Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 787 (2003) 11–18
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Multi-dimensional liquid phase based separations in proteomics

Hong Wang, S. Hanash*

University of Michigan, Department of Pediatrics, Ann Arbor, MI 48109-0656, USA

Abstract

This review covers recent developments towards the implementation of multi-dimensional (MuD) liquid phase based systems for proteome investigations. Although two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used as a standard approach in proteomics, its drawbacks including the limited dynamic range and molecular mass range, together with lack of on-line integration with biological mass spectrometery (Bio-MS) have limited its widespread use and applications in proteomics. In the meantime, various liquid-phase based multi-dimensional separation techniques have been explored. Especially, with the emergence of the combination of nanoflow capillary high-performance liquid chromatography (cHPLC) and Bio-MS, attention is again refocused on utilizing multi-dimensional liquid-phase based separation of proteins. Some remarkable applications of on-line analysis of intact proteins and on-column digested proteins, and the emergence of approaches such as multiple HPLC–electrospray ionization tandem MS and capillary array electrophoresis-matrix assisted laser desorption ionization MS, have stimulated thinking towards developing a automated multi-dimensional system (MuDSy) that integrates liquid phase based separation, digestion and identification of proteins in complex biological mixtures.

Keywords: Reviews; Proteomics

Contents

1. Introduction 12
2. Proteomics 12
   2.1. Goal of proteomics 12
   2.2. Approaches to quantitative proteome analysis 13
3. Multi-dimensional chromatography system 13
   3.1. Peak capacity 13
   3.2. Working mode 13
   3.3. Multi-dimensional chromatographic separation of proteins 14
   3.4. Multi-dimensional chromatography separation of peptides 15
      3.4.1. 2D-AC–RPLC mode 16
      3.4.2. 2D-IEC–RPLC mode 16
4. Conclusions 16
5. Nomenclature 17
References 17

*Corresponding author. University of Michigan, Department of Pediatrics, 1150 West Medical Center Drive, MSRBI, Room A520, Ann Arbor, MI 48109-0656, USA. Tel.: +1-734-763-9311; fax: +1-734-647-8148.
E-mail address: shanash@umich.edu (S. Hanash).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00335-5

1. Introduction

As an orthogonal highly resolving separation technique, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) introduced more than a quarter century ago [1,2] has firmly held a central place in proteomics research. Its major utility is to serve as a platform in which protein mixtures derived from complex proteome systems, such as cell-lines, tissues or biological fluids are isolated on the basis of two different physicochemical properties of protein: isoelectric point (p*I*) and relative molecular mass. With the rapid development in instrumentation and software, the integration of 2D-PAGE with biological mass spectrometery (Bio-MS) [3–9] has become an ideal core technique for global proteome profiling of the most functional compartment encoded for in the genome [10–12]. The quantitative image-based analysis of separated proteins performed using informatics tools has been extensively applied to biomedical investigations [13–15]. While 2D-PAGE still provides an unparalleled resolving power, its drawbacks have limited its use. For example, its limited dynamic range and ability to resolve small and large proteins have been the important limitations. Proteins that occur in low copy number in whole cell lysates (e.g., signaling proteins and transcription factors) and hydrophobic proteins (e.g., the membrane-associated and transmembrane proteins) have not been adequately investigated using this approach [16]. Another serious limitation of 2D-PAGE is that it cannot be directly coupled with Bio-MS which necessitates off-line protein digestion. These drawbacks have undermined the prospects for 2D-PAGE as a dominant separation technique in proteomics and have stimulate the development of alternative technologies. In particular, with the emergence of techniques such as nanoflow capillary high-performance chromatography (cHPLC) in conjunction with electrospray ionization tandem MS (HPLC–ESI-MS–MS), attention is refocusing on developing comprehensive multidimensional liquid phase based separation techniques. The possibility of multimodular combinations of HPLC, isoelectric focusing (IEF), chromatofocusing (CF) and capillary electrophoresis (CE) provides numerous options for developing liquid phase based strategies for the separation of complex mixtures of proteins and peptides [17–41].

Promising approaches have been described, and have included HPLC–ESI-MS for intact protein analysis [24–26,42], HPLC–ESI-MS–MS for on-column digested protein identification [43–45], high-efficiency multiple cHPLC–ESI-MS for high-throughput proteome studies [36], 2D-cHPLC separation systems coupled with 96-capillary array electrophoresis (CAE) for peptide mapping [37], 2D separations with strong cation-exchange (SCX) reversed-phase HPLC (RP-HPLC), with the eluted peptides from RP-HPLC column directly dropped onto the sample target of matrix-assisted laser desorption ionization quadrupole time-of-flight mass spectrometer (MALDI-Q-TOF) for quantitative proteome analysis [46] and CAE–MALDI-TOF-MS using Mylar (polyester) tape as the interface on which the eluted peptide is deposited [47]. The above cited approaches represent a step toward the goal of developing automated multi-dimensional systems (MuDSys) that include liquid phase based protein separations, protein digestion and MS identification system. Moreover, MS provides yet another dimensional separation based on the mass of proteins and peptides.

Strategies for combining different liquid phase based separation including HPLC–HPLC, HPLC–CE, IEF–HPLC and CF–HPLC, hybrid combinations of liquid phase based separation techniques with 1D-PAGE or 2D-PAGE, are reviewed

2. Proteomics

2.1. Goal of proteomics

The proteome is defined as the ‘‘protein complement encoded by a given genome’’, or ‘‘protein equivalent of a genome’’ [48]. The proteome in the multicellular organism is defined as the protein complement of a genome expressed in a tissue or a cell population. Proteomics encompasses expression proteomics and functional proteomics. Expression proteomics involves the identification and quantification of all proteins encoded in the genome and assessment of their cellular localization and their post-translational modification. Functional proteomics involves the assay of protein interaction, the determination of the role of individual proteins in

specific pathways and cellular structures, the elucidation of protein structure and function.

2.2. Approaches to quantitative proteome analysis

Expression proteomics relies heavily on the analysis of resolved proteins either through their separation followed by their quantitative analysis or through the ability of individual proteins to bind to a ligand such as an antibody. The combined application of highly resolving separation techniques (e.g., 2D-PAGE), intelligent Bio-MS and advanced informatics tools have provided a basic approach to characterize proteins in complex proteome systems. There are potentially more than 15 000 proteins expressed in a given human cell type at any time at different level of concentration with a dynamic range of fivefold. Theoretically, 2D-PAGE can provide 10 000 detectable protein spotx [49], but only a few hundred to a few thousand proteins can be identified. The lack of fast, high resolving, high reproducibility and automated protein separation for complex proteome system has limited the pace of proteomics.

Obviously, separation techniques play a critical role in acquiring high quality quantitative data. Several multi-dimensional liquid phase based separation techniques have been investigated as an alternative approach to 2D-PAGE. Some of the comprehensive multidimensional liquid separation techniques for proteins and peptides are listed in Table 1.

Table 1
Major applications of comprehensive multidimensional chromatography separation of proteins and peptides during last decade

Sample	Type of chromatography	Refs.
Protein	SEC–CE	[17]
	SEC–RPLC	[22,27]
	AC–RPLC	[23]
	IEF–RPLC	[24]
	CF–RPLC	[26,42]
	IEC–RPLC	[25]
Peptide	RPLC–CE	[17,37]
	SEC–RPLC	[29]
	IEC–RPLC	[32,38,52]
	AC–RPLC	[39,30,35,41,40]

AC: Affinity chromatography; CE: capillary electrophroesis; CF: chromatofocusing; IEF: isoelectric focusing; RPLC: reversed-phase liquid chromatography; SEC: size-exclusion chromatography.

3. Multi-dimensional chromatography system

3.1. Peak capacity

Orthogonal multidimensional chromatography systems separate proteins or peptides based on a variety of properties, such as p*I* and relative molecular mass, size and charged state, hydrophobicity and charged state.

According to the mathematical model introduced by Giddings [50], The peak capacity is expressed as follows:

$$P_{\mathrm{mD}} = P_1 \times P_2 \times P_3 \ldots$$

where, P_{mD}, P_1, P_2, P_3 are the peak capacity of the separation system in the multi-dimensional separation system, the first dimension, the second dimension and the third dimension, respectively.

Most applications have used a 2D chromatography system, such as ion-exchange chromatography coupled with reversed-phase liquid chromatography (IEC–RPLC), and size-exclusion chromatography coupled with capillary electrophoresis (SEC–CE).

If the peak capacity of each dimension is 60, the total peak capacity for a 2D system will be 3600. It is clear that such a 2D system still hardly meets the separation requirements for complex proteomes. Thus, an efficient upstream purification or pre-fractionation step need to be considered with the use of such a 2D system (see Fig. 1).

3.2. Working mode

There are usually two working modes used in a 2D system: a profiling mode and a target mode. The former is used to fractionate all components in complex mixtures (such as the profiling of soluble proteins in whole cell extracts), the latter is used to isolate a single or a small group of components from complex mixtures (such as the tagged proteins on affinity columns).

For the profiling mode, SEC or IEC can be used as the pre-fractionation step. For the target mode, affinity chromatography (AC) can be used as the upstream purification step.

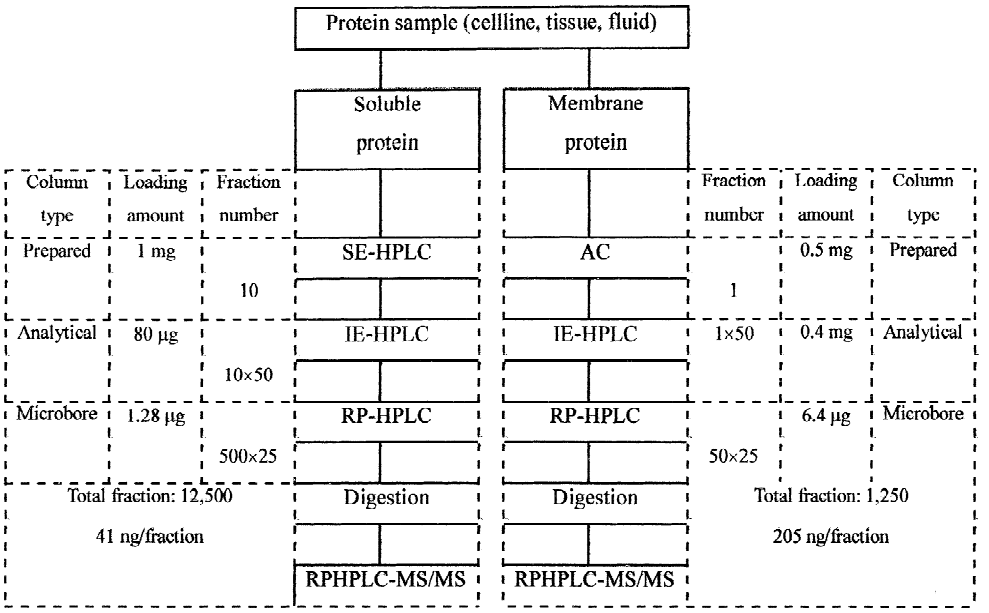

Fig. 1. Flow chart of multi-dimensional system (MuDSy). The loading amount is calculated with 80% of recovery to each dimensional separation.

Zhang and Smith [27] separated modified human lens β-crystallins by using SEC as the pre-fractionation step, followed by 2D-RPLC–IEC separation.

3.3. Multi-dimensional chromatographic separation of proteins

Comprehensive orthogonal 2D separation techniques for proteins and peptides have been proposed over a decade ago [51]. Various coupled-column approaches to protein separation, such as 2D-SEC–CE [17] and 2D-SEC–RPLC [22] were developed. Protein mixtures in *Escherichia coli* lysates were separated using a 2D-SEC–RPLC system. The protein fractions eluted from SEC were automatically subjected to RPLC to further separate proteins of a similar size on the basis of their various hydrophobicities. The protein fractions separated by RPLC were collected into 96-well microtiter plates and subjected to identification by MS.

Coutre et al. [23] analyzed membrane proteins in *E. coli* by using AC, followed by on-line RPLC–ESI-MS to directly characterize the interested membrane protein—OmpA (P02934) just with relative molecular mass measured by ESI-MS. They found that OmpA has not been post-translation modified. Feng et al. [25] reported the use of IEC to isolate recombinant protein (crotony-acyl carrier protein, an enzymatically acylated protein substrate) followed by the on-line eight-channel parallel RPLC–ESI-MS to monitor the protein purification process with a high-throughput analysis.

Our group [24] proposed a novel 2D-IEF–RPLC system to resolve large numbers of cellular proteins with the use of isoelectric focusing (Rotofor, Bio-Rad) as the first dimension. A total of 20 fractions with a p*I* range of 3.2–9.5 were collected and further resolve using non-porous RPLC. Eluted chromatographic peaks were represented by bands of different intensity in a computer reconstructed 2D image. This application demonstrated an interesting alternative approach to the 2D-PAGE technique for profiling protein expression. Recently, we developed an improved 2D-IEC–RPLC approach to 2D-IEF–RPLC. The rigid macroporous polystyrene–divinylbenzene (PS–DVB) matrix-based packing was used to re-

place the silica-based medium. Fast separations with a lower back pressure was achieved. This system has been applied to the comparative proteome analysis of different tumor cell lines. A partial composite of the 2D-IEC–RPLC patterns is shown in Fig. 2.

3.4. Multi-dimensional chromatography separation of peptides

Highly effective 2D-LC separations of peptides have been developed over the past few years ex-

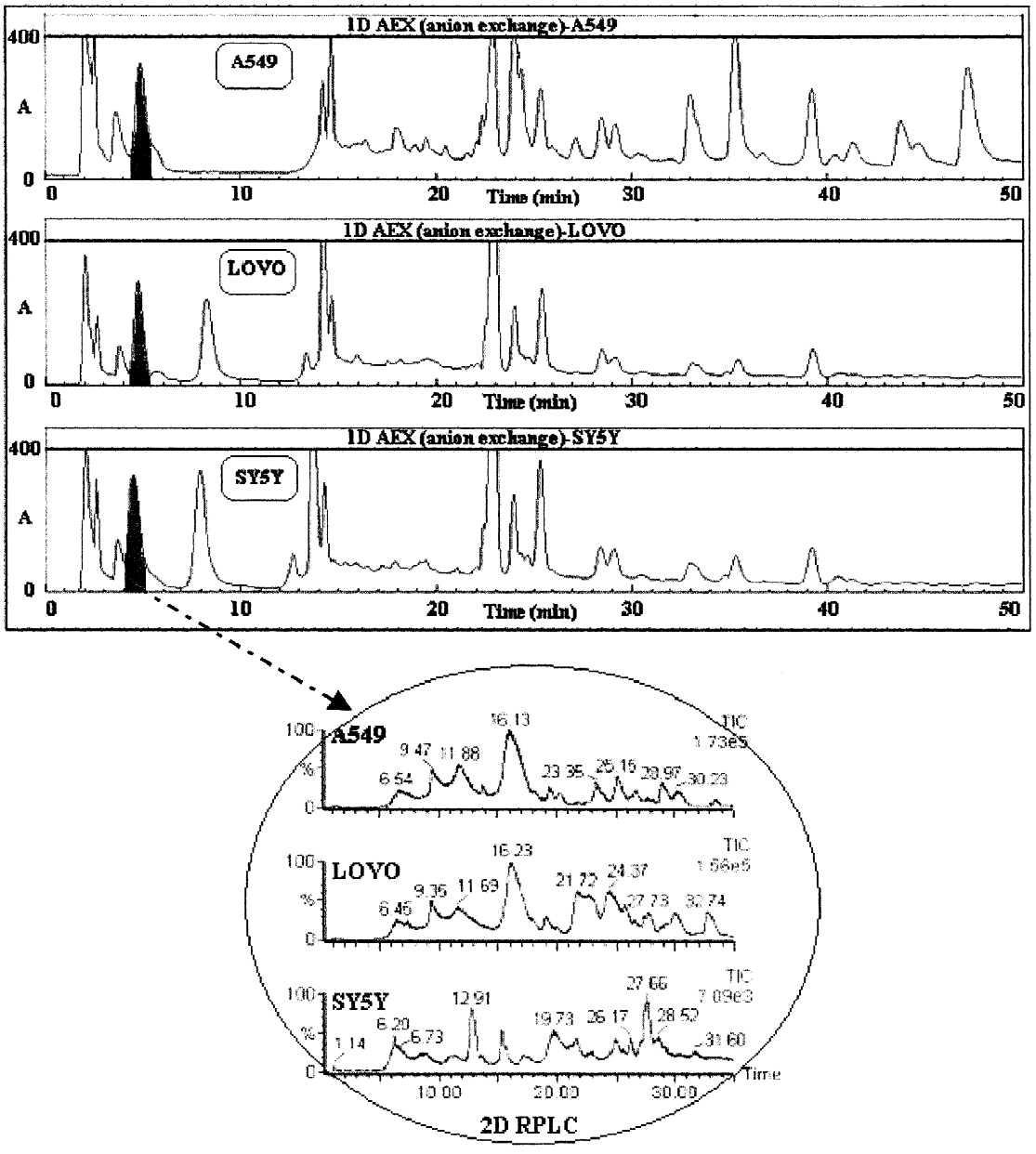

Fig. 2. Separations of three cancer cell lines (A549, LOVO and SY5Y) by 2D-AEX–RPLC. AEX: 150×4.6 mm I.D., flow-rate: 0.8 ml/min; RPLC: 200 mm×508 μm I.D., flow-rate: 50 μl/min.

emplified by the use of nano flow cRPLC–ESI-MS–MS. The separation of complex digested protein mixtures is achieved by nano flow cRPLC with on-line mass spectral detection using automated acquisition modes whereby conventional MS and MS–MS spectra are collected in a data-dependent manner, followed by automated data processing and database search to produce final identification of the parent protein(s). There are two major types of chromatography combinations: 2D-AC–RPLC and 2D-IEC–RPLC.

3.4.1. 2D-AC–RPLC mode

This is a major method used for the analysis of specific peptides, such as phosphopeptides. Phosphorylation of serine, threonine, and tyrosine represents a major post-translational modification of proteins. It is of important biological significance to identify specific amino acid residues that are phosphorylated since protein phosphorylation–dephosphorylation is the predominant mechanism of signal transduction in mammalian cells.

Adamczyk et al. [34] introduced a new approach to the characterization of serine and threonine phosphopeptides in proteins, based on the conversion of phosphoserine and phosphothreonine residues to *S*-(2-mercaptoethyl) cysteinyl or β-methyl-*S*-(2-mercaptoethyl) cysteinyl residues by $Ba(OH)_2$-catalyzed β-elimination and 1,2-ethaneditiol (EDT) addition, followed by reversible biotinylation of modified proteins. After trypsin digestion, the digest products were purified through an affinity column and the derivatized peptides analyzed by RPLC–ESI-MS–MS. This technique was successfully demonstrated using α-casein, β-casein, and ovalbumin as model proteins.

Giggs et al. [35] described an automated multi-dimensional liquid chromatography system including on-line protein digestion, AC and RPLC–ESI-MS. Protein digestion was performed in a auto-sampler and then the digest mixtures were loaded onto an immobilized enzyme column and the digests were directly transferred to an AC column and the captured phosphorylated peptides were transferred to an RPLC column coupled with ESI-MS–MS. The entire process was controlled by software and total analysis took only 2 h.

3.4.2. 2D-IEC–RPLC mode

Other combinations used to analyze peptides in complex digests include IEC–RPLC and CE–RPLC [33]. Washburn et al. have developed a multi-dimensional protein identification technology (MuD-PIT) to profile the whole yeast proteome [52]. The core part of this MuD-PIT is an orthogonal biphasic capillary column packed with two different types of packings. One is the strong cation-exchange (SCX) material. The other is reversed-phase material. The whole protein mixture was digested without performing any pre-separation, and the total digested protein mixture was loaded onto the biphasic column coupled with ESI-MS–MS. A total of 1484 proteins were detected and identified. This approach provides more protein identification than previously achieved by 2D-PAGE, although quantitative protein data could not be ascertained which represents a drawback of this MuD-PIT technique.

Issaq et al. [37] used RPLC as the first dimension and 96-array capillary electrophoresis (ACE) as the second dimension to analyze tryptic digests. Total analysis time was less than 1 h. Griffin et al. [46] reported a novel approach to quantitative proteome analysis by using a multi-dimensional separation system directly coupled with MALDI-Q-TOF-MS–MS. Proteins were first labeled by an isotope coded affinity tag reagent, and then were trypsin digested. The labeled peptides were purified by using SCX-AC–RPLC separation system. The eluted peptides from RPLC column were directly deposited onto a MALDI sample target. The sample spots were analyzed with MALDI-Q-TOF-MS–MS. Both quantification and identification information for proteins expressed in yeast cells were acquired during a single run. The major advantage of this new approach is its high throughput quantitative proteomics feature.

4. Conclusions

Various multi-dimensional liquid phase based techniques have shown promise as alternatives to 2D gels for high-throughput implementation in proteomics. In the years to come there remains a substantial need for improvements in all aspects of the protein separation process. Other high resolution

separation techniques include capillary electrochromatography (CEC) which should be yet considered as an additional separation mode, since CEC itself is an orthogonal separation technique that could be combined with cHPLC and CE [53,54]. The ultimate goal is a turn-key system that allow injection of a complex protein mixture at one end and display for the levels of abundance and modification states of its constituent proteins. Such a system may well be in hand before the end of this decade.

5. Nomenclature

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
AC	Affinity chromatography
AEX	Anion-exchange chromatography
Bio-MS	Biological mass spectrometry
CAE	Capillary array electrophoresis
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CF	Chromatofocusing
cHPLC	Capillary high-performance liquid chromatography
ESI-MS–MS	Electrospray ionization tandem mass spectrometry
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
MALDI	Matrix-assisted laser desorption ionization
MuD-PIT	Multi-dimensional protein identification technology
MuDSy	Multi-dimensional system
P	Peak capacity of a chromatography column
PS–DVB	Polystyrene–divinylbenzene
Q-TOF	Quadrupole time-of-flight
RPLC	Reversed-phase liquid chromatography
SEC	Size-exclusion chromatography
SCX	Strong cation-exchange

References

[1] J. Klose, Hum. Genet. 26 (1975) 231.
[2] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[3] S.M. Hanash, Electrophoresis 21 (2000) 1202.
[4] T. Rabilloud, Proteomics 2 (2002) 3.
[5] M. Karas, F. Hillenkamp, Anal. Chem. 60 (1988) 2299.
[6] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.
[7] M. Mann, R.C. Hendrickson, A. Pandey, Annu. Rev. Biochem. 70 (2001) 437.
[8] S.D. Patterson, R. Aebersold, Electrophoresis 16 (1995) 1791.
[9] C.H. Wheeler, S.L. Berry, M.R. Wilkins, J.M. Corbett, K. Ou, A.A. Golley, I.H. Smith, K.L. Williams, M.J. Dunn, Electrophoresis 7 (1996) 580.
[10] S.M. Hanash, Clin. Chem. Lab. Med. 38 (2000) 805.
[11] S.M. Hanash, Curr. Opin. Mol. Ther. 3 (2001) 538.
[12] S.M. Hanash, M.P. Bobek, D.S. Rickman, T. Williams, J.M. Rouillard, R. Kuick, E. Puravs, Proteomics 2 (2002) 69.
[13] P. Josef, V. Jiří, Electrophoresis 18 (1999) 3483.
[14] F.L. Peter, M.M. James, L. Yegappan, J.S. Matthew, L.P. James, V.H. Peter, C.T. Gregory, W.P. Alan, Electrophoresis 18 (1999) 3492.
[15] C. Hoogland, J.C. Sanchez, D. Walther, V. Baujard, O. Baujard, L. Tonella, D.F. Hochstrasser, R.D. Appel, Electrophoresis 18 (1999) 3568.
[16] G.L. Corthals, V.C. Wasinger, D.F. Hochstrasser, J.C. Sanchez, Electrophoresis 21 (2000) 1104.
[17] J.P. Larmann Jr., A.V. Lemmo, A.W.J. Moore, J.W. Jorgenson, Electrophoresis 14 (1993) 434.
[18] D.J. Rose, G.J. Opiteck, Anal. Chem. 66 (1994) 2529.
[19] A.W. Moore Jr., J.W. Jorgenson, Anal. Chem. 67 (1995) 3448.
[20] A.W. Moore Jr., J.W. Jorgenson, Anal. Chem. 67 (1995) 3456.
[21] J. MacNair, G.J. Opiteck, J.W. Jorgenson, M.A. Moseley III, Rapid Commun. Mass Spectrum. 11 (1997) 1279.
[22] G.J. Opiteck, S.M. Ramirez, J.W. Jorgenson, M.A. Moseley III, Anal. Biochem. 258 (1998) 349.
[23] J.I. Coutre, J.P. Whitelegge, A. Gross, E. Turk, E.M. Wright, H.R. Kaback, K.F. Faull, Biochemistry 39 (2000) 4237.
[24] D. Wall, M. Kachman, S. Gong, R. Hinderer, S. Parus, D. Misek, S. Hanash, D. Lubman, Anal. Chem. 72 (2000) 1099.
[25] B. Feng, A.H. Patel, P.M. Keller, J.R. Slemmon, Rapid Commun. Mass Spectrom. 15 (2001) 821.
[26] B.E. Chong, R.L. Hamler, D.M. Lubman, F.R. Miller, Rapid Commun. Mass Spectrom. 15 (2001) 291.
[27] Z. Zhang, D.L. Smith, J.B. Smith, Proteomics 1 (2001) 1001.
[28] L.A. Holland, J.W. Jorgenson, R.J. Anderegg, Anal. Chem. 67 (1975) 3275.
[29] G.J. Opiteck, K.C. Lewis, J.W. Jorgenson, R.J. Anderegg, Anal. Chem. 69 (1997) 1518.
[30] A. Apffel, J.A. Chakel, W.S. Hancock, C. Souders, T. M'Timkulu, E. Pungor Jr., J. Chromatogr. A 750 (1996) 35.
[31] G.J. Opiteck, J.W. Jorgenson, Anal. Chem. 69 (1997) 2283.
[32] J.P.C. Vissers, R.E.J. van Soest, J.P. Chervet, C.A. Cramers, J. Microcol. Sep. 11 (1999) 277.
[33] H.J. Issaq, K.C. Chan, G.M. Janini, G.M. Muschik, Electrophoresis 20 (1999) 1533.
[34] M. Adamczyk, J.C. Gebler, J. Wu, Rapid Commun. Mass Spectrom. 15 (2001) 1481.

[35] L. Giggs, C. Sioma, F.E. Regnier, J. Chromatogr. A 924 (2001) 359.
[36] Y. Shen, N. Tolic, R. Zhao, L. Pasa-Tolic, L. Li, S.J. Berger, R. Harkewicz, G.A. Anderson, M.E. Belov, R.D. Smith, Anal. Chem. 73 (2001) 3011.
[37] H.J. Issaq, K.C. Chan, C.S. Liu, Q. Li, Electrophoresis 22 (2001) 1133.
[38] D. Wolter, M.P. Washburn, J.R. Yates III, Anal. Chem. 73 (2001) 5683.
[39] M.T. Davis, J. Beierle, E.T. Bures, M.D. McGinley, J. Mort, J.H. Robinson, C.S. Spahr, W. Yu, R. Leuethy, S.D. Patterson, J. Chromatogr. B 752 (2001) 281.
[40] M.B. Goshe, T.D. Veenstra, E.A. Panisko, T.P. Conrads, N.H. Angell, R.O. Smith, Anal. Chem. 74 (2002) 607.
[41] S. Wang, X. Zhang, F.E. Reginier, J. Chromatogr. A 949 (2002) 153.
[42] B.E. Chong, R.L. Hamler, D.M. Lubman, S.P. Ethier, A.J. Rosenspire, F.R. Miller, Anal. Chem. 73 (2001) 1219.
[43] G. Marie, L. Serani, O. Laprevote, Anal. Chem. 72 (2000) 5423.
[44] M. Margalione, Rapid Commun. Mass Spectrom. 15 (2001) 1383.
[45] S. Wang, F.E. Regnier, J. Chromatogr. A 913 (2001) 429.
[46] T.J. Griffin, S.P. Gygi, B. Rist, R. Aebersold, A. Loboda, A. Jilkine, W. Ens, K.G. Standing, Anal. Chem. 73 (2001) 978.
[47] J. Preisler, P. Hu, T. Rejtar, E. Moskovets, B.L. Karger, Anal. Chem. 74 (2002) 17.
[48] V.C. Wasinger, S.J. Cordwell, A. Cerpa-Poljak, J.X. Yan, A.A. Gooley, M.R. Wilkins, M.W. Duncan, R. Harris, K.L. Williams, I. Humphery-Smith, Electrophoresis 16 (1995) 1090.
[49] J. Klose, U. Kobalz, Electrophoresis 16 (1995) 1034.
[50] J.C. Giddings, J. High Resolut. Chromatogr. 10 (1987) 319.
[51] M.M. Bushey, J.W. Jorgenson, Anal. Chem. 62 (1990) 978.
[52] M.P. Washburn, D. Wolters, J.R. Yates, Nat. Biotechnol. 19 (2001) 242.
[53] K. Walhagen, K.K. Unger, M.T.W. Hearn, J. Chromatogr. A 887 (2000) 165.
[54] C.M. Tellez, K.D. Cole, Electrophoresis 21 (2000) 1001.