenediaminetetraacetic acid (EDTA), and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) at pH 7.5. The cells were lysed using a French Press (Aminco, Silver Spring, MD, USA) at 6.9 MPa and then centrifuged (26 000 × g, 4 °C, 30 min). The supernatant was filtered using 0.2-μm poly(vinylidene difluoride) (PVDF) filters (Millipore, Billerica, MA, USA). DNase I and RNase A were added to the cell lysate to reach the concentrations of 0.2 and 0.05 mg/mL, respectively, and the lysate was incubated at 4 °C for 2 h with agitation. The lysate was then dialyzed using a 3000 MW cutoff membrane (Pierce Biotechnology, Rockford, IL, USA) against 1 L of dialysis buffer in a cold room for three times of duration 2, 2, and 12 h. A bicinchoninic acid (BCA) assay from Pierce was used to measure the protein concentration after dialysis. Before chromatofocusing, the cell lysate was dialyzed against the presaturation buffer for 1 h, and then filtered through a 0.2-μm polysulfone filter from Millipore.

### 2.4. 2D-PAGE

Each protein fraction was precipitated with trichloroacetic acid (TCA) at a 16% (m/v) final concentration and centrifuged (15 000 g,

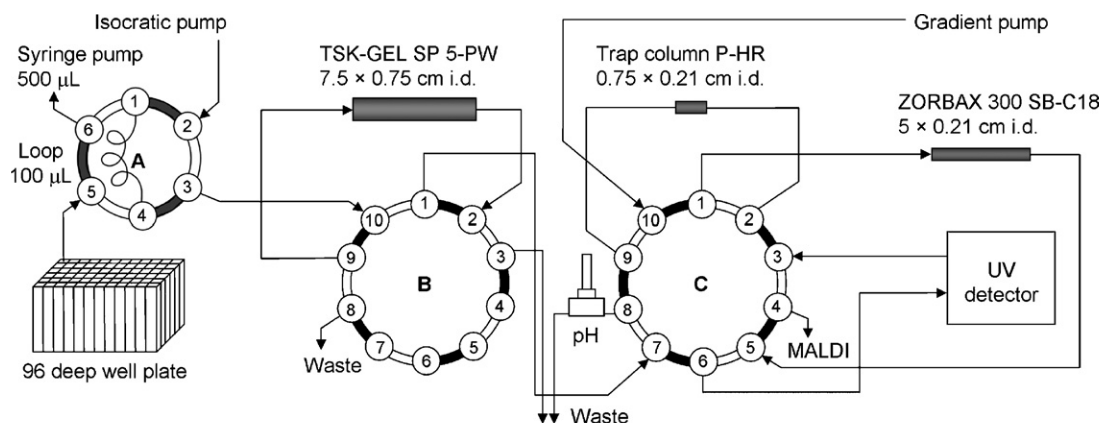


**Fig. 2.** The 2D-HPLC system, including an autosampler (Famos), two 10-port valves and one isocratic pump incorporated into one instrument (Switchos), and one gradient pump and UV detector incorporated into another instrument (Ultimate).

4 °C, 10 min) to collect the protein pellet. The protein pellet was washed with −20 °C acetone twice to remove excess TCA, and then was resuspended in 125 μL isoelectric focusing rehydration buffer. The rehydration buffer for fractions 1–4 of the SDC effluent contained 7 M urea, 2 M thiourea, 50 mM DTT, 1% (m/v) $C_7B_zO$ detergent from Sigma–Aldrich), 0.1% (m/v) pH 3–11 non-linear immobilized pH gradient (IPG) buffer (GE Healthcare, Piscataway, NJ, USA). The rehydration buffer for fractions 5 and 6 of the SDC effluent contained 7 M urea, 2 M thiourea, 50 mM DTT, 1% (m/v) $C_7B_zO$, 0.1% (m/v) pH 3–5 and 0.1% (v/v) 3–10 linear IPG buffer (Bio-Rad Labs, Hercules, CA, USA). The dissolved proteins were applied to an IPG strip rehydration tray to rehydrate on 7 cm linear pH gradient IPG strips (pH 3–10) overnight at room temperature. The IPG strips were focused in an isoelectric focusing cell from Bio-Rad Labs using first a linear gradient from 50 to 250 V in 20 min, then a linear gradient from 250 to 4000 V in 2 h, then a constant voltage at 4000 V for 4.75 h, and then a step change to a constant voltage at 500 V for 1 h. The focused IPG strips were equilibrated with equilibration buffer at room temperature for 15 min with agitation, and then applied to a 12% (m/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) mini-gel, which was run at 150 V for 1 h.

### 2.5. Silver staining method

The gel was fixed in a solution of 50% (v/v) methanol and 10% (v/v) acetic acid in deionized water for 30 min, then fixed in a solution of 5% (v/v) methanol and 1% (v/v) acetic acid in water for 15 min, and then washed for 5 min 3 times with deionized water. The gel was sensitized by incubated it for 90 s in 0.8 mM $Na_2S_2O_3$, then rinsed for 30 s three times with deionized water, then stained by incubating it for 30 min with 0.2% (m/v) $AgNO_3$, which was made immediately before use, and then rinsed for 30 s twice with deionized water. The gel was developed for up to 10 min with 0.566 M $Na_2CO_3$, 0.016 mM $Na_2S_2O_3$, and 500 μL formaldehyde in 1 L of deionized water.

### 2.6. 2D-HPLC

2D-HPLC was implemented using a LC Packings instrument composed of the Famos, Switchos, and Ultimate modules from Dionex, as shown in Fig. 2. Samples were filtered using 0.2-μm polysulfone filters and stored in a 96 deep well plate thermostated at 4–8 °C. A 6-port valve (Famos valve A) was used to inject the sample, and two 10-port valves (Switchos valves B and C) were used to coordinate the first dimension, second dimension, and trap columns. The Switchos isocratic pump was used to deliver the chromatofocusing buffers to the first-dimension ion-exchange column, and the Ultimate gradient pump was used for the second-dimension reversed-phase column. A UZ-view flow cell with 180 nL volume and 10 mm path length was used for detection. As shown in Fig. 2, one unique feature of the 2D method design is that one UV–vis detector is able to monitor the effluent from both the first- and second-dimension columns. A PVDF 50-μL internal volume flow cell was connected to the column outlet through valve C so that the pH of the effluent could be measured using a Model 450CD pH electrode (Sensorex, Garden Grove, CA, USA), a Model 520A pH meter (Orion, Beverly, MA, USA), and a Model UCI-50 universal interface box from Dionex. The entire process was fully automated and controlled by Chromeleon software version 6.6 from Dionex.

### 2.7. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS

The MS analysis was performed on a BRUKER Daltonics Autoflex MALDI-TOF instrument (Bruker Daltonics, Billerica, MA, USA) to determine peptide masses from 1000 to 2500 Da in a positive linear mode. The $N_2$ laser provided light at 337 nm for laser desorption and ionization. The matrix was $\alpha$-cyano-4-hydroxycinnamic acid (20 mg/mL) dissolved in 50% (v/v) acetonitrile, 50% (v/v) water (HPLC grade), and 0.1% (v/v) trifluoroacetic acid (TFA), which was mixed with sample in a 1:1 ratio (v/v) on the target spot and then allowed to air-dry before analysis. A mixture of angiotensin I (MW 1296.69) and renin substrate (MW 1758.93) was used as an external calibration.

## 3. Results and discussion

### 3.1. Application of SDC as a prefractionation method in 2D-PAGE

To investigate SDC as a prefractionation method, a LNCaP prostate cancer cell lysate was separated using a six-step pH gradient formed on a strong-base ion-exchange column under nondenaturing conditions. The results of the chromatofocusing procedure are shown in Fig. 3, and several of the fractions shown were further separated using full-size-range (pH 3–10) 2D-PAGE gels. As shown in Fig. 4, the majority of the proteins in each pH front were approximately clustered in the proper p*I* region on the 2D-PAGE, although some protein spots are located outside the expected p*I* region, evidently due to differences between the p*I* and $pI_{app}$ values for some of the proteins. In addition, fractions 5 and 6 from Fig. 3 were also separated on narrow-p*I*-range (pH 5–8) 2D-PAGE gels, and the results obtained from both full-size-range and narrow-p*I*-range 2D-PAGE are also shown in Fig. 4. As evident by a qualitative

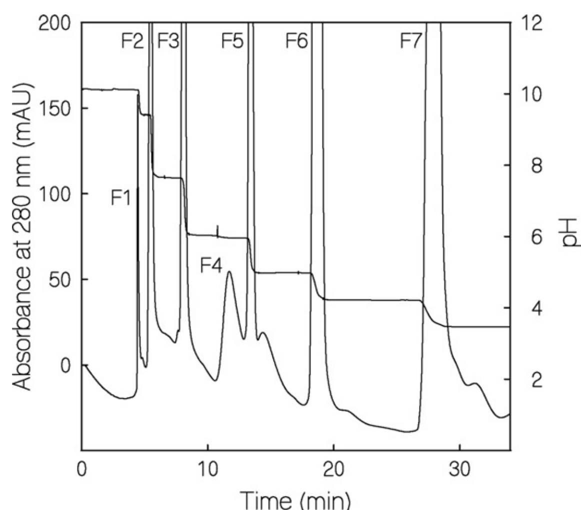


**Fig. 3.** LNCaP prostate cancer cell lysate (2.1 mg total mass) prefractionated using a pH gradient formed on a ProPac SAX-10 column (25 cm × 0.4 cm I.D.). The presaturation buffer contained 20 mM NaOH, 25 mM glycine, and 0.02% (m/v) *n*-octyl-$\beta$-D-glucopyronoside, and was at pH 10.2. The elution buffer containing 20 mM NaOH, 30 mM tricine, 20 mM 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES), 9 mM acetic acid, 8 mM formic acid, 0.02% (m/v) *n*-octyl-$\beta$-D-glucopyronoside was titrated with HCl to pH 3.5. The flow rate was 0.5 mL/min, and the UV absorbance was monitored at 280 and 260 nm. The numbers indicate the fractions collected.

comparison of the results obtained for fractions 5 and 6, better resolution was achieved when using the narrow-p*I*-range 2D-PAGE.

### *3.2. Application of SDC in a 2D-PAGE method for peptide separation*

Most applications of "shotgun" proteomics employ a tryptic digest of a protein mixture. As discussed by Kang and Frey [48], since trypsin cleaves polypeptides at the carboxylic end of lysine and arginine residues, essentially all tryptic peptides have a charge of at least +2 at pH 3 so that they can be bound to a strong-acid cation-exchange column at this pH and then eluted at a higher pH as their residues become titrated. Consequently, to demonstrate 2D-HPLC incorporating SDC for peptide separations, and to investigate the amount by which a first dimension SDC separation enhances the resolution obtained using reversed-phase chromatography as the second dimension, a cation-exchange column packing was employed for SDC along with a simple, model tryptic digest of the four proteins $\beta$-lactoglobulin A (p*I* = 5.1), myoglobin (p*I* = 7.3), cytochrome *c* (p*I* = 10.8), and lyszoyme (p*I* = 11.4). Since the majority of the tryptic peptides obtained from these proteins eluted in the acidic pH region, the pH gradient was designed to consist of three narrow-pH-range fronts in the acidic region: one from pH 3.0 to 4.6, one from pH 4.6 to 5.2, and one from pH 5.2 to 6.1, and one broad-pH-range front covering the whole basic region from pH 6.1 to 9.0.

Several sequential steps were used to accomplish 2D-HPLC as follows, and as shown in Fig. 2. Step 1: valves A, B, and C were set at the 6-1, 1-2, and 1-2 positions, and the first dimension column was equilibrated at pH 3 with presaturation buffer containing 20 mM *N*,*O*-dimethylhydroxylamine–HCl for 3 h at 0.2 mL/min. Step 2: valves A, B, and C were set at the 6-1, 10-1, and 1-2 positions, and the tubing before the ion-exchange column was purged with elution buffer containing 20 mM *N*,*N*-dimethylhydroxylamine–HCl, 17.5 mM BIS–TRIS, 42 mM ethanolamine, and 12 mM formic acid at pH 9.0 for 4 min at 0.5 mL/min. Step 3: valves A, B, and C were set at the 1-2, 1-2, and 10-1 positions, the sample was injected into the first dimension column, the first pH front exited the column and was captured by the trap column in 26 min when the flow rate 0.2 mL/min, and the second-dimension reversed-phase column was equilibrated with buffer A containing 5% (v/v) acetonitrile, 95% (v/v) distilled water, and 0.1% (v/v) TFA at 0.25 mL/min for 30 min. Step 4: valves A, B, and C were set at the 6-1, 10-1, and 10-1 positions, and the trap column was washed with buffer A at 0.5 mL/min for 6 min to remove chromatofocusing buffer. Step 5: valves A, B, and C were set at the 6-1, 10-1, and 1-2 positions, and the components captured by the trap column were further separated on the second-dimension reversed-phase column with a gradient starting from buffer A and finishing with buffer B, which contained 100% (v/v) acetonitrile and 0.07% (v/v) TFA. The gradient consisted of 0–16%

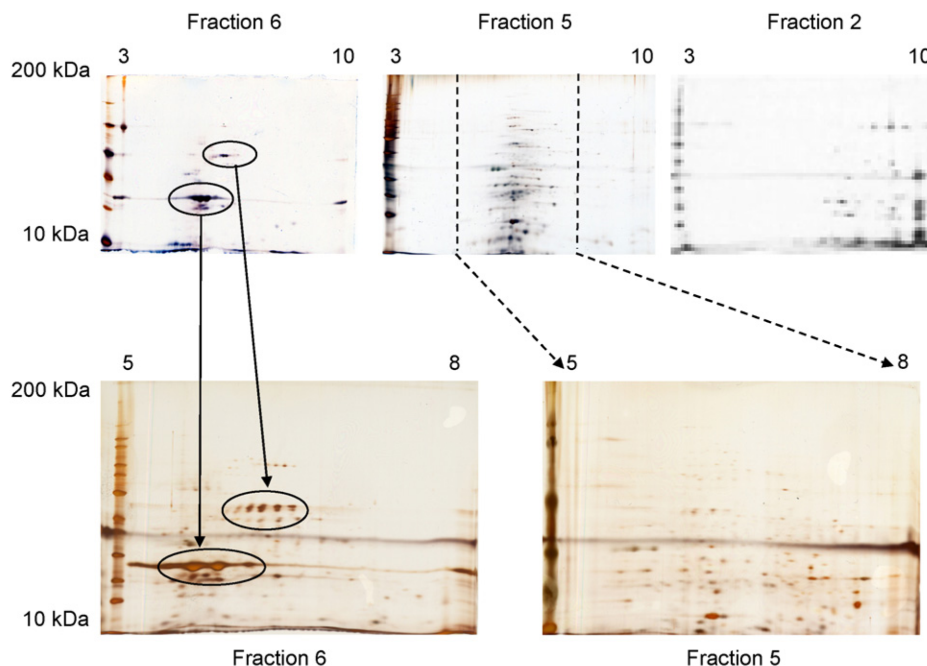


**Fig. 4.** Top: 2D-PAGE gels (pH 3–10) of LNCaP prostate cancer cell lysate fractions 2, 5, and 6 (50 μg) separated on a ProPac SAX-10 column (25 cm × 0.4 cm I.D.), as shown in Fig. 3. Protein standards are shown on the left side of the gels. Bottom: Narrow-p*I*-range 2D-PAGE gels (pH 5–8) of LNCaP prostate cancer cell lysate fractions 5 and 6 (50 μg) obtained on a ProPac SAX-10 column, as shown in Fig. 3.

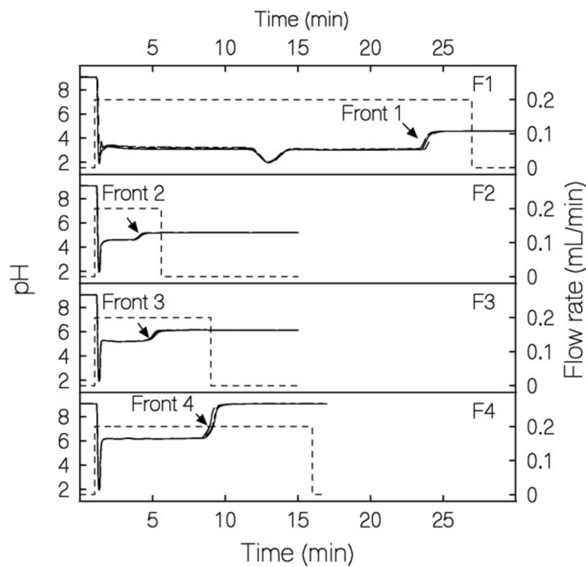


**Fig. 5.** Flow rate and pH gradient profile obtained in the "stop-and-go" mode of operation used in the first dimension SDC formed on a TSK-GEL SP-5PW (75 cm × 0.75 cm I.D.) column during 2D-HPLC. The flow rate is shown as the dashed curve and the pH gradient is shown for four runs to demonstrate the repeatability of the method: – (run 1), - - - (run 2), – – – (run 3), – – – – (run 4). Note that the pH gradients for the four runs that were performed coincide closely so that in most locations only a single curve is visible.

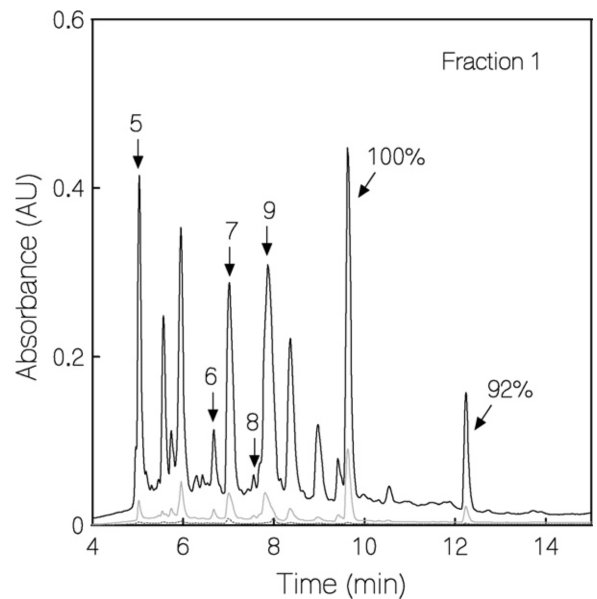


**Fig. 7.** The tryptic digest fraction from the first pH front (pH 3.0–4.6) in SDC separated on the second-dimension RPC column. Other conditions are the same as in Fig. 6A.

buffer B in 2 min, 16–35% buffer B in 10 min, 35–100% buffer B in 2 min, and 100% buffer B for 4 min at 0.25 mL/min. During this procedure, the tubing connected to the first dimension column was washed with elution buffer at 0.1 mL/min. Steps 3–5 were repeated three times to elute one-by-one the fractions focused on the subsequent pH fronts, which exited the column at 4.6, 8, and 15 min at the flow rate 0.2 mL/min. The corresponding equilibration times for the second-dimension reversed-phase column were 15, 15, and 17 min at the flow rate 0.5 mL/min.

Fig. 5 illustrates the operation of the SDC procedure used where, after each pH front exits the first-dimension column, the flow through that column is temporarily stopped as described above to permit the processing of eluted peptides on the second-dimension reversed-phase chromatography column. Although the pH gradient was operated in this "stop-and-go" mode, the whole 2D-HPLC process was fully automated as shown in Fig. 2, and all the pH fronts were highly repeatable, as demonstrated by the four independent runs shown in the figure.

In order to provide a proper comparison basis, the tryptic digest sample was first separated solely using one-dimensional reversed-phase chromatography (1D-RPC) as shown in Fig. 6A. The same sample was then separated using 2D-HPC incorporating SDC, as shown in Figs. 7–10, which correspond to the second-dimension RPC chromatograms for the four fractions from the first dimension. The figures in particular illustrate three specific cases of enhanced resolution obtained in the 2D method. In the first case, as judged

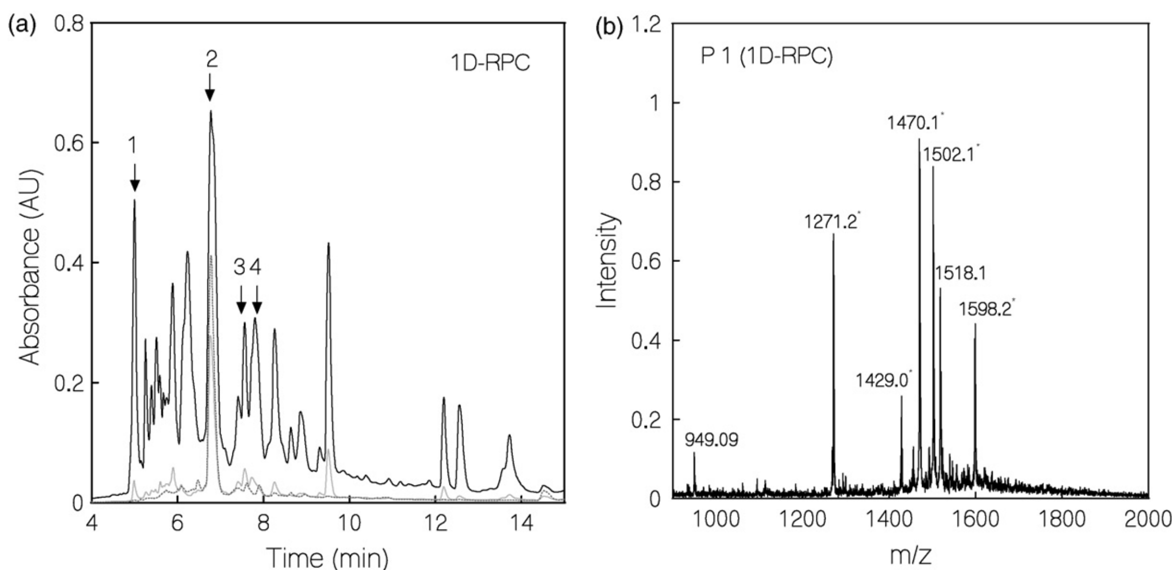


**Fig. 6.** (A) Tryptic digest of four proteins (12.5 μg each of cytochrome *c*, myoglobin, lysozyme, and β-lactoglobulin A) separated on the RPC column. The buffers and gradient used are the same as described in Section 3.2. The flow rate was 0.25 mL/min, and the absorbances were monitored at 215, 280, and 415 nm, which are denoted by very dark, light, and intermediate dark curves, respectively. (B) Mass spectrum of peak 1 from (A). The relative molecular mass with a superscript • indicates that the measured value agrees with the calculated value based on the amino acid sequence of the peptide.

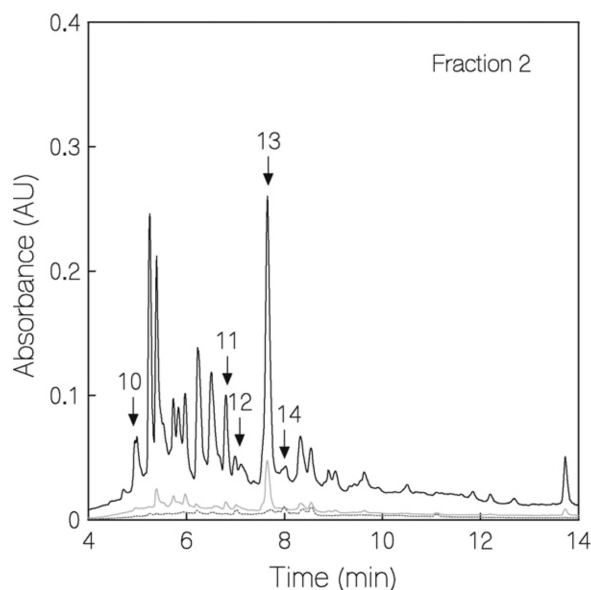


**Fig. 8.** The tryptic digest fraction from the second pH front (pH 4.6–5.2) in SDC separated on the second dimension RPC column. Other conditions are the same as in Fig. 6A.

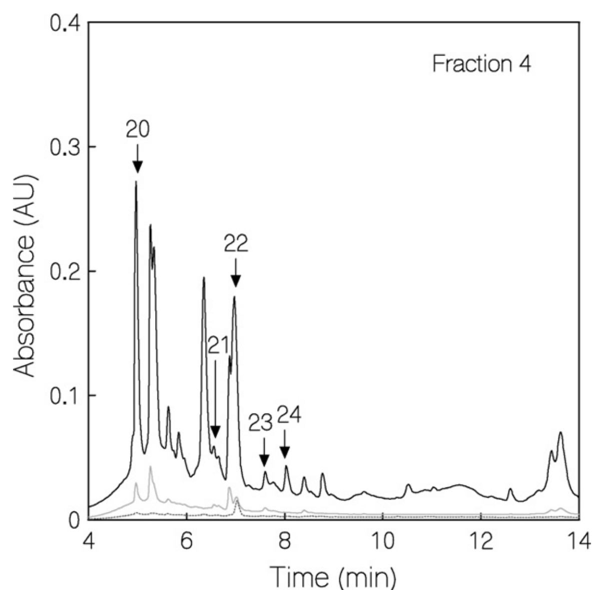


**Fig. 10.** The tryptic digest fraction from the fourth front (pH 6.1–9.0) in SDC separated on the second dimension RPC column. Other conditions are the same as those in Fig. 6A.

by the retention time of the peak maxima, peak 1 shown for 1D-RPC was resolved into four peaks in 2D-HPLC, which are denoted as peak 5, 10, 15, and 20 shown in Figs. 7–10. In the second case, as again judged by the retention time, peak 2 shown for 1D-RPC was resolved into eight peaks in 2D-HPLC, which were denoted as 6, 7, 11, 12, 16, 17, 21, and 22 shown in Figs. 7–10. Note that the heme-group containing peptide present in peak 2 in Fig. 6A, which is the only peptide that has a substantial absorbance at 415 nm, is also present mainly in fraction 3 shown in Fig. 9, which indicates a minimal amount of fraction cross contamination for this peptide. In the third case, peaks 3 and 4 shown for 1D-RPC were both resolved into four peaks in 2D-HPLC, as shown in Figs. 7–10, where peaks 8, 13, 18, and 23 were resolved from peak 3, and peaks 9, 14, 19, and 24 were resolved from peak 4.

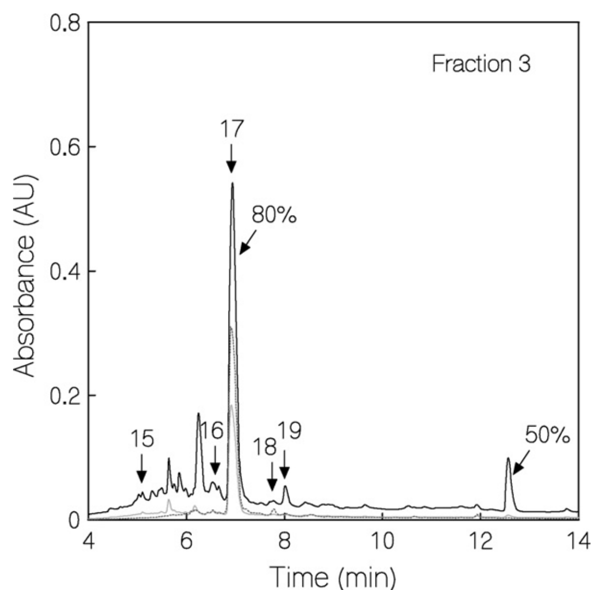


**Fig. 9.** The tryptic digest fraction from the third front (pH 5.2–6.1) in SDC separated on the second dimension RPC column. Other conditions are the same as in Fig. 6A.

To confirm the trends described above more completely, peptides contained in each peak mentioned were further analyzed using MALDI-TOF MS. In the first case mentioned above, MS data from peak 1 for 1D-RPC, as shown in Fig. 6B, were used to identify five tryptic peptides, listed as nos. 3, and 6–9 in Table 1, by comparing the measured and theoretical relative molecular mass of the peptides, with the latter determined assuming that trypsin cleaves a polypeptide chain at the carboxyl side of arginine or lysine residue, and allowing for three missed cleavage sites. As shown by the MS data in Fig. 11, these same five known peptides were separated into different fractions in 2D-HPLC with minimal fraction cross-contamination. Also, in the second case mentioned above, results from MS (data not shown) suggest that only two major tryptic peptides from the four model proteins were detected by MS from peak 2 in 1D-RPC, which are the peptides listed as nos. 2 and 10 in Table 1. In contrast, besides these two peptides, five additional peptides, listed as nos. 1, 4, 5, 11 and 12 in Table 1 having the same retention time in RPC as peak 2 from the 1D-RPC experiment, were detected in 2D-HPLC, with four of these peptides being eluted in the second and fourth pH fronts of the SDC first dimension, whereas the two major peptides were eluted in the first and third pH fronts. These obser-

**Table 1**
The amino acid sequences of peptides whose measured relative molecular mass matches the theoretically calculated value. The source proteins for these peptides were lysozyme, cytochrome *c*, $\beta$-lactoglobulin A and myoglobulin.

| No. | Peptide sequence | Source protein | Calculated relative molecular mass | Measured relative molecular mass |
|---|---|---|---|---|
| 1 | WWCNDGR | Lysozyme | 936.4 | 936.7 |
| 2 | EDLIAYLK | Cytochrome *c* | 964.5 | 963.9, 964.3, 964.5 |
| 3 | LFTGHPETLEK | Myglobin | 1271.7 | 1271.2, 1272.3 |
| 4 | GYSLGNWVCAAK | Lysozyme | 1268.6 | 1266.6 |
| 5 | TEREDLIAYLK | Cytochrome *c* | 1350.7 | 1350.6 |
| 6 | FESNFNTQATNR | Lysozyme | 1428.7 | 1429.0, 1429.8 |
| 7 | TGQAPGFTYTDANK | Cytochrome *c* | 1470.7 | 1470.1, 1470.9 |
| 8 | HPGDFGADAQGAMTK | Myglobin | 1502.7 | 1502.1, 1502.9 |
| 9 | KTGQAPGFTYTDANK | Cytochrome *c* | 1598.8 | 1598.2, 1599.5 |
| 10 | IFVQKCAQCHTVEK | Cytochrome *c* | 1633.8 | 1633.7, 1634.0 |
| 11 | IVSDGNGMNAWVAWR | Lysozyme | 1675.8 | 1675.2 |
| 12 | KIVSDGNGMNAWVAWR | Lysozyme | 1803.9 | 1803.1 |

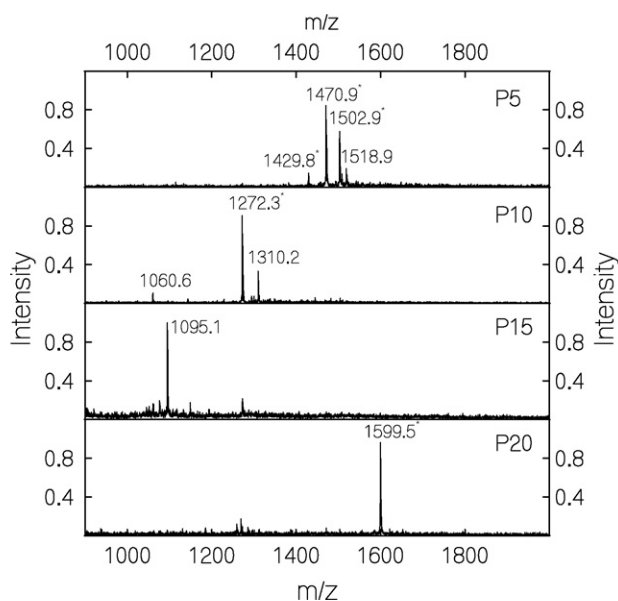


**Fig. 11.** Mass spectra obtained from peaks 5, 10, 15, and 20 in Figs. 7–10, and denoted as P5, P10, P15, and P20, respectively. The relative molecular mass with a superscript • indicates that the measured value agrees with the calculated value based on the amino acid sequence of the peptide.

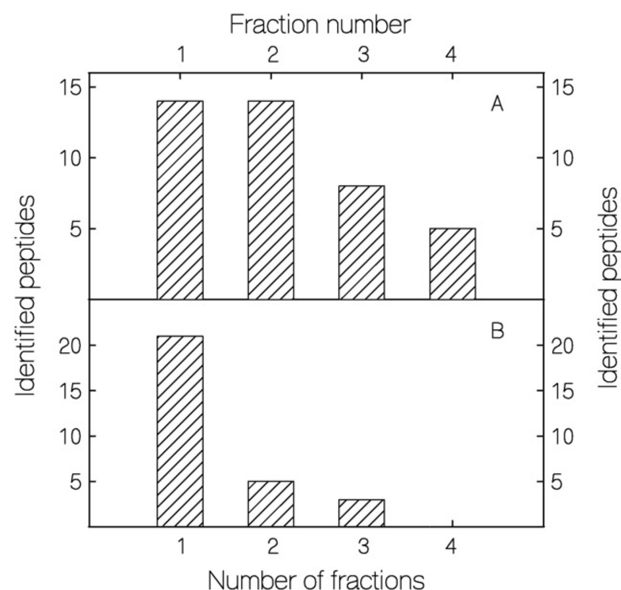


**Fig. 12.** Top: Number of the tryptic peptides identified in each SDC fraction. Bottom: Number of the tryptic digest peptides present simultaneously in a particular number of SDC fractions.

vations indicate that certain low-abundance peptides, such as the missed-cleavage peptides with molecular masses of 1350.6 from cytochrome *c* and 1803.1 from lysozyme, that were not detected in 1D-RPC, did became detectable in 2D-HPLC, possibly due to the selective concentration effect of the SDC method and the reduced ion suppression for the minor components that results in MALDI-TOF MS when major components are removed. Similar results (data not shown) were obtained for the third case described above pertaining to peak 3 and 4 in Fig. 6A. The amino acid sequences of the tryptic peptides observed for the cases considered above whose sequences were deduced from their calculated relative molecular masses are listed in Table 1.

In addition to the three cases described above, several additional peaks observed in Figs.7–10 were analyzed by MALDI-TOF MS, and the resulting number of tryptic peptides identified in each SDC fraction is summarized in the top part of Fig. 12, where it can be seen that relatively few peptides were observed in the basic region of the pH gradient. The MS data obtained also permitted a determination of the levels of peptide cross-contamination in the SDC fractions. As the results show in the bottom part of Fig. 12, 73% of the peptides identified by MS were present in just a single fraction, while 17% and 10% of the peptides were present in two and three fractions, respectively. No peptide was found to be present in all four of fractions. Although cross-contamination is generally minimized in SDC as described earlier, when a large number of components are present, it is unavoidable that some peptides will be distributed into more than one fraction. Furthermore, some of the fraction cross-contamination observed may be due to the formation of peptide association complexes in the fluid phase [49], as opposed to deficiencies in the basic chromatographic method. Nevertheless, the degree of fraction cross-contamination shown in Fig. 12 is comparable to, and possibly less than, the p*I*-range fraction cross-contamination observed when using MCE-IM for tryptic peptide fractionation [50].

To evaluate the recovery of peptides in the 2D-HPLC method, four well resolved peaks observed in both 1D-RPC and in the second dimension reversed-phase column in 2D-HPLC, were chosen as the basis for the calculation. In addition, all four peptides in this peptide group, including the heme-group containing peptide, were mainly located on a single pH front. For the heme-group containing peptide, the peak area was calculated using the UV absorbance at 415 nm, and the recovery was calculated as the ratio of the peak area in the second dimension reversed-phase chromatography to that observed in 1D-RPC. The results for each peptide are denoted by the percentages given in Figs. 7 and 9, and the average recovery of all four peptides was 80%.

To evaluate the repeatability of the 2D-HPLC system described above, the relative standard deviation (RSD) of both retention time and peak heights in four identical 2D-HPLC runs were compared. Based on calculations for a total of 50 peaks present in four fractions for each run, the RSD of the retention time is 0.7% on average, while the RSD of the peak height is 14% on average based on calculations for a total of 40 peaks. These results suggest that the 2D-HPLC method investigated here is sufficiently repeatable so that peak heights are likely to be directly useful as indicators of protein expression levels when comparing two different samples.

## 4. Extensions

The chromatographic techniques described in this study can be considered as prototype methods to which a number of potentially useful modifications can be made. For example, higher protein solubilities, and consequently larger numbers of observed protein spots on subsequently used 2D gels, might be achieved by incorporating denaturants such as 8 M urea in the eluent, although such additives may also lower the chromatographic performance due to the resulting higher eluent viscosity and lower eluite diffusivities. In this regard, it can be noted that Sahab et al. [51] recently described a batch contacting version of chromatofocusing pre-fractionation that employed high urea concentrations. In the present case, the use of silica-based columns composed of micropellicular particles of small size may permit a denaturing solvent to be employed with a limited reduction in chromatographic performance by permitting operation at very high pressures.

One potentially useful modification of the 2D-HPLC method for peptide separation investigated here is to employ a mixed aqueous-organic eluent to reduce associations between peptides so that fraction cross contamination is reduced [48,49]. Sheng et al. [6] recently employed this method for the chromatofocusing of pro-

teins using a traditional polyampholyte elution buffer. In addition, Shen [52] has shown that as many as 10 pH steps can be produced in a single SDC procedure, and it has been shown by others that the use of ultra-high-pressure, reversed-phase chromatography, or monolithic reversed-phase columns, can achieved peak capacities in the range of 500–1500 [53], so that a maximum total peak capacity for the 2D method explored here can reasonably be expect to be 5000–15 000. This peak capacity, as well as the selective concentration characteristics of SDC, can be further increased if more than one eluent fraction is collected at each pH front. By using these modifications, the technique described here is likely to have a sufficient peak capacity for a variety of proteomics methods [47]. As a final potential modification, it was shown in Part I that the scale of the method can be reduced by a corresponding reduction in the diameters of the columns used, so that sub-millimeter diameter columns can be employed of the type commonly interfaced directly to electrospray ionization MS.

## 5. Conclusions

A novel SDC method in which proteins or peptides are eluted and/or displaced with a multistep, retained pH gradient was investigated along with a computer-aided design method based on local-equilibrium theory. In Part I of this study, it was shown that SDC can produce a selective concentration effect for minor components in a fraction, that it is repeatable and highly scalable, able to achieve good mass recoveries, and is able to reasonably differentiate proteins in terms of their p*I* values. In Part II of this study, SDC was used to prefractionation a LNCaP prostate cancer cell lysate prior to using SDS-PAGE, and also prior to using both normal-p*I*-range and narrow-p*I*-range 2D-PAGE. The results suggest that SDC may have uses as a prefractionation method prior to a further gel-based separation that is a low cost, rapid, and highly scalable. The method was also used as the first dimension coupled with reversed-phase chromatography as the second dimension to form a novel 2D-HPLC method for peptide separations that was shown to have performance advantages over the use of 1D-RPC.

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