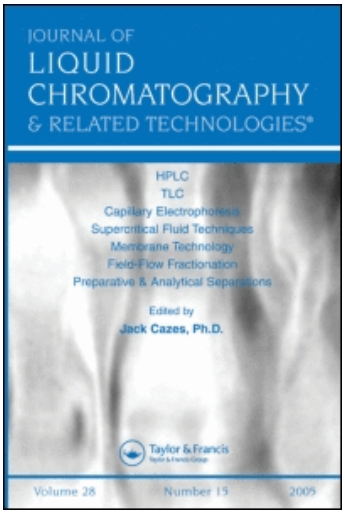

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Integration of Chromatography and Peptide Mass Modification for Quantitative Proteomics

Thomas C. Hunter[a]; Michael P. Washburn[a]
[a] Proteomics, Torrey Mesa Research Institute, San Diego, California, USA

Online publication date: 23 July 2003

To cite this Article Hunter, Thomas C. and Washburn, Michael P.(2003) 'Integration of Chromatography and Peptide Mass Modification for Quantitative Proteomics', Journal of Liquid Chromatography & Related Technologies, 26: 14, 2285 — 2301
To link to this Article: DOI: 10.1081/JLC-120023247
URL: http://dx.doi.org/10.1081/JLC-120023247

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 14, pp. 2285–2301, 2003

Integration of Chromatography and Peptide Mass Modification for Quantitative Proteomics

Thomas C. Hunter* and Michael P. Washburn

Proteomics, Torrey Mesa Research Institute, San Diego, California, USA

ABSTRACT

In the past few years the field of quantitative proteomics has generated intense interest by promising to determine the protein expression differences of 100s, and eventually 1000s, of proteins between two samples. A wide variety of methods have been reported, which alter protein/peptide masses in a fashion compatible with mass spectrometry. Each method modifies the masses of proteins/peptides at different points in an experimental scheme, providing a researcher with a variety of tools to choose from. When these methods are coupled with chromatography based approaches to proteomics, the relative abundances of 100s of proteins may currently be determined. As chromatography based proteomics advances and is coupled with the protein/peptide mass modification methods described herein, the hope is that comprehensive quantitative

*Correspondence: Thomas C. Hunter, Proteomics, Diversa Corp., 4955 Directors Place, San Diego, CA 92121, USA; E-mail: thunter@diversa.com.

DOI: 10.1081/JLC-120023247
1082-6076 (Print); 1520-572X (Online)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

proteomic analyses will be possible on cells from any organism grown in culture or extracted from a tissue.

Key Words: Multi-dimensional chromatography; MudPIT; Quantitative proteomics.

INTRODUCTION

While two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) remains the most widely implemented separation method in proteomics,[1–3] there is growing interest in alternate approaches to 2D-PAGE. Several biases of 2D-PAGE result in limited capabilities of the technology to detecting and identifying low abundance proteins,[4–6] proteins with extremes in isoelectric point and molecular weight,[7,8] and very hydrophobic proteins.[9,10] Even though two-dimensional differences in-gel electrophoresis[11] has allowed for improved quantitative proteomic analyses via 2D-PAGE,[11,12] the shortcomings of 2D-PAGE continue to stimulate research in alternate approaches to separation of complex proteomes for either qualitative or quantitative analyses.

Chromatographic approaches to the separation of proteomes are being widely pursued as an alternate methodology to 2D-PAGE. While a variety of multidimensional chromatographic approaches to separating proteins and peptides have been described,[3,13–16] proteomic analyses via multidimensional protein identification technology (MudPIT) have resulted in the detection and identification of 1484 proteins from *Saccharomyces cerevisiae* (yeast)[17] and more than 2400 proteins from both *Oryza sativa* (rice)[18] and *Plasmodium falciparum* (malaria).[19] Multidimensional protein identification technology was first described as DALPC by Link et al. as a method for the rapid analysis of *S. cerevisiae* ribosomes.[20] Subsequent methodological improvements[21] resulted in the detection and identification of 1484 proteins from the proteome of *S. cerevisiae*.[17] In MudPIT a complex peptide mixture is prepared from a whole cell lysate of a sample and directly loaded onto a biphasic microcapillary column packed with reversed phase and strong cation exchange packing materials. The MudPIT column is interfaced with an HPLC pump and mass spectrometer, allowing for the nearly simultaneous separation of peptides and generation of mass spectral information necessary for subsequent peptide identification.

Initially, the main criticism of MudPIT and of many of the multidimensional approaches being investigated was that they were not quantitative by nature. However, there has been intensive research into quantitative proteomic methods, which can be directly implemented into a chromatographic based separation method with the intent of eventually detecting, identifying,

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

and quantifying thousands of proteins from any given sample. Effectively, each quantitative proteomic method is a peptide mass modification method. In a quantitative proteomic analysis, cells from a variety of organisms may be grown under at least two different conditions expected to alter protein expression levels. In order to determine the relative abundance of a peptide from the same protein from two different cell states by mass spectrometry, the masses of identical proteins from each sample need to be modified in such a manner that the same peptide from the same protein but from each different sample has a unique mass to charge ratio (m/z). This way, each peptide has a unique intensity that can be compared to the other in order to determine the relative abundance of the proteins from the two samples. Proteins or peptides from each sample, therefore, need to be labeled as "light" and "heavy" in order to carry out this process. In a quantitative proteomic analysis, the mass spectrometer serves the dual purpose of determining the relative abundances of two peptides from the same protein from two different cell states, and of generating the necessary information to determine the identity of the peptide and, therefore, the protein from the original sample. At the end of the experiment, therefore, a researcher obtains a list of proteins from two different cell states and the relative abundance of each protein between each cell state.

Several peptide mass modification methods, compatible with chromatography, have been described in the past few years under the general heading of quantitative proteomics. These methods can be placed into three general classes: metabolic labeling,[22–31] site specific amino acid modification of proteins or peptides,[32–41] or C-/N-terminal labeling.[42–48] All of these methods have different entry points into a quantitative proteomic scheme, with metabolic labeling introducing the label into the samples at the earliest time in an experiment, and site-specific peptide labeling introducing the label at the latest point in a scheme. In principle, experimental error introduced by differential sample handling should be minimized by using metabolic labeling strategies vs. other approaches, but this has not been proven. The goal of this review is to introduce the reader to each of these methods and to describe their implementation, either proven or potential, into a quantitative proteomic scheme utilizing non-gel based separations of proteins or peptides.

Metabolic Labeling

Full metabolic labeling, with stable isotopes of proteins and selective incorporation of stable isotopes into proteins, are widely used methods in structural biology to prepare samples for analysis by nuclear magnetic resonance (NMR) analysis.[49–51] In full metabolic labeling, cells whose growth media can be controlled are grown under two conditions; one with a

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

media with naturally abundant isotopes (referred to as "light" media) and the other with a media enriched for a heavy isotope like ^{15}N (referred to as "heavy" media) (Fig. 1). By carrying out a full metabolic labeling experiment, each amino acid from each protein then has a distinct mass from its counterpart grown in the other media. An alternate approach to metabolic labeling is to use rare isotope depleted media and normal media for the comparison of two cell states.[23] However, this method is limited in its use to very high-resolution mass spectrometers like Fourier transform ion cyclotron resonance mass spectrometry (FTICR), because when a media is depleted of rare isotopes there is very little shift in m/z of peptides since there is little rare isotope in a normal media to begin with.

As mentioned earlier, metabolic labeling is the earliest entry point into a quantitative proteomic scheme (Fig. 1). Early implementations of metabolic labeling in quantitative proteomics used gel electrophoresis and spot excision as the protein isolation method.[22,24] Oda et al. identified proteins that were altered in expression between two strains of *S. cerevisiae* grown in ^{14}N or ^{15}N media, and determined the phosphorylation levels of a specific protein in the same sample.[22] While this method used one-dimensional gel electrophoresis and relatively few proteins were detected, identified, and quantified, it demonstrated the potential impact of metabolic labeling in quantitative proteomics.

Since these descriptions, quantitative proteomics by full metabolic labeling has been used in chromatography based proteomics approaches, rather than electrophoretic separations. Full metabolic labeling has been demonstrated using reversed phase-liquid chromatography (RP-LC) coupled directly FTICR on *D. radiodurans*[25,28] and mouse B16 cells,[25,30] and using MudPIT on *S. cerevisiae*.[31] Since the information for relative abundance determination and peptide identity determination is contained within the mass spectrometry data, electrophoresis based approaches are not necessary when alternate separation methods like RP-LC or MudPIT are used.

As an alternative to whole proteome metabolic labeling, isotopically enriched single amino acids may be used for the selective metabolic labeling of a cell type for a quantitative proteomic analysis (Fig. 1). Again, these approaches to cell labeling have been widely used in the structural biology field, and have recently begun to be applied to quantitative proteomics. Jiang et al. have described the single amino acid isotopic enrichment of *S. cerevisiae* with D_{10}-Leu in an attempt to overcome slow growth problems with metabolically labeled minimal media.[27] Ong et al. have carried out a similar approach where mammalian cell cultures were prepared with D_3-Leu or normal Leu.[29] In both of these manuscripts, the authors used gel electrophoresis to separate out the protein bands and, upon band isolation, analyzed the protein spot identity and relative abundance of proteins via mass spectro-

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

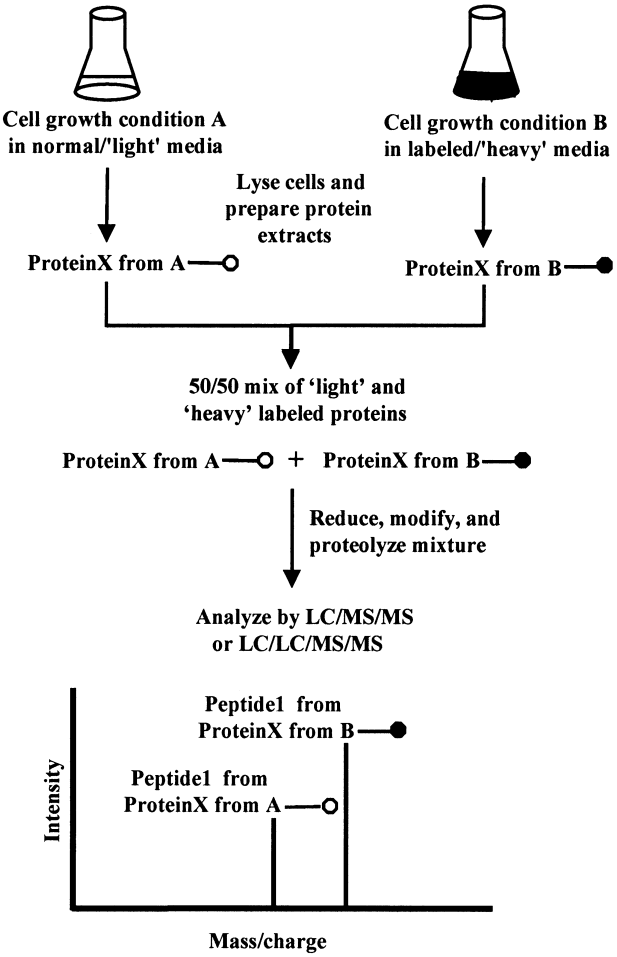

Figure 1. Quantitative Proteomic Analysis via Metabolic Labeling. In metabolic labeling, cells may be grown to either completely enrich all the proteins from one growth condition using as isotope like ^{15}N[22,24,25,28,30,31] or cells may be grown to specifically enrich proteins in media containing a stable isotope labeled single amino acid.[26,27,29] In both cases, once the cellular growth is completed the labeling process is completed. After cell lysis and generation of complex protein mixtures, a protein mixture made of 50% protein from one cell growth and 50% protein from the second cell growth is generated. From this point forward a single sample has been generated which will be prepared for mass spectrometry analysis.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

metry.[27,29] Very few proteins were detected, identified, and quantified in each analysis, limiting the biological impact of each study. However, both of these approaches are amenable to chromatography-based approaches to proteomics, since the relative abundance measurement comparing the protein abundance from two samples is made in the mass spectrometer. For example, Berger et al. have recently described the comparative analysis of *S. cerevisiae* cultured in media containing either ^{13}C-Lys or unlabeled lysine by RP-LC/FTICR.[26] To date, the focus of the published research of single amino acid stable isotope labeling has been limited to method development rather than biological inquiry. Generally, this is also the case for full metabolic labeling. However, both isotopic labeling methodologies have the potential to be applied to a wide variety of biological problems.

Post-Translational Labeling

Site specific amino acid modification of proteins or peptides[32–41] or C-/N-terminal labeling[42–48] are post-translational labeling approaches. By using a post-translational approach to quantitative proteomics, one can analyze a wider variety of systems than with metabolic labeling, since one does not need to exquisitely control growth media. Using post-translational methods, one should be able to analyze tissues from mammalian systems, whereas this cannot be done with metabolic labeling strategies. The general strategy for post-translational labeling schemes is to: (1) differentially label samples with isoforms of the labeling agent; (2) mix the differentially labeled samples; (3) chromatographic separation of the labeled mixture; and (4) determine the ratio of peptide isoforms by mass spectrometry.

The primary challenge in the development of these methods is the need to minimize the resolution of the peptide isoforms during chromatographic fractionation and mass spectrometric analysis.[52,53] Therefore, the methods must be simple, and robust and produce a population of differentially labeled peptide isoforms that retain the same physical–chemical characteristics with respect to chromatographic retention time and ionization, in order to obtain an accurate measure of the relative abundance of the peptide isoforms pairs. Furthermore, the differential label must produce spectra such that algorithms, such as SEQUEST,[54] which correlate observed spectra with theoretical spectra, are capable of making proper identifications.

Specific Amino Acid Modification

Foremost among the post-translational labeling methods and quantitative proteomics methods in general, is an approach based upon a class of reagents

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

termed isotope-coded affinity tags (ICATs).[32] An ICAT reagent consists of three functional elements: (1) a cysteine specific reactive group; (2) an isotopically labeled region; and (3) a method for affinity purification of the labeled proteins or peptides (Fig. 2). The four major steps in ICAT analysis are shown in Fig. 3. Briefly, the cysteine residues in the samples of interest are reduced and derivatized with either an isotopically light or heavy version of the ICAT reagent. The derivatized samples are then combined and digested with a protease resulting in both labeled and unlabeled peptide fragments. The labeled peptide fragments are then purified by affinity chromatography, fractionated by reversed-phase chromatography, and analyzed by tandem mass spectrometry, which provides both qualitative analysis and the relative abundance of the peptide isoforms in the samples. It has been estimated that in yeast there are approximately 350,000 tryptic peptides, and that approximately 30,000 of these peptides contain at least one cysteine residue.[42] The reduction in sample complexity that occurs using the ICAT system may be advantageous when dealing with highly complex samples. However, this method will preclude analysis of non-cysteine containing proteins and 100% of any single protein.

Since the first version of ICAT, there have been a number of permutations and improvements to the core technology. One such adaptation has been the introduction of a solid-phase capture of cysteinyl peptides from complex mixtures.[41] In this format, the most significant changes are the use of aminopropyl-coated glass beads and an O-nitrobenzyl photo-labile linker for UV mediated release of the captures cysteinyl peptides.[41] The preliminary study of this system indicated that a greater number of proteins were identified using this system in a comparative study with the avidin-based system.

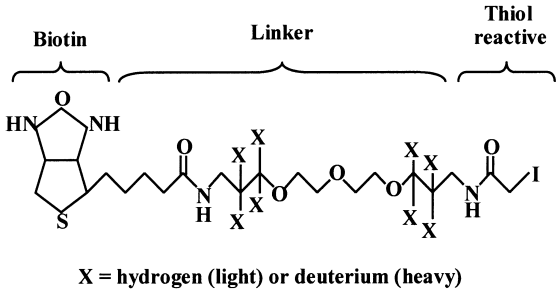

Figure 2. Structure of Isotope Coded Affinity Tags. The ICAT reagent consists of a biotin group linked to a cysteine reactive group.[32] The linker may be deuterated eight times or protonated at each site allowing for the generation of D_0- or D_8-ICAT. The differential masses of the linker group allow for the use of ICAT in a quantitative proteomic scheme.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

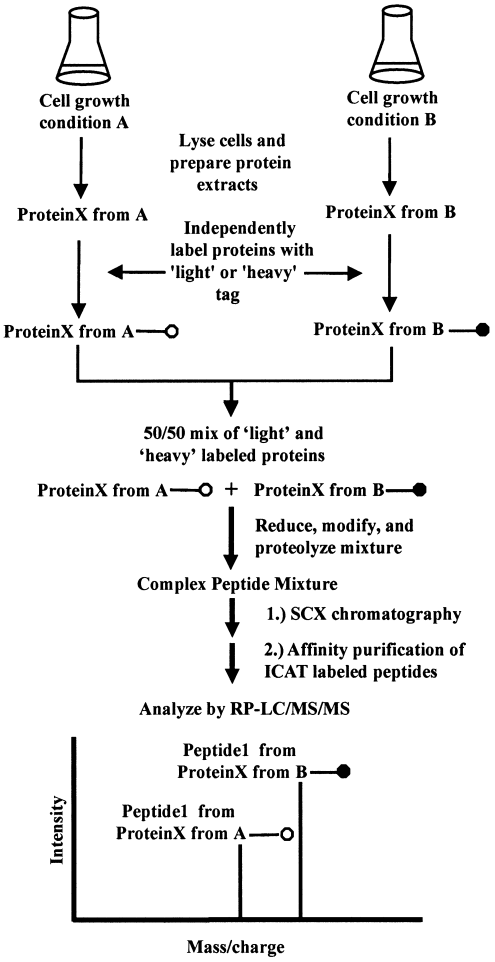

Figure 3. Quantitative Proteomic Analysis via Isotope Coded Affinity Tags. As with all quantitative proteomic methods, the key is to introduce a sample into the mass same protein but from different growth conditions. When using ICAT, whole cell lysatspectrometer that has a different m/z for the same peptide from these two different cell growths or tissue types may be prepared and upon the generation of the complex protein mixtures, the cysteines of each sample are differentially labeled.[32,33] After ICAT labeling, a protein mixture made of 50% protein from one cell growth or tissue type and 50% protein from the second cell growth or tissue type is generated. Typically, ICAT is carried out after sample mixing by running off-line strong cation exchange chromatography and collecting some number of fractions.[33,40] After affinity purification to capture ICAT labeled peptides, each fraction is analyzed by RP-LC/MS/MS where the RP-LC is directly coupled to the mass spectrometer.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

However, it was not established if the prior proteolytic digestion of proteins for the bead solid-phase analysis played a role in the greater number of proteins identified.[41] Another significant modification has been the incorporation acid-labile linker groups to the original ICAT platform.[39] This system termed "ALICE" for acid-labile isotope-coded extractants uses the basic structure ICAT structure with the addition of an acid-labile linker region. In this method, peptides were released from the biotin conjugate by incubation in trifluoroacetic acid. Again, this method used a prior proteolytic digestion of the proteins before capture and labeling, presumably to reduce steric hindrance resulting from the relatively bulky nature of the reagent.[39]

There are several alternatives to the residue-specific modification of cysteine, which include methods for differential modification of lysine[35,36] and O-phosphorylated serine residues.[37] The phosphoprotein isotope coded affinity tag (PhIAT) method has been shown capable of enriching and identifying mixtures of low-abundance phosphopeptides.[37] The PhIAT method uses a chemical modification of phosphorylated serine and threonine residues to cysteine before introduction of a standard ICAT reagent.[37] The mass-coded abundance tag (MCAT) approach uses a residue specific modification lysine residues by O-methylisourea to introduce a differential tag[36] (Fig. 4). In addition, 2-methoxy-4,5-dihydro-1H-imidizole has also been used to modify lysine residues for the purpose of introducing a differential mass tag.[35]

Each site specific amino acid modification method suffers from potential problems such as difficulty in spectral interpretation, and unwanted side reactions. One of the primary problems with the introduction of mass tags such as MCAT is the difference observed in the retention time between the

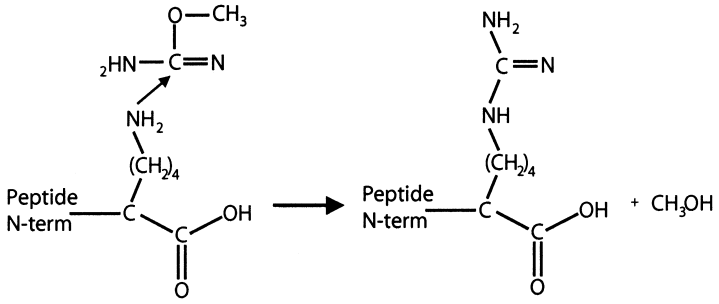

Figure 4. Lysine modification via O-methylisourea. In the Mass Coded Abundance Tagging reaction, C-terminal lysines are modified post-digestion by O-methylisourea to generate homoarginine.[36] Labeling of one sample with O-methylisourea and mixing this with an unlabeled sample allows for the determination of the relative abundance of peptides from a mixture.

peptide isoforms during reversed phase separations. It has been shown that these changes in retention time can lead to very large errors in the quantitative analysis.[52,53]

In spite of these limitations, the ICAT methodology has been successfully applied to a variety of biological questions including studies of several cell types, organelles, and different classes of proteins. Quantitative proteomic analysis via ICAT has been coupled with cDNA array analysis to investigate the galactose utilization pathway in *S. cerevisiae*[34] and to investigate the mRNA and protein expression changes brought about by culturing *S. cerevisiae* in either galactose or ethanol.[38] In addition, the ability of ICAT to detect protein expression changes of peripheral and integral membrane proteins has been demonstrated by analyzing the effect on 12-phorbol 13-myristate acetate on the microsomes of HL-60 cells.[33] A recent study has used ICAT for a proteomics analysis of Myc oncoprotein function in mammalian cells.[40] Expression differences among many functionally related proteins were identified, including proteases, protein synthesis pathways, adhesion molecules, cytoskeletal networks, and signaling pathways.[40] Of all quantitative proteomic strategies, ICAT is the most mature, as demonstrated by the successful use of ICAT in biologically driven analyses.

C-/N-Terminal Labeling Strategies

An alternative post-translational modification strategy to site-specific amino acid labeling is the global modification of all proteolytic peptides in a mixture. In general, these methods target the amino and carboxyl groups that are generated during enzymatic proteolysis. Labeling of carboxyl groups occurs through incorporation of ^{18}O from $H^{18}O$ during proteolytic hydrolysis[43,45,48] (Fig. 5). Incorporation of two ^{18}O into C-termini during proteolysis introduces a 4 Da mass shift to labeled peptides relative to an unlabeled analog. The label has been shown to be stable under MS conditions with very little back exchange of ^{18}O label with buffer.[43] This method has been most successful when used in conjunction with ESI-quadropole (QTOF) instruments. However, there are limitations to the rate and efficiency of incorporation for the two ^{18}O labels which varies between enzymes (trypsin and chymotrypsin), labeling is also not universally consistent from peptide to peptide, and there can be issues in spectral interpretation.[47] A C-terminal modification method more amenable to lower resolution ion-trap type instruments is methyl esterification of carboxyl groups, using either methanolic HCl or the deuterated analog.[42] This method is a mixed-mode method falling into two of the three general classes of quantitative proteomic methods since aspartic acids, glutamic acids, and C-termini are modified.[42] Alternatively

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

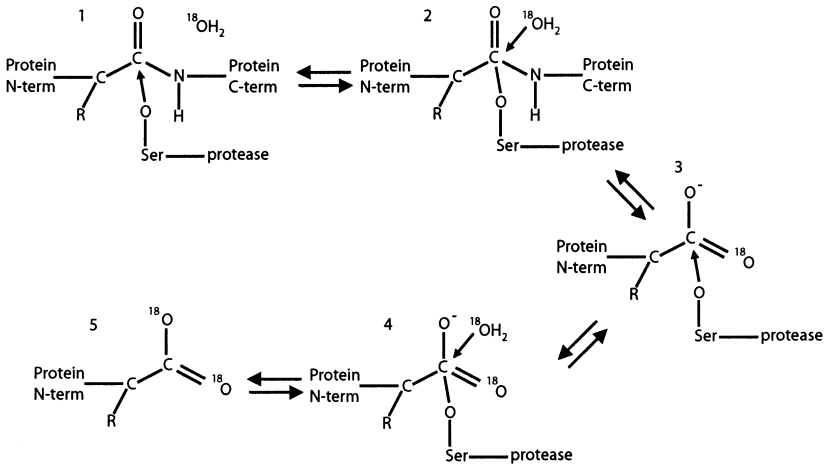

Figure 5. C-terminal digestion modification with ^{18}O. ^{18}O may be incorporated into the C-terminus of a peptide during digestion with enzymes like trypsin, endoproteinase Lys-C and endoproteinase Glu-C.[43] A simplified version of this reaction scheme is shown. (1) To begin, the peptide needs to be digested in $^{18}OH_2$ in order to then incorporate ^{18}O. The serine in the active site of the proteases listed attacks the carbonyl carbon in a peptide bond. (2) Next, $^{18}OH_2$ attacks the protein-protease intermediate also at the carbonyl carbon displacing the NH group on the peptide bond. (3) As a result, a peptide with a single ^{18}O has been generated. (4) A repeat of steps 1 and 2 is needed to drive the reaction to completion as shown in (5) where two atoms of ^{18}O have now been incorporated into the peptide C-terminus. Labeling of one sample with ^{18}O by digesting in $^{18}OH_2$ and mixing this with the other sample digested in ^{18}O depleted water allows for the determination of the relative abundance of peptides from a mixture.

there are methods available for N-terminal labeling of tryptic peptides with *N*-hydroxysuccinimide[44] or 1-Nicotinoyloxysuccinimide esters.[46] In fact, coupling ^{18}O labeling and N-terminal labeling methods for protein expression profiling produced the more comprehensive results than when either method was used alone.[47]

Challenges Ahead

Most of the descriptions of peptide mass modification methods as quantitative proteomic tools have focused on method development and "proof of concept" studies with limited biological applications. One potential reason for this is the challenge posed by the data analysis. Site-specific amino acid modification methods such as ICAT alter the mass of peptides by a

defined mass that can be scanned for in the raw mass spectrometry data,[33] which simplifies relative abundance determinations. In principle, this approach should be able to be implemented when stable isotope labeling with single amino acids. For example, when D_{10}-Leu, for example, is used in the growth media there should be isotopic peak pairs 10 amu apart for +1 peptides, 5 amu apart for +2 peptides, and 3.33 amu apart for +3 peptides. When full metabolic labeling is employed, however, finding the peak pairs is difficult unless one knows the identity of one of the two peptides and you calculate the predicted m/z of the other isotopic peak pair based on the number of nitrogen atoms in a particular peptide.[31] Wang et al. have proposed a method termed inverse metabolic labeling, where each of two experimental samples is labeled with heavy and light reagents and analyzed with the corresponding both "heavy" or "light" fraction from the other experimental sample.[55,56] By then comparing the mass spectra obtained from each pair, wise comparison of "heavy" and "light" samples, peptides whose abundances are altered between samples are readily observed.[55,56] While this method assists in the identification of proteins with expression changes, software would be needed to implement this analysis on highly complex mixtures. Advanced software is needed to carry out an analysis where a ^{15}N labeled *S. cerevisiae* cell culture, for example, is compared to a ^{14}N labeled cell culture because the m/z separation of a pair of ^{14}N and ^{15}N peptides varies depending on the peptide sequence. While this has been done in conjunction with developing MudPIT into a quantitative proteomic tool,[31] currently there are no commercially available sources for software to carry out this task.

CONCLUSIONS

Quantitative protoemic approaches utilizing chromatography rather than electrophoresis are garnering great interest in the proteomic and biological communities. A variety of approaches are currently available to a researcher and can generally be compared to each other as having different entry points into a quantitative proteomic scheme. The proof of concept of many quantitative proteomic methods have been established, but the implementation of these methods as part of a biological inquiry has been rarely demonstrated, except in the case of ICAT.[32–34,38] The quantitative proteomic methods described in this review have the potential to profoundly impact biology, but several issues remain. If quantitative proteomic methods are to truly carry out comprehensive analyses where 1000s of proteins are detected, identified, and quantified from any given sample, continued method development for improved separations of complex mixtures is essential since the largest quantitative proteomic analysis published to date identified and quantified

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

528 proteins.[40] Furthermore, there has been little literature based discussion and presentation of data describing the sample-to-sample reproducibility of any of the methods described in this review, and, as mentioned previously, software is needed to analyze the chromatograms generated from quantitative proteomic analyses of highly complex samples.[31,33] These issues will need to be dealt with in order for any method to become widely accepted by the biological community.

REFERENCES

1. Hanash, S.M. Biomedical applications of two-dimensional electrophoresis using immobilized pH gradients: current status. Electrophoresis **2000**, *21* (6), 1202–1209.
2. Pandey, A.; Mann, M. Proteomics to study genes and genomes. Nature **2000**, *405* (6788), 837–846.
3. Washburn, M.P.; Yates, J.R. Novel methods of proteome analysis: multidimensional chromatography and mass spectrometry. Proteomics: A Curr. Trends Suppl. **2000**, *1*, 28–32.
4. Fountoulakis, M.; Takacs, M.F.; Berndt, P.; Langen, H.; Takacs, B. Enrichment of low abundance proteins of Escherichia coli by hydroxyapatite chromatography. Electrophoresis **1999**, *20* (11), 2181–2195.
5. Fountoulakis, M.; Takacs, M.F.; Takacs, B. Enrichment of low-copy-number gene products by hydrophobic interaction chromatography. J. Chromatogr. A **1999**, *833* (2), 157–168.
6. Gygi, S.P.; Corthals, G.L.; Zhang, Y.; Rochon, Y.; Aebersold, R. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proc. Natl. Acad. Sci. USA **2000**, *97* (17), 9390–9395.
7. Oh-Ishi, M.; Satoh, M.; Maeda, T. Preparative two-dimensional gel electrophoresis with agarose gels in the first dimension for high molecular mass proteins. Electrophoresis **2000**, *21* (9), 1653–1669.
8. Corthals, G.L.; Wasinger, V.C.; Hochstrasser, D.F.; Sanchez, J.C. The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis **2000**, *21* (6), 1104–1115.
9. Molloy, M.P.; Herbert, B.R.; Slade, M.B.; Rabilloud, T.; Nouwens, A.S.; Williams, K.L.; Gooley, A.A. Proteomic analysis of the Escherichia coli outer membrane. Eur. J. Biochem. **2000**, *267* (10), 2871–2881.
10. Santoni, V.; Molloy, M.; Rabilloud, T. Membrane proteins and proteomics: un amour impossible? Electrophoresis **2000**, *21* (6), 1054–1070.
11. Unlu, M.; Morgan, M.E.; Minden, J.S. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis **1997**, *18* (11), 2071–2077.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

12. Zhou, G.; Li, H.; DeCamp, D.; Chen, S.; Shu, H.; Gong, Y.; Flaig, M.; Gillespie, J.W.; Hu, N.; Taylor, P.R.; Emmert-Buck, M.R.; Liotta, L.A.; Petricoin, E.F., 3rd; Zhao, Y. 2D differential in-gel electrophoresis for the identification of esophageal scans cell cancer-specific protein markers. Mol. Cell. Proteomics **2002**, *1* (2), 117–124.
13. Wall, D.B.; Kachman, M.T.; Gong, S.; Hinderer, R.; Parus, S.; Misek, D.E.; Hanash, S.M.; Lubman, D.M. Isoelectric focusing nonporous RP HPLC: a two-dimensional liquid-phase separation method for mapping of cellular proteins with identification using MALDI-TOF mass spectrometry. Anal. Chem. **2000**, *72* (6), 1099–1111.
14. Kachman, M.T.; Wang, H.; Schwartz, D.R.; Cho, K.R.; Lubman, D.M. A 2-D liquid separations/mass mapping method for interlysate comparison of ovarian cancers. Anal. Chem. **2002**, *74* (8), 1779–1791.
15. Mawuenyega, K.G.; Kaji, H.; Yamauchi, Y.; Shinkawa, T.; Saito, H.; Taoka, M.; Takahashi, N.; Isobe, T. Large-scale identification of caenorhabditis elegans proteins by multidimensional liquid chromatography-tandem mass spectrometry. J. Proteome Res. **2002**, *ASAP Article.*
16. Peng, J.; Elias, J.E.; Thoreen, C.C.; Licklider, L.J.; Gygi, S.P. Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. J. Proteome Res. **2002**, *ASAP Article.*
17. Washburn, M.P.; Wolters, D.; Yates, J.R., 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. **2001**, *19* (3), 242–247.
18. Koller, A.; Washburn, M.P.; Lange, B.M.; Andon, N.L.; Deciu, C.; Haynes, P.A.; Hays, L.; Schieltz, D.; Ulaszek, R.; Wei, J.; Wolters, D.; Yates, J.R., 3rd. Proteomic survey of metabolic pathways in rice. Proc. Natl. Acad. Sci. USA **2002**, *99* (18), 11969–11974.
19. Florens, L.; Washburn, M.P.; Raine, J.D.; Anthony, R.M.; Grainger, M.; Haynes, J.D.; Moch, J.K.; Muster, N.; Sacci, J.B.; Tabb, D.L.; Witney, A.A.; Wolters, D.; Wu, Y.; Gardner, M.J.; Holder, A.A.; Sinden, R.E.; Yates, J.R.; Carucci, D.J. A proteomic view of the Plasmodium falciparum life cycle. Nature **2002**, *419* (6906), 520–526.
20. Link, A.J.; Eng, J.; Schieltz, D.M.; Carmack, E.; Mize, G.J.; Morris, D.R.; Garvik, B.M.; Yates, J.R., 3rd. Direct analysis of protein complexes using mass spectrometry. Nat. Biotechnol. **1999**, *17* (7), 676–682.
21. Wolters, D.A.; Washburn, M.P.; Yates, J.R., 3rd. An automated multidimensional protein identification technology for shotgun proteomics. Anal. Chem. **2001**, *73* (23), 5683–5690.
22. Oda, Y.; Huang, K.; Cross, F.R.; Cowburn, D.; Chait, B.T. Accurate quantitation of protein expression and site-specific phosphorylation. Proc. Natl. Acad. Sci. USA **1999**, *96* (12), 6591–6596.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

23. Pasa-Tolic, L.; Jensen, P.K.; Anderson, G.A.; Lipton, M.S.; Peden, K.K.; Martinovic, S.; Tolic, N.; Bruce, J.E.; Smith, R.D. High throughput proteome-wide precision measurements of protein expression using mass spectrometry. J. Am. Chem. Soc. **1999**, *121*, 7949–7950.
24. Lahm, H.W.; Langen, H. Mass spectrometry: a tool for the identification of proteins separated by gels. Electrophoresis **2000**, *21* (11), 2105–2114.
25. Conrads, T.P.; Alving, K.; Veenstra, T.D.; Belov, M.E.; Anderson, G.A.; Anderson, D.J.; Lipton, M.S.; Pasa-Tolic, L.; Udseth, H.R.; Chrisler, W.B.; Thrall, B.D.; Smith, R.D. Quantitative analysis of bacterial and mammalian proteomes using a combination of cysteine affinity tags and 15N-metabolic labeling. Anal. Chem. **2001**, *73* (9), 2132–2139.
26. Berger, S.J.; Lee, S.W.; Anderson, G.A.; Pasa-Tolic, L.; Tolic, N.; Shen, Y.; Zhao, R.; Smith, R.D. High-throughput global peptide proteomic analysis by combining stable isotope amino acid labeling and data-dependent multiplexed-MS/MS. Anal. Chem. **2002**, *74* (19), 4994–5000.
27. Jiang, H.; English, A.M. Quantitative analysis of the yeast proteome by incorporation of isotopically labeled leucine. J. Proteome Res. **2002**, *1* (4), 345–350.
28. Lipton, M.S.; Pasa-Tolic, L.; Anderson, G.A.; Anderson, D.J.; Auberry, D.L.; Battista, J.R.; Daly, M.J.; Fredrickson, J.; Hixson, K.K.; Kostandarithes, H.; Masselon, C.; Markillie, L.M.; Moore, R.J.; Romine, M.F.; Shen, Y.; Stritmatter, E.; Tolic, N.; Udseth, H.R.; Venkateswaran, A.; Wong, K.K.; Zhao, R.; Smith, R.D. Global analysis of the Deinococcus radiodurans proteome by using accurate mass tags. Proc. Natl. Acad. Sci. USA **2002**, *99* (17), 11049–11054.
29. Ong, S.E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D.B.; Steen, H.; Pandey, A.; Mann, M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics **2002**, *1* (5), 376–386.
30. Pasa-Tolic, L.; Harkewicz, R.; Anderson, G.A.; Tolic, N.; Shen, Y.; Zhao, R.; Thrall, B.; Masselon, C.; Smith, R.D. Increased proteome coverage for quantitative peptide abundance measurements based upon high performance separations and DREAMS FTICR mass spectrometry. J. Am. Soc. Mass. Spectrom. **2002**, *13* (8), 954–963.
31. Washburn, M.P.; Ulaszek, R.; Deciu, C.; Schieltz, D.M.; Yates, J.R., 3rd. Analysis of quantitative proteomic data generated via multidimensional protein identification technology. Anal. Chem. **2002**, *74* (7), 1650–1657.
32. Gygi, S.P.; Rist, B.; Gerber, S.A.; Turecek, F.; Gelb, M.H.; Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. **1999**, *17* (10), 994–999.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

33. Han, D.K.; Eng, J.; Zhou, H.; Aebersold, R. Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. Nat. Biotechnol. **2001**, *19* (10), 946–951.
34. Ideker, T.; Thorsson, V.; Ranish, J.A.; Christmas, R.; Buhler, J.; Eng, J.K.; Bumgarner, R.; Goodlett, D.R.; Aebersold, R.; Hood, L. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science **2001**, *292* (5518), 929–934.
35. Peters, E.C.; Horn, D.M.; Tully, D.C.; Brock, A. A novel multifunctional labeling reagent for enhanced protein characterization with mass spectrometry. Rapid Commun. Mass Spectrom. **2001**, *15*, 2387–2392.
36. Cagney, G.; Emili, A. De novo peptide sequencing and quantitative profiling of complex protein mixtures using mass-coded abundance tagging. Nat. Biotechnol. **2002**, *20* (2), 163–170.
37. Goshe, M.B.; Veenstra, T.D.; Panisko, E.A.; Conrads, T.P.; Angell, N.H.; Smith, R.D. Phosphoprotein isotope-coded affinity tags: application to the enrichment and identification of low-abundance phosphoproteins. Anal. Chem. **2002**, *74* (3), 607–616.
38. Griffin, T.J.; Gygi, S.P.; Ideker, T.; Rist, B.; Eng, J.; Hood, L.; Aebersold, R. Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae. Mol. Cell. Proteomics **2002**, *1* (4), 323–333.
39. Qiu, Y.; Sousa, E.A.; Hewick, R.M.; Wang, J.H. Acid-labile isotope-coded extractants: a class of reagents for quantitative mass spectrometric analysis of complex protein mixtures. Anal. Chem. **2002**, *74* (19), 4969–4979.
40. Shiio, Y.; Donohoe, S.; Yi, E.C.; Goodlett, D.R.; Aebersold, R.; Eisenman, R.N. Quantitative proteomic analysis of Myc oncoprotein function. Embo. J. **2002**, *21* (19), 5088–5096.
41. Zhou, H.; Ranish, J.A.; Watts, J.D.; Aebersold, R. Quantitative proteome analysis by solid-phase isotope tagging and mass spectrometry. Nat. Biotechnol. **2002**, *20* (5), 512–515.
42. Goodlett, D.R.; Keller, A.; Watts, J.D.; Newitt, R.; Yi, E.C.; Purvine, S.; Eng, J.K.; von Haller, P.; Aebersold, R.; Kolker, E. Differential stable isotope labeling of peptides for quantitation and de novo sequence derivation. Rapid. Commun. Mass Spectrom. **2001**, *15* (14), 1214–1221.
43. Yao, X.; Freas, A.; Ramirez, J.; Demirev, P.A.; Fenselau, C. Proteolytic 18O labeling for comparative proteomics: model studies with two serotypes of adenovirus. Anal. Chem. **2001**, *73* (13), 2836–2842.
44. Ji, J.; Chakraborty, A.; Geng, M.; Zhang, X.; Amini, A.; Bina, M.; Regnier, F. Strategy for qualitative and quantitative analysis in proteomics based on signature peptides. J. Chromatogr. B Biomed. Sci. Appl. **2000**, *745* (1), 197–210.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

45. Mirgorodskaya, O.A.; Kozmin, Y.P.; Titov, M.I.; Korner, R.; Sonksen, C.P.; Roepstorff, P. Quantitation of peptides and proteins by matrix-assited laser desorption/ionization mass spectrometry using 18O-labeled internal standards. Rapid Commun. Mass Spectrom. **2000**, *14*, 1226–1232.
46. Munchbach, M.; Quadroni, M.; Miotto, G.; James, P. Quantitation and facilitated de novo sequencing of proteins by isotopic N-terminal labeling of peptides with a fragmentation-directing moiety. Anal. Chem. **2000**, *72* (17), 4047–4057.
47. Liu, P.; Regnier, F. An isotope coding strategy for proteomics involving both amine and carboxyl labeling. J. Proteome Res. **2002**, *1* (5), 443–450.
48. Reynolds, K.J.; Yao, X.; Fenselau, C. Proteolytic 18O labeling for comparative proteomics: evaluation of endoproteinase Glu-C as the catalytic agent. J. Proteome Res. **2002**, *1* (1), 27–33.
49. Hansen, A.P.; Petros, A.M.; Mazar, A.P.; Pederson, T.M.; Rueter, A.; Fesik, S.W. A practical method for uniform isotopic labeling of recombinant proteins in mammalian cells. Biochemistry **1992**, *31* (51), 12713–12718.
50. Dotsch, V.; Wagner, G. New approaches to structure determination by NMR spectroscopy. Curr. Opin. Struct. Biol. **1998**, *8* (5), 619–623.
51. Goto, N.K.; Kay, L.E. New developments in isotope labeling strategies for protein solution NMR spectroscopy. Curr. Opin. Struct. Biol. **2000**, *10* (5), 585–592.
52. Zhang, R.; Sioma, C.S.; Wang, S.; Regnier, F.E. Fractionation of isotopically labeled peptides in quantitative proteomics. Anal. Chem. **2001**, *73* (21), 5142–5149.
53. Zhang, R.; Sioma, C.S.; Thompson, R.A.; Xiong, L.; Regnier, F.E. Controlling deuterium isotope effects in comparative proteomics. Anal. Chem. **2002**, *74* (15), 3662–3669.
54. Eng, J.; McCormack, A.L.; Yates, J.R., III. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. **1994**, *5*, 976–989.
55. Wang, Y.K.; Ma, Z.; Quinn, D.F.; Fu, E.W. Inverse 15N-metabolic labeling/mass spectrometry for comparative proteomics and rapid identification of protein markers/targets. Rapid Commun. Mass Spectrom. **2002**, *16* (14), 1389–1397.
56. Wang, Y.K.; Ma, Z.; Quinn, D.F.; Fu, E.W. Inverse 18O labeling mass spectrometry for the rapid identification of marker/target proteins. Anal. Chem. **2001**, *73* (15), 3742–3750.

Received October 9, 2002
Accepted March 27, 2003
Manuscript 6114

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016