RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2001; **15**: 291–296

RCM

Chromatofocusing nonporous reversed-phase high-performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry of proteins from human breast cancer whole cell lysates: a novel two-dimensional liquid chromatography/mass spectrometry method

Bathsheba E. Chong[1], Fang Yan[1], David M. Lubman[1]* and Fred R. Miller[2]
[1]Department of Chemistry, The University of Michigan, Ann Arbor, MI 48109-1055, USA
[2]Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, USA

Received 22 December 2000; Accepted 26 December 2000

SPONSOR REFEREE: Professor Aaron Timperman, Department of Chemistry, West Virginia University, PO Box 6045, Morgantown, WV 26506-6045, USA

A novel two-dimensional two-column liquid chromatography/mass spectrometry (LC/MS) technique is described in this work, where chromatofocusing (CF) has been coupled to nonporous reversed-phase (NPS-RP) HPLC to separate proteins from human breast epithelial whole cell lysates. The liquid fractions from NPS-RP-HPLC are readily amenable to direct on-line analysis using electrospray ionization orthogonal acceleration time-of-flight mass spectrometry (ESI-TOFMS). A key advantage of this technique is that proteins can be 'peeled off' in the liquid phase from the CF column according to their isoelectric points (*pI*) in the first chromatographic separation dimension. The NPS-RP-HPLC column further separates these *pI*-focused fractions based upon protein hydrophobicity as the second chromatographic dimension. The third dimension involves on-line molecular weight determination using ESI-TOFMS. As a result, this method has the potential to be fully automated. In addition, a 2-D protein map of *pI* versus molecular weight is generated, which is analogous to a 2-D gel image. Thus, this technique may provide a means to study differential expression of proteins from whole cell lysates. **Copyright © 2001 John Wiley & Sons, Ltd.**

Chromatofocusing (CF) is a protein separation technique originally introduced by Sluyterman and co-workers between 1977 and 1981.[1–5] During the CF process, a weak ion-exchange column of suitable buffering capacity is equilibrated with a buffer that defines the upper pH of the separation gradient. A second 'focusing' buffer is then applied to elute bound proteins, roughly in order of their isoelectric (*pI*) points. The pH of the focusing buffer is adjusted to a pH that defines the lower limit of the pH gradient. The pH is formed internally during isocratic elution with a single focusing buffer where no external gradient-forming device is required. The pH gradient is formed as the eluting buffer titrates the buffering groups on the ion exchanger. Peak widths in the range of 0.05 of a pH unit and samples containing several hundred milligrams of protein can be processed in a single step.[6–8] CF has been used to fractionate and enrich *H. influenza* proteins,[9,10] characterize and purify immunoglobulin G in blood,[11] quantify the BRCA1 gene in sporadic breast carcinoma,[12] and purify therapeutic proteins from *E. coli* extracts,[13] to name a few of its applications. CF is a powerful analytical tool involving protein surface charge and an effective preparative technique for protein isolation.

A key advantage of using CF is its ability to isolate proteins in a specific pH zone of interest so that proteins can be 'peeled off' a fraction of a pH unit at a time. This peeling off procedure allows proteins to be focused in their respective *pI* regions and eluted accordingly. This makes CF ideal as a first dimension of separation. These *pI*-focused fractions can then be conveniently analyzed by a second chromatographic separation to resolve the proteins in each *pI* range. Although in other work Jorgenson and co-workers performed 2-D liquid separations of proteins using size-exclusion chromatography (SEC) or ion exchange followed by RP-HPLC,[14,15] these methods cannot provide *pI* information in a 2-D map. It should be emphasized that the *pI* information is important because it is a physical property that is listed in the databases and used for protein identification. Also the use of *pI* is important for separation of different isoforms of proteins.

**Correspondence to*: D. M. Lubman, Department of Chemistry, The University of Michigan, Ann Arbor, MI 48109-1055, USA.
Contract/grant sponsor: National Institutes of Health; Contract/grant number: ROIGM49500.
Contract/grant sponsor: National Science Foundation; Contract/grant number: DBI-9987220.
Contract/grant sponsor: PG Research Foundation, Inc.

Copyright © 2001 John Wiley & Sons, Ltd.

The novel aspect of this work is the use of nonporous reversed-phase (NPS-RP) HPLC as a second dimension of separation following the initial *pI* separation of proteins using CF. The advantages of using NPS-RP-HPLC involve speed of separation, recovery and resolution relative to porous media, where proteins from bacterial and human whole cell lysates have been separated in 10–25 min.[16–20] The NPS packing material consists of 1.5-μm diameter spherical silica beads coated with C18, which alleviates problems associated with proteins sticking inside the pores. Ultimately, the eluent from NPS-RP-HPLC is analyzed directly on-line by electrospray ionization orthogonal acceleration time-of-flight mass spectrometry (ESI-TOFMS) to obtain the molecular weight and relative abundance information of the isolated protein peaks. This process results in a two-dimensional separation/mass mapping method that provides information analogous to classical 2-D gel methods, where the protein profile is mapped in terms of *pI* versus molecular weight. A significant advantage of the method though, is the ability to obtain a relatively accurate molecular weight of intact proteins to provide a mass map of the protein content of a cell.

EXPERIMENTAL

Sample preparation

In this work, we applied this novel technique to rapidly profile proteins from human breast epithelial whole cell lysates of MCF10A cell lines. Cells from MCF10A are normal, but immortalized, and originated from a fibrocystic patient.[21] Proteins were extracted from MCF10A cells using a chemical lysing procedure. The lysis buffer consists of 6M guanidine hydrochloride, 20 mM *n*-octyl *β*-D-glucopyranoside and 50 mM Tris. Cells were vortexed rigorously and kept overnight at −20 °C. They were subsequently centrifuged at 17 000 rpm for 20 min. The supernatant was removed from the cell debris and re-centrifuged at high speed to further remove any particulate. For the best reproducible results, the lysate should be used within 48 h. The sample preparation and CF separation involve the use of guanidine hydrochloride or urea and a nonionic detergent (*n*-octyl *β*-D-glucopyranoside) which are compatible with the analyses by NPS-RP-HPLC and ESI-TOFMS. These agents improved the solubilization of large proteins.

Protein separation by CF

Buffers for CF are (A) imidazole-HAC, 0.1% guanidine hydrochloride, 0.05% *n*-octyl *β*-D-glucopyranoside, pH 7.2, and (B) polybuffer 74 (diluted 1:10), 0.1% guanidine hydrochloride, 0.05% *n*-octyl *β*-D-glucopyranoside, pH 4. The CF column is a Mono P HR 5/20 (Amersham Pharmacia Biotech, Uppsala, Sweden) with a flowrate of 1 mL/min at room temperature. The column dimensions are 5 mm i.d. and 20 cm length. Prior to injection, the lysate is equilibrated with buffer A with a loading time of 20 min. The sample loadability for the CF column is >10 mg of protein. The separation profile is monitored at 280 nm while the pH gradient is monitored using a pH flowcell meter (Amersham Pharmacia).

The CF column is equilibrated with buffer A to define the upper pH range (7 in this case) of the pH gradient. The second 'focusing' buffer B is then applied to elute bound proteins, in the order of their isoelectric (*pI*) points. The pH of buffer B is 4, which defines the lower limit of the pH gradient. The pH gradient is formed as the eluting buffer B titrates the buffering groups on the ion exchanger.

Protein separation by NPS-RP-HPLC

The *pI*-focused liquid fractions from CF are collected and then analyzed in the second dimension using NPS-RP-HPLC. The use of the NPS-RP-HPLC columns (Eichrom Technologies, Darien, IL, USA) is the novel aspect here whereby they are used as the second orthogonal separation dimension after CF. These NPS columns are excellent for protein separation due to their high protein recovery, speed and efficiency. The loadability of the columns is typically 50 μg of protein which is lower than that of porous columns. To achieve optimal protein separation, the columns must be maintained at a high temperature (60 °C). The resolution and loadability can be enhanced by using multiple NPS columns in series.[22] In these experiments, ODS1 columns (Eichrom Technologies) of 4.6 × 33 mm dimensions were used.

Chemicals

The chemicals involved in this study were used without prior purification. Acetone (HPLC grade) was obtained from Fisher (Fair Lawn, NJ, USA). Acetonitrile, guanidine hydrochloride (gu-HCl), α-cyano-4-hydroxycinnamic acid (α-CHCA), trifluoroacetic acid (TFA), formic acid (FA), *n*-octyl *β*-D-glucopyranoside (OCG), and imidazole-HAC were from Aldrich (Milwaukee, WI, USA). Trypsin was acquired from Promega (Madison, WI, USA). Polybuffer 74 was from Amersham Pharmacia Biotech (Uppsala, Sweden). Distilled and deionized water was obtained from a Milli-Q reagent grade purification system from Millipore (Bedford, MA, USA).

Protein detection using mass spectrometry

Eluents from the NPS-RP-HPLC separation can be directly analyzed by MS to determine their molecular weight, identity and relative abundance. In this case, the eluted proteins were detected simultaneously by ESI-TOFMS (Model LCT, Micromass, Ltd., Manchester, UK). The MS parameters were set as follows: source capillary 3000 V, sample cone 45 V, RF lens 800 V, extraction cone 2 V, desolvation temperature 300 °C, and source temperature 120–150 °C. The Beckman HPLC system (as described above) was interfaced with the LCT using the NPS column separations. The solvents for the mobile phase were (solvent A) Milli-Q water with 0.1% TFA + 0.2 to 0.3% FA and (solvent B) acetonitrile with 0.1% TFA + 0.2 to 0.3% FA with a flow rate of 0.5 mL/min where the temperature of the NPS column was maintained at 65 °C in a Timberline column heater. The gradient profile used for solvent B was generally as follows: 5% for 1.5 min; 5 to 20% in 2 min; 20 to 60% in 20 min; 60 to 100% in 1 min, 100% for 2 min, 100 to 5% in 1 min, 5% for 2 min. The 0.5 mL/min was split to a 1:1 ratio before entering the electrospray source. The chromatograms generated were deconvoluted using the MaxEnt software (Micromass).

Copyright © 2001 John Wiley & Sons, Ltd.

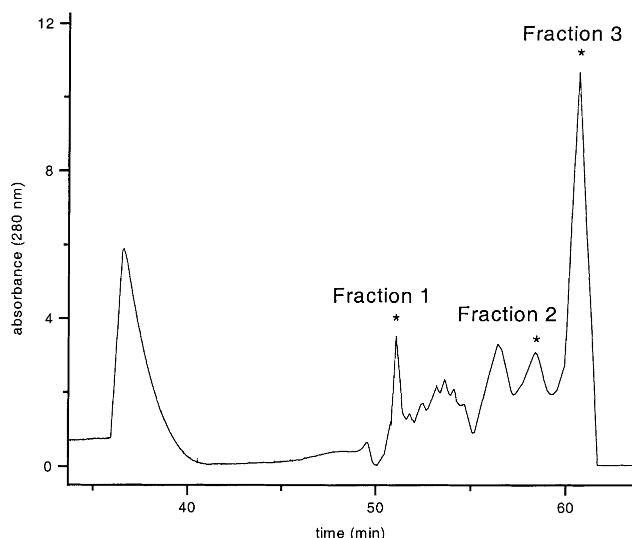

Figure 1. Chromatofocusing profile of MCF10A human breast epithelial whole cell lysates.

Software and data presentation

The data generated by CF-NPS-RP-HPLC/ESI-TOFMS can be presented as a 2-D map or a 2-D image similar to that of the traditional 2-D gels. The chromatograms (or TICs) are integrated, and the deconvoluted mass spectra are converted into the ASCII format before being plotted vertically, using a 256-step gray scale, such that peaks are represented as darkened bands against a white background. The 256 steps are then divided by the number of proteins, which in turn will determine the width of each protein band. For example, if there were 40 proteins, then each protein band would have a width of 6.4 on this scale (i.e. = 256/40). The fewer the proteins, the wider the bands. Thus, the present software format does not actually correspond to the resolution of the method. This scale can also be produced in a variety of color formats. Therefore, this 2-D map provides information on *pI* and molecular weight as well as the relative abundance of separated proteins. This map can also be adjusted to zoom into a specific area of interest for a more detailed image of all the bands therein.

RESULTS AND DISCUSSION

Figure 1 shows the CF protein profile of MCF10A whole cell lysates using Polybuffer 74 over a pH range of 7 to 4 using UV absorption detection. Liquid fractions were collected from the column every minute and the fractions were further

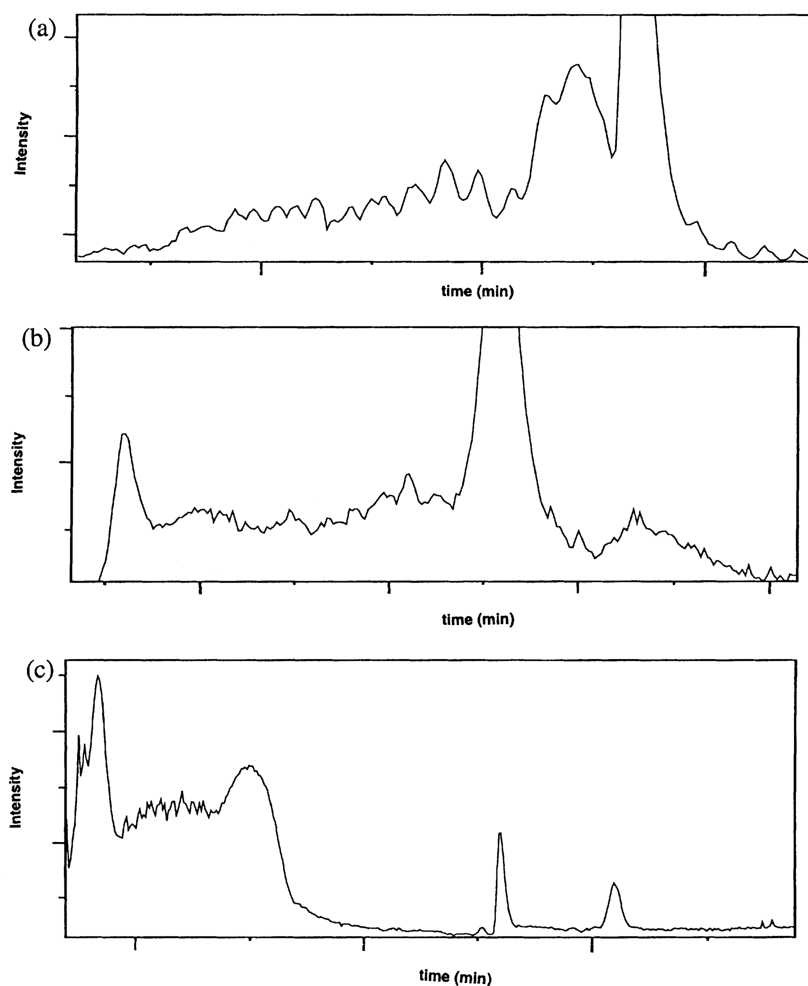

Figure 2. Reversed-phase separation of CF fractions with ESI-TOF detection: (a) fraction 1 (pH 6.75–6.55), (b) fraction 2 (pH 5.50–5.25), and (c) fraction 3 (pH 5.20–4.90).

Copyright © 2001 John Wiley & Sons, Ltd.

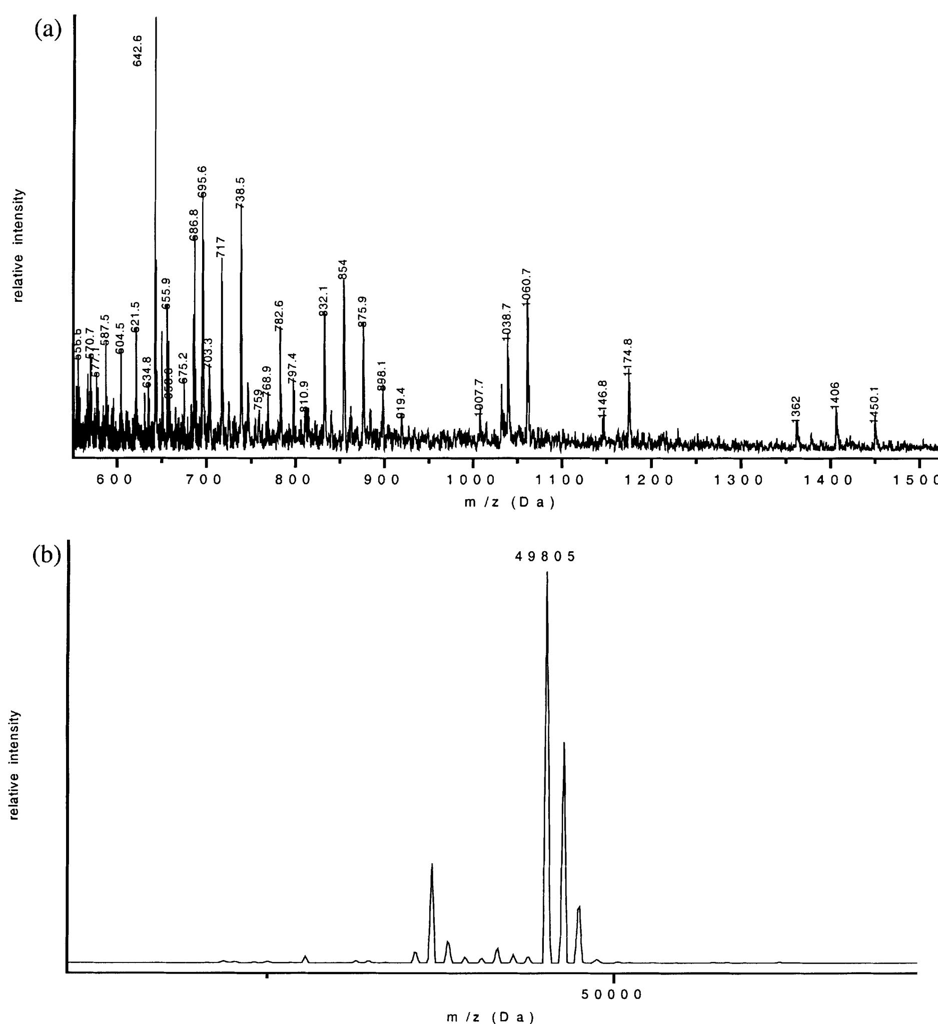

Figure 3. (a) A multiply charged protein ladder from fraction 1 after integrating the TIC profile, and (b) the deconvoluted molecular weight at 49.8 kDa using MaxEnt.

analyzed on-line by NPS-RP-HPLC-ESI-TOFMS. Figure 2 shows the TIC profiles of three selected fractions, which were chosen for further analysis. The ESI source generates a distribution of multiply charged species for the large proteins detected from the cell lysate, as shown in Fig. 3(a) for a protein peak selected from fraction 1. These spectra must be deconvoluted by MaxEnt software (Micromass) to obtain the corresponding molecular weight of the intact protein, as shown in Fig. 3(b). The proteins have not been identified, but it appears as if at least two proteins are present in this figure. This procedure was performed for the more expressed proteins in the TICs of Fig. 2 and the molecular weight of each protein determined.

Figure 4 shows a 2-D image of the *pI* versus the molecular weight of each protein from the TICs of the three CF fractions analyzed. An advantage of this format is that it displays proteins in a format similar to that of 2-D gels, which is the method generally used to separate and identify proteins. In each fraction there are typically 40 proteins detected which are displayed according to their relative abundance using a gray scale representation, where the bands vary in intensity depending on the amount of protein present. Most of the proteins included in the map are more abundant proteins that were readily analyzed by MaxEnt software. Also, the dynamic range of the computer program is limited to 256 steps so that low level proteins would not be observed unless the plot is rescaled. Nonetheless, a detailed analysis would show protein present in regions of the map where in the present figure no protein is shown.

The present method provides several potential advantages for analysis of proteins from cell lysates. These include the separation and detection of proteins in the liquid phase, which allows the use of on-line detection of proteins based upon intact molecular weight by ESI-MS. This can provide a means of obtaining an exact molecular weight map of the protein content of a cell analogous to 2-D gel electrophoresis. In this work, a molecular weight map of intact proteins accurate to better than 100 parts per million can be obtained as compared to the 2-D gel where molecular weight measurement may only be +/−10% accurate. In the 2-D map of Fig. 4 the bands do not show the high resolution of the mass data, but only indicate the relative positions of protein bands. The high resolution mass data can be obtained directly from the deconvoluted mass peaks. In addition, because the map depends on molecular weight measurement at a specific *pI*, the issue of reproducibility in

Copyright © 2001 John Wiley & Sons, Ltd.

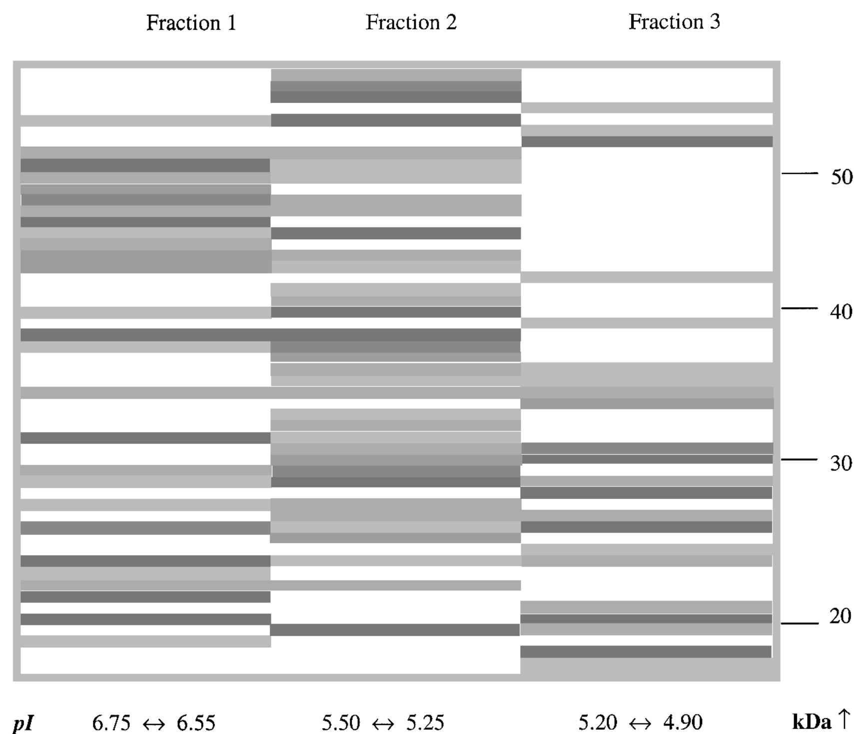

Figure 4. 2-D image of protein molecular weight of three MCF10A fractions. The right bar shows molecular weight scale (kDa) while the peak intensity is depicted in shades of gray. The *pI* of each fraction is also shown.

the separation, as in 2-D gels, is no longer critical. Mass maps from different cell samples can be compared at a particular *pI* according to the molecular weight of the proteins.

A second advantage of this method is the use of *pI* separation in the first dimension followed by molecular weight detection. The method is analogous to the 2-D gel where *pI* and molecular weight are used to separate and map proteins. In the 2-D liquid separation, *pI* and exact molecular weight can be used to identify subtle changes in proteins based upon differential changes in *pI* and molecular weight due to post-translational modifications (PTM) of proteins. A third advantage of the method is the potential for total automation of the method. In this work each *pI* fraction can be 'peeled off' the CF column by controlling the titration rate of the elution buffer. The fractions can in principle be automatically injected into the NPS-RP-HPLC column with detection performed on-line by ESI-MS. A major advantage of this method thus becomes the ability to interface the liquid separation to analysis by MS. Furthermore, a fourth advantage of the method becomes the capability for relatively rapid analysis. The CF step may require 1–2 h, while the on-line analysis of each fraction may take 10–25 min depending on the resolution required. A total protein map could be obtained in several hours.

The major disadvantage of the method in its current form is the data analysis, which has not yet been automated. The deconvolution of each protein ESI multiply charged spectrum and the optimization of the most probable mass may require a significant amount of time. In the present work, we have generally chosen the more abundant proteins in several fractions for analysis. However, analysis of the low level proteins would require significantly more time and many of the low level proteins may be lost in the background noise if they coelute with high abundance proteins. Further development of the software will be required to make this method practical. The molecular weight map can be used to study differential changes in proteins based upon shifts in *pI* and changes in molecular weight. However, identification of proteins will still require digestion and peptide mapping following collection of the proteins from the liquid eluent of NPS-RP-HPLC separations. Such identification is in progress in present work.

In conclusion, CF coupled with NPS-RP-HPLC has been used to separate proteins from whole cell lysates of human breast epithelial cell lines. CF combines the advantage of high capacity ion-exchange procedures with separation based upon pH using isoelectric focusing. A key advantage of this method is that the focused protein bands can be 'peeled off' the column according to their *pI* in the first chromatographic dimension by controlling the pH of the elution buffer. This capability for selectively peeling off a protein of a selected pH range provides a method for readily interfacing CF to a second dimension for separation. These *pI*-focused fractions in the liquid phase are then readily separated using a NPS-RP-HPLC column in the second dimension based upon hydrophobicity. Eluents from NPS-RP-HPLC can be analyzed directly on-line by ESI-TOFMS for protein molecular weight determination. It is demonstrated that CF-NPS-RP-HPLC/ESI-TOFMS is a two-dimensional two-column on-line method for analysis of proteins from cell lysates, which can provide a protein map based upon *pI* and molecular weight.

Copyright © 2001 John Wiley & Sons, Ltd.

Acknowledgements

We gratefully acknowledge partial support of this work by the National Institutes of Health Grant #R01GM49500, the National Science Foundation Grant #DBI-9987220, and the PG Research Foundation, Inc. We thank Dr. Tim Barder of Eichrom Technologies, Inc. (USA) for generously providing the nonporous columns. We also thank Drs. Allan Simpson and AnnaCarin Eriksson of Amersham Pharmacia Biotech (Sweden) for providing the CF columns and polybuffers. In addition, we thank Dr. Tim Riley of Micromass, Ltd. (USA) for the loan of the LCT unit.

REFERENCES

1. Sluyterman LAAE, Wijdeness J. *Proc. Int. Symp. Electrofocusing and Isotachophoresis*, Radola BJ, Graesslin D (eds). Walter de Gruyter: Berlin, 1977; 463–466.
2. Sluyterman LAAE, Elgersma O. *J. Chromatogr.* 1978; **150**: 17.
3. Sluyterman LAAE, Wijdeness J. *J. Chromatogr*. 1978; **150**: 31.
4. Sluyterman LAAE, Wijdeness J. *J. Chromatogr*. 1981; **206**: 429.
5. Sluyterman LAAE, Wijdeness J. *J. Chromatogr*. 1981; **206**: 441.
6. Hutchens TW, Wiehle RD, Shahabi NA, Wittliff JL. *J. Chromatogr.* 1983; **266**: 115.
7. Hutchens TW. *Protein Purification*, Janson J and Ryden L. (eds). VCH: New York, 1989; 149–174.
8. Li CM, Hutchens TW. *Methods in Molecular Biology*, Vol. 11: *Practical Protein Chromatography*, Kenney A. and Fowell S., (eds). The Humana Press: Totowa, 1992; 237–248.
9. Fountoulakis M, Langen H, Gray C, Takacs B. *J. Chromatogr. A*, 1998; **806**: 279.
10. Langen H, Takacs B, Evers S, Berndt P, Lahm HW, Wipf B, Gray C, Fountoulakis M. *Electrophoresis*, 2000; **21**: 411.
11. Abu Al-Soud W, Jonsson LJ, Radstrom P. *J. Clin. Microbiol.* 2000; **38**: 345.
12. Rio PG, Maurizis JC, de Latour MP, Bignon YJ, Bernard-Gallon DJ. *Int. J. Cancer* 1999; **80**: 823.
13. Logan KA, Lagerlund I, Chamow SM. *Biotechnol. Bioeng.* 1999; **62**: 208.
14. Opiteck GJ, Lewis KC, Jorgenson JW, Anderegg RJ. *Anal. Chem.* 1997; **69**: 1518.
15. Opiteck GJ, Ramirez SM, Jorgenson JW, Mosley MA. *Anal. Biochem.* 1998; **258**: 344.
16. Chen YJ, Wall DB, Lubman DM. *Rapid Commun. Mass Spectrom.* 1998; **12**: 1994.
17. Wall DB, Lubman DM, Flynn SJ. *Anal. Chem.* 1999; **71**: 3894.
18. Chong BE, Lubman DM, Miller FR, Rosenspire AJ. *Rapid Commun. Mass Spectrom*. 1999; **13**: 1808.
19. Wall DB, Kachman MT, Gong SY, Hinderer R, Parus S, Misek DE, Hanash SM, Lubman DM. *Anal. Chem.* 2000; **72**: 1099.
20. Chong BE, Kim JK, Lubman DM, Tiedje JM, Kathariou S. *J. Chromatogr. B* 2000; **748**: 167–177.
21. Soule HD, Maloney TM, Wolman SR, Peterson WD Jr, Brenz R, McGrath CM. Russo J, Pauley RJ, Jones RF, Brooks SC. *Cancer Res.* 1990; **50**: 6075.
22. "Liquid Chromatography: Tandem System Rivals 2-D Gels," *High Tech Separation News*, 1999; **12**: 3.

Copyright © 2001 John Wiley & Sons, Ltd.