

PROTEOMIC TOOLS FOR QUANTITATION BY MASS SPECTROMETRY

Jennie Lill*

ActivX Biosciences, 11025 North Torrey Pines Rd., La Jolla, California
92037

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Techniques for the quantitation of proteins and peptides by mass spectrometry (MS) are reviewed. A range of labeling processes is discussed, including metabolic, enzymatic, and chemical labeling, and techniques that can be employed for comparative and absolute quantitation are presented. Advantages and drawbacks of the techniques are discussed, and suggestions for the appropriate uses of the methodologies are explained. Overall, the metabolic incorporation of isotopic labels provides the most accurate labeling strategy, and is most useful when an internal standard for comparative quantitation is needed. However, that technique is limited to research that uses cultured cells. © 2003 Wiley Periodicals, Inc., Mass Spec Rev

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I. INTRODUCTION

This article reviews the different protein and peptide quantitation strategies that have been described and employed by the proteomic community. These quantitation strategies are being used to solve increasingly challenging analytical and sequencing problems, including the measurement of protein/protein complexes (Coates & Hall, 2003), the measurement of post-translational modifications (Itoh et al., 2002; Li et al., 2002), the presentation of natural cell-surface ligands (Skipper et al., 1999; Clark et al.,

*Correspondence to: Jennie Lill, ActivX Biosciences, 11025 North Torrey Pines Rd, La Jolla, CA 92037. E-mail: Jenniel@activx.com

2001), and the characterization of low abundant proteins. Over the past decade, mass spectrometry (MS) has proven to be the most effective analytical tool to characterize the complex and dynamic entity that constitutes a proteome (Arnott, Shabanowitz, & Hunt, 1993; Kuster & Mann, 1998). Extended and often multidimensional chromatographic separations, as well as techniques that exploit the high dynamic range of newer mass spectrometers, are required to characterize complex protein mixtures that may be expressed at different levels. With the rapid advancement of bioanalytical instruments, as well as sequencing and quantitation software, proteomic MS is in an era of exponential growth.

In addition to the initial identification of phenotypic expression and protein characterization, a key parameter in proteomic analysis is the ability to quantify proteins of interest. Quantitation remains a vital analytical component of target validation for proteomic analysis, and of the determination of translational effects that affect protein production and function. Unfortunately, ion-current intensities of mass spectrometric signals alone may not always correlate precisely, accurately, or directly with the amount of analyte present in a sample. This lack of correlation is true regardless of the ionization technique or instrument employed, especially when measuring subtle differences in expression.

During liquid chromatography–mass spectrometry (LC–MS) analyses, the level or reproducibility may decrease after several chromatographic analyses. Suppression of ionization because of species of higher abundance can skew quantitation when using either micro-electrospray ionization (ESI) techniques or matrix-assisted laser desorption ionization (MALDI), the two most commonly employed ionization techniques in modern proteomics. Additionally, proteins and peptides may not crystallize homogeneously during matrix preparation, even with the addition of reagents such as nitrocellulose (Preston, Murray, & Russell, 1993). This factor may cause problems for quantitation because non-homogeneous crystallization may introduce a bias into the quantitative analysis. Hence, internal standards must be implemented to circumvent these issues and enable accurate quantitation. The choice of standards is essential. Ideally, they should be as physically and chemically identical as possible to the proteins being quantified. Many techniques have been explored and implemented for the routine quantitation of proteins (Oda et al., 1999; Yan et al., 1999; Washburn et al., 2002). For relative quantitation (i.e., the direct comparison of the abundance of proteins or peptides between two or more samples), these techniques are widely available. However, choosing a technique that provides the least bias and the highest compatibility in terms of preparation and mass spectrometric analysis for the samples in question is crucial.

Discrepancies between mRNA levels and protein expression are now documented, veering quantitation efforts in drug discovery towards proteomics-based profiling (Gygi et al., 1999a). Quantitation of proteins was historically undertaken, using the following methods: specific antibodies (e.g., Western blotting) (Zhou, Pyne, & Tikoo, 2001), autoradiography after metabolic labeling, fluorography, and the use of protein stains (D'Andrea et al., 1999). However, many of these methods are neither high-throughput nor accurate (Oda et al., 1999). In the last decade, protein detection through two-dimensional (2-D) gel analysis, followed by the identification of proteins by direct excision and processing from the gel, became a popular proteomic tool. Although many groups still characterize proteomic samples this way, new proteomic separation, and quantitation techniques continue to emerge. For example, multidimensional liquid chromatographic techniques (Opitck et al., 1997; Clark et al., 2001), particularly those that include the use of mixed-bed biphasic chromatographic columns (Link et al., 1999), are gaining popularity for high-throughput full-proteome characterization. Other sophisticated techniques designed to specifically enrich a subset of the proteome have also been described. Of particular interest is a method that employs activity-based probes that allow the selection and covalent modification of specific enzymatic classes. Activity-based profiling allows the characterization of low-abundant enzymatic species that typically remain undetected when using conventional analytical techniques such as 2-D gel electrophoresis (2-D PAGE) (Cravatt & Sorensen, 2000; Patricelli et al., 2001).

As implied above, although the technique remains popular among many researchers, 2-D PAGE alone does not allow for the quantitation of all proteins. Many proteins, such as hydrophobic membrane-associated proteins or proteins of low abundance, fail to be detected with 2-D PAGE. Also, the presence of multiple protein forms because of post-translational modification may also cause difficulties in quantitative analysis that result from multiple spots per protein or multiple proteins per spot. Therefore, this technology is being superseded by analytical approaches that use on-line chromatographic techniques in conjunction with *in vivo* and *in vitro* metabolic and chemical quantitation tools. On-line chromatographic separations of peptides are less restrictive. Therefore, the inconsistent extraction of the protein from an impervious matrix does not enter into the analytical equation. Absolute quantitation (i.e., determining the accurate molarity, total protein, or copy number per cell/sample) may also be necessary for some analyses. In those cases, traditional analytical skills, using standard curves and standard addition must be employed.

This review concentrates on novel proteomic quantitation methodologies, as well as popular techniques for proteins from cell lines, fluid samples, and tissue samples.

Peptidic species, including those peptides derived from proteolytic digestion, as well as those peptides that are naturally expressed as peptide ligands, are also considered. The main focus of the review will be on relative (rather than absolute) quantitation. Each technology is described, and the relative merits and limitations are discussed.

II. RELATIVE QUANTITATION

Most proteomic applications to drug discovery are concerned with the relative abundances of proteins. Comparison of diseased and “control” cell lines or tissues, or of perturbed and non-perturbed samples, is generally necessary to suggest drug targets or to deduce the effects of drug candidates. In this type of analysis, the determination of relative amounts of protein, or of relative degrees of post-translational modifications associated with the proteins of interest, may be necessary. To further explore quantitation

techniques available for such investigations, one first must consider the source of the biological materials of interest. For example, more flexibility for quantitative comparison of proteins exists for proteins from cell lines grown in tissue culture than for proteins from tissue samples of clinical origin. Typically, to compare samples quantitatively, the qualitative and quantitative analyses are both performed in parallel. In contrast, when absolute quantitation is needed, samples are compared to a well-established standard. Several methodologies for comparatively quantitating proteins and peptides are discussed below. The use of internal standards in quantitative proteomic analysis is summarized in Table 1.

A. 2-D Gel Electrophoresis

Conventional proteomics relies heavily on 2-D PAGE integrated with mass spectrometric analysis to characterize and compare protein samples quantitatively (Patton, 1999;

TABLE 1. Summary of Quantitation Techniques Described for Proteomics

LABELING TECHNIQUE	OVERVIEW OF METHODOLOGY	ADVANTAGES	LIMITATIONS
Metabolic Labeling	<i>Growth on general or specific isotope source</i>	<i>Earliest incorporation Tailored for specific residues. >2 samples per comparison possible.</i>	<i>Growth on expensive media is required.</i>
Chemical Labeling of Thiol groups (ICAT & ALICE)	<i>Cysteine-residue modification followed by selective capture</i>	<i>Less complex samples Commercially available.</i>	<i>Non-specific binding at capture stage. Tag complicates MS analysis</i>
Enzymatic isotopic labeling	<i>C-terminal modification during proteolytic cleavage</i>	<i>Versatile Easy to implement</i>	<i>Small isotope shift. Late incorporation of isotope</i>
Derivatization of the N- or C- terminus of peptides		<i>Universally applicable. Modifications generally incorporated into many search algorithms.</i>	<i>No selectivity; therefore, reduced dynamic range.</i>
Lysine-specific labeling		<i>Abundant residues are modified.</i>	<i>Reduced dynamic range.</i>
Phosphopeptide labeling	<i>β-elimination from P-Ser or P-Thr followed by addition of labeling reagent</i>	<i>Monitors residues important for metabolic control.</i>	<i>Lacks P-Tyr Chemistry difficult.</i>
Differential mass mapping	<i>Compare a mass map of chromatographically separated proteins.</i>	<i>Simple</i>	<i>Reproducible hi-resolution separations required to minimize suppression affects.</i>

Yan et al., 1999; Newsholme et al., 2001). Protein mixtures derived from tissue samples, fluid sample, or cell cultures are separated by 2-D PAGE, which is comprised of the first-dimension isoelectric focusing (IEF) of denatured proteins, followed by a second-dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins are visualized by non-specific staining techniques. After staining, the gel images are quantitated by densitometry, whereby differences in staining intensity correlate with differences in protein abundance.

Although the reproducibility and sensitivity of quantitative measurements based on 2-D PAGE have seen significant improvement, 2-D PAGE is not without flaw. As a first caveat, a full complement of the proteome is seldom seen; 2-D PAGE is notorious for its inability to detect many membrane-bound proteins because of their low solubility during the first (IEF) dimension separation. In addition, the dynamic range of the staining method is, at best, three orders of magnitude. Consequently, proteins of low abundance cannot be detected. Further, proteins that remain outside the range of separation, such as proteins with either an extremely high or low pI, are not visible with 2-D PAGE. A second caveat is that, despite two dimensions of separation, each gel band may contain multiple proteins, or some proteins may be found in multiple gel bands. Staining intensities vary from protein to protein and from analysis to analysis; this variability lowers the confidence in comparative quantifications of samples separated on different gels. Further, proteins may form multiple spots, or in the worst case, a broad streak on the gel. This effect is often because of post-translational modifications, and skews the analysis. A final caveat is that the coverage of the protein sequence (i.e., the fraction of the protein's amino acid sequence that is actually observed in the mass spectrometric analysis) after extraction of peptides by enzymatic digestion from 2-D PAGE is generally low. In such cases, even though the protein identity is unequivocal, the quantitation is inevitably unreliable, and subtle differences in protein concentration become impossible to measure.

As an alternative approach to standard 2-D PAGE, Sechi & Chait described the use of deuterated and non-deuterated acrylamide to modify cysteine residues in proteins prior to separation. In that method, quantitative information could be obtained both through conventional densitometry and through mass shifts in the isotopically labeled cysteine-containing peptides (Sechi & Chait, 1998; Sechi, 2002). Further, such mass spectrometric analyses can be used to determine whether more than a single protein is represented in a given band because the quantitation is based on mass rather than only on visualization.

In many research groups, quantitative 2-D PAGE is being replaced by newer methods of multidimensional separation (e.g., on-line multidimensional liquid chroma-

tographic separations) coupled with one of the quantitative methodologies described below. Such methods are easier to automate, so that their application is quickly becoming routine (Peng & Gygi, 2001).

B. Metabolic Isotopic Labeling

Metabolic labeling exploits the incorporation of isotopic labels (generally a stable isotope-incorporated amino acid) during the processes of cellular metabolism (which includes *de novo* amino acid biosynthesis) and during protein synthesis, where exogenous amino acids are specifically introduced into nascent proteins. Labeling through metabolic incorporation is one of the most accurate procedures for quantitation of proteins from a cell line because each peptide produced by the cell and cleaved by enzymatic digestion will have an isotopically identical internal standard.

1. ^{15}N

A simple and universal method to metabolically incorporate an isotopic reagent involves growing one cellular group in an isotopically depleted media enriched in ^{15}N , and the other in standard ^{14}N -rich media. After harvesting cells, the two sets are equalized with respect to cell weight/number, and are pooled. Because degradation or non-specific binding may occur in many steps of the process, the pooling of the samples ensures that the depletions affect both sample sets equally, and that the processing occurs in an unbiased manner. In this way, errors in sample processing do not affect the final quantitation. This approach has been demonstrated by many groups, and has been proven to be an accurate quantitation methodology (Oda et al., 1999; Washburn et al., 2002).

2. ^{13}C Enrichment and Depletion

The above technique can also be used with ^{13}C -isotopically modified media, although this method has not been reported extensively. Stocklin et al. (2002) described a novel technique in which positive- and negative-labeling by isotope dilution was exploited for the quantitation of peptides. This strategy was described in a pharmacokinetic context in which one sample was grown in ^{12}C at 99.98% enrichment (^{13}C -depleted, negative labeling) and the other in ^{13}C -enriched (^{12}C -depleted, positive labeling) media, and an internal standard was monitored alongside a pharmacokinetically derived peptide. As well as aiding quantitation, this technique also aided mass spectrometric interpretation, because these two labeled peptides showed non-natural isotopic distributions when analyzed by

MALDI-TOF, and were clearly distinguished between standards. For this method and the ^{15}N -incorporation strategy described above, software tools, for example, TurboSequest and Xcalibur (Thermo Finnigan, San Jose, CA), have been adapted to allow the easy identification and quantitation when these isotope strategies are employed.

3. Select Isotopic Amino Acid Incorporation

Many researchers have described the use of selected stable isotope-incorporated amino acids as tools for quantifying protein/peptide levels between two given cell lines (Meiring et al., 2001; Ong et al., 2002; Zhu et al., 2002). Again, researchers who exploit actively metabolizing cell lines can employ this methodology because selected stable isotope-incorporated amino acids are incorporated into the proteins from the cell culture media in the same manner as described above for the ^{15}N technique. Here, instead of isotopically labeling the entire pool of amino acids with ^{15}N , selected amino acids are labeled. Several isotopic amino acids have been used in this manner, such as (5,5,5- $^2\text{H}_3$)leucine and (^{15}N)methionine. Again, one sample set is grown in the non-isotopic growth media whereas the sample for comparison is grown in depleted growth media that contains one or more isotopic amino acid residues. After harvesting the cells, the two sample sets (of equal weight/cell number) can again be pooled, and biochemical preparation, separation, and mass spectrometric analysis performed. If MS is the method of detection, then the differences (Δ) in mass between the heavy and light isotopes can be monitored. When analyzing a total proteome, it is best to choose an abundant amino acid, although this choice can be modified to a specific amino acid residue if a particular peptide has been pre-selected. For peptidic cell ligands, such as those that participate in the immune response [e.g., major histocompatibility complex (MHC) class I], the “anchor” residues, which are conserved recognition elements based on the subtype of the receptor, can be targeted for amino acid substitution (Meiring et al., 2001).

Ong et al. coined the term *Stable Isotope Labeling by Amino acids in Cell culture* (SILAC). In this method, specific amino acids are incorporated metabolically *in vivo* into all proteins as described above (Ong et al., 2002). This group showed that, in their example, the substitution of an isotopic amino acid in the growth media does not significantly alter cell growth, cell morphology, doubling time, or the ability to differentiate, and hence can be used as a powerful quantitative tool for proteomic analysis. However, it is necessary to be confident in the isotopic composition of the target cells. Typically, the cells are passaged several times in an isotopically homogeneous media to enforce isotopically uniform intracellular label-

ing during the translation process. Ong et al. describe the complete incorporation of selected stable isotope-incorporated amino acids after five doublings in the cell lines and proteins that were studied. Of course, the necessary number of passages in the presence of isotopically enriched media may vary from cell line to cell line for complete metabolic incorporation of the isotope, and longer incubation times may be necessary for the complete labeling of proteins that turn over slowly.

Isotopic incorporation of ^{15}N or a selected amino acid is ideal for quantitating proteins or natural cell ligands from cell lines because it is relatively inexpensive, analytically accurate, and fairly simple to carry out. Because isotopes are incorporated at the earliest possible stage, experimental error is minimized. Further, mass spectrometric analysis does not discriminate between control and comparative samples because both proteins have equal ionization efficiencies. In addition, peptide-bond fragmentation because of collision-activated dissociation (CAD) is not altered appreciably, even when considering the primary isotope effect on amide bond fragmentation when ^{15}N is used. The benefit of using an isotopically depleted media enriched with ^{15}N is that it does not rely upon just one amino acid residue for labeling, as can be the case for many stable-isotope incorporation labeling techniques or the incorporation of single amino acids. Also, because of the increasing popularity of using metabolic labeling for quantitation, bioinformatic software is now available to sequence and quantify the mass spectrometric data that result from this technique. Incorporating a single selected stable isotope-incorporated amino acid has the advantage over the ^{15}N technique in that the isotope mass shift Δ (during MS/MS fragmentation to *b*- and *y*-ions) can be predefined. This ability facilitates sequencing efforts.

Metabolic labeling is limited because it relies on proteomic samples derived from cell culture. Other samples of interest, for example, clinical tissue samples, are not amenable to this technique. Therefore, either proteolytic-labeling or chemical-labeling techniques might need to be explored.

C. Chemical Labeling

When metabolic labeling is not suited to an analysis, these chemical-labeling techniques offer an excellent alternative quantitative tool.

1. Labeling During Proteolysis: ^{18}O Incorporation During Enzymatic Cleavage

Proteolysis incorporates an oxygen atom from the solvent into the C-terminus. Thus, heavy (^{18}O) and light (^{16}O) isotopic oxygen incorporation during proteolytic cleavage has been used for the comparative quantitation of proteins

(Yao et al., 2001; Stewart, Thomson, & Figeys, 2001; Mirgorodskaya et al., 2002). In that method, one set of proteins is cleaved in “heavy” (H_2^{18}O) water, and the other set is cleaved similarly in “light” (H_2^{16}O) water. Typically, trypsin is used, although other proteolytic enzymes may be used as well. In this method, samples are pooled later in the process (after digestion). This method leads to a somewhat higher variability. As with other methods, quantitation by mass spectroscopy is the final step in the analysis.

Systematic studies have shown that proteolytic enzymes incorporate different levels of isotope from water during digestion. For instance, Asp-N and chymotrypsin incorporate one ^{18}O atom into the peptide C terminus, whereas trypsin, Glu-C, and Lys-C incorporate either one or two ^{18}O atoms (Reynolds, Yao, & Fenselau, 2002). Again, quantitation is based on isotope ratios. Analysis of this type of data can be ambiguous. The mass Δ between each sample for the incorporation of a single ^{18}O is only 2 Daltons (Da). That mass difference produces small changes in m/z , especially for multiply charged species (Fig. 1). Although modern mass spectrometers are capable of measuring small mass differences with very high accuracy, the presence of natural isotopes (e.g., ^{13}C) in all samples can interfere with an accurate measurement. A mass shift of >4 Da would be the minimum needed to move away from such ^{13}C interferences.

To help overcome this ambiguity, a protocol with inverse ^{18}O -labeling has been described. In that protocol, the first analysis is performed as described above, but a second analysis is also performed by reversing the isotopic labels. The two sets of data are compared. For isotopic incorporation during proteolysis to have occurred, the results should show an increase in the “heavy” peak in the first analysis, mirrored by an increase in the “light” peak in the second analysis. This method allows easier detection of

relative protein quantities because it minimizes any ambiguity in the data interpretation (Wang et al., 2001).

Overall, that method is advantageous because the oxygen atoms of the C-terminal carboxylates do not exchange with solvent in the absence of enzymatic action. Further, because an isotope is not incorporated during protein synthesis, this technique can be applied universally. One drawback to this type of quantitation is that isotopic distinction occurs relatively late in sample processing. Typically, prior to enzymatic digestion, clean-up and chromatographic procedures are performed that may result in unequal losses because of sample handling and non-specific binding. This technique is also not applicable to all types of proteomic analyses. For example, naturally expressed ligands, such as the MHC-associated peptides (Bonner et al., 2002) are too small to be cleaved enzymatically. The advantage of such a subtle technique is, as for the *in vivo* isotopic quantitation methodologies above, that such small changes of sample mass equate to less chromatographic drift; those small changes provide accurate quantitation between sample pairs (because of isotope effects on retention times). Also, the fragmentation of peptides during CAD that occurs in the same manner for labeled and non-labeled species aids *de novo* sequencing.

2. Isotopic Tags—Isotope-Codes Affinity Tag Reagents (ICAT)

A favorite target for chemical labeling is the thiol group of cysteine. The first chemical-labeling strategies were based on the labeling of cysteines by iodoacetamide derivatives. Isotope-coded affinity tag (ICAT) reagents label proteins at the alkylation step of sample preparation (Gygi et al., 1999). Labeled proteins are either enzymatically or chemically digested prior to analysis (Griffin et al., 2001), or are

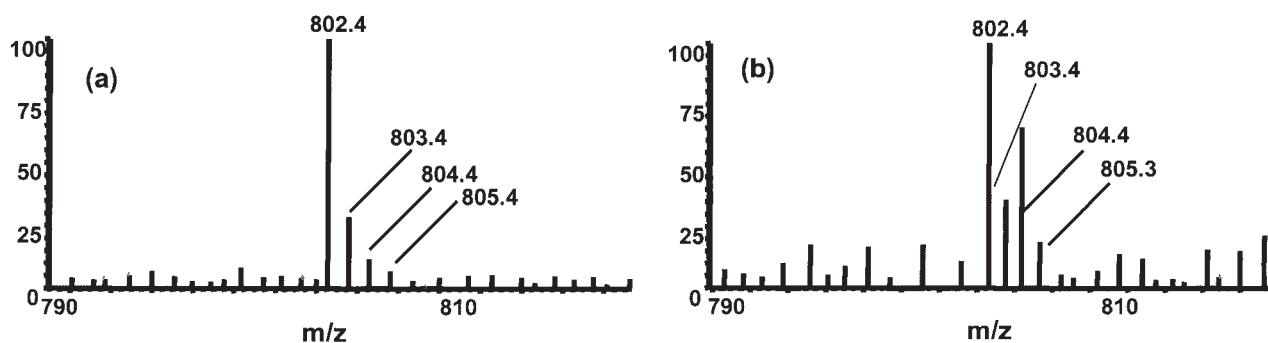


FIGURE 1. **a:** Mass spectrum obtained for the tryptically derived peptide IAALAGCK, seen at m/z 802.4, for the $[\text{M} + \text{H}]^+$ ion, obtained through digestion of Carboxylesterase 1 (*Mus musculus*) in O^{16} -enriched water. These ions represent the typical peptidic isotopic-distribution seen to occur because of carbon isotopes. **b:** Shows the same peptide after tryptic digestion in the presence of O^{18} -enriched water. The peak at m/z 804.4 is enhanced, and the distribution of ion intensity is altered.

separated on an SDS gel system prior to digestion. MS-based detection, either with MALDI or ESI, is performed, and quantitation is based on analyzing either peak areas or signal intensity, or a combination of both.

The original ICAT reagents contain a biotin affinity tag to allow the enrichment of cysteine-containing peptides with avidin affinity column chromatography. By selecting cysteine-containing peptides, a large mass spectrometric dynamic range is available compared to similar analyses on total proteomic samples. These first-generation ICAT reagents are either deuterated (D_8) or non-deuterated (D_0) to give a mass Δ of 8 Da. The added mass of biotin increases the mass of the analyte significantly (+442 Da), and results in incomplete sequence information for some peptides. Incomplete sequences may result from peptide fragmentation because of the 1/3 mass cut-off rule when using ion trap mass spectrometers as the detector. Also, the isotope effect on retention times for the two species is often significant and causes the two species to elute at different times. When two peptides co-elute, the same ionization stresses are applied to both peptides because of the presence of identical co-eluting background species. Peptides that do not co-elute can bias quantitation slightly because the different backgrounds that are present at different elution times can result in different ionizations. Further, the reliance on biotin/avidin-binding systems may lead to additional complications, such as non-specific binding peptides and incomplete elution (Moseley, 2001).

These issues have been addressed with the new, commercially available ICAT reagents (Applied Biosystems, Framingham, MA). An acid-cleavable linker has been incorporated to allow the removal of the biotin affinity tag before MS and MS/MS analysis. Also, ^{13}C instead of 2H is used in the heavy ICAT reagent. This compound improves quantitation accuracy, because the isotope shift in reversed-phase separations is virtually eliminated, and the added mass is minimized (the first generation added 442 Da, whereas the new generation adds 227 Da to labeled peptides). Further, the use of chemical affinity to capture labeled peptides allows more stringent washes to eliminate contaminants such as small molecules and detergents. Another ICAT reagent in which an *O*-benzyl-based photocleavable linker is used instead of the acid-labile version has recently been described. This modification further reduces the size of the mass addition to +170 Da by using a leucine-based tag (Zhou et al., 2002). However, this method differs significantly in comparison to conventional ICAT in that the solid-support method labels peptides and captures them after proteolysis instead of labeling the intact protein.

Overall, the negative aspects of ICAT include the cost, the requirement for a cysteine residue in the peptide sequence, and tag fragmentation. Tag fragmentation in the bulkier labels complicates the MS/MS spectra because

labeled ions fragment further under routine CAD conditions. The lighter tag in the new immobilized reagent does not fragment as much as the heavier tag. With modern search algorithms that include the use of ICAT modifications, these additional non-peptidic ions do not pose a problem. However, in *de novo* sequencing, these ions make the spectra exceedingly difficult to interpret.

Other commercially available reagents have also been used for ICAT-type quantitation. Conrads et al. employed the commercially available reagent, iodoacetyl PEO-biotin (Pierce, Rockford, IL) to isolate and quantify cysteine-containing peptides. A combination of the ^{15}N -labeling approach with the chemical-labeling approach mentioned above was employed to quantify proteins from a mouse melanoma cell line. Labeling was applied at two time-points. As with the ICAT reagents, cysteine-containing peptides were selected with the iodoacetyl reagent. Combining this feature with proteomes that were labeled metabolically reduced the bias that derived from labeling at a later stage of biochemical preparation (Conrads et al., 2001). Finally, all of the reagents employed for ICAT-type quantitation suffer from the identification of significantly fewer proteins than by conventional analysis, because only cysteine-containing peptides are selected. In particular, this method reduces the identification of proteins that have been post-translationally modified (MacCoss & Yates, 2001).

3. Isotopic Tags—Acid-Labile Isotope-Coded Extractants (ALICE)

Qui et al. (2002) synthesized labeling reagents that, like the ICAT reagents, are stable-isotope-incorporated for quantitation and contain thiol-reactive groups that label cysteines. Unlike ICAT, these reagents have an acid-labile linker that connects the cysteine-containing peptides to a non-biological polymer support. These reagents are termed ALICE reagents, for “acid-labile isotope-coded extractants.” This methodology reportedly overcomes the irreversible binding of peptides that affects the avidin affinity column used with the first-generation commercial ICAT reagents. With ALICE, peptides are eluted from the resin in high yield, using mild conditions that are amenable to subsequent analysis by MS. Further, the reagents impart only a small additional mass (“light” ALICE is 219.1 Da, “heavy” version 220.2 Da), and are less susceptible to fragmentation under standard CAD conditions than ICAT reagents.

The addition of the isotopic tags described above can be applied readily to proteins from most biological samples. Again, the only prerequisite is that at least one cysteine residue is present in each protein of interest. Because the addition of the isotopic tag occurs relatively early in the analytical process, the quantitative bias because of sample loss is minimized.

4. Lysine-Specific Labeling

Another alternative amino acid to cysteine for labeling for quantitative purposes is the lysine residue. A technique to differentially label lysine residues by labeling the ϵ -nitrogens with 2-methoxy-4,5-dihydro-1 *H*-imidazole, converting them to 4,5-dihydro-1 *H*-imidazol-2-yl lysine without labeling N- or C-terminal peptides, was described (Peters et al., 2001). A mass-labeling scheme for quantitation, as well as an enhanced mass spectrometric ionization with MALDI-TOF because of the high basicity of the modification, was observed. $^1\text{H}_4$ - or $^2\text{H}_4$ -forms of the reagent were used. This technique offers an alternative method for quantitation that relies on the presence of a lysine residue, rather than the cysteine residue, as described above.

5. Phosphoserine- and Phosphothreonine-Specific Labeling

The identification and quantitation of post-translationally modified proteins is currently one of the most prevalent topics in proteomics. The phosphorylation state of the proteome is particularly important because of the role of protein kinases and phosphatases in biological regulation and disease etiology. Weckwerth, Willmitzer, & Fiehn (2000) first described a method for the comparative quantitation and identification of phosphoproteins that used stable isotope-labeling and LC/MS. Quantitation was achieved by the β -elimination of phosphate from phosphoserine or phosphothreonine to form dehydroalanine or dehydroamino-2-butyric acid, respectively, followed by the addition of ethanethiol and/or ($^2\text{H}_5$) ethanethiol to the α,β -unsaturated amide.

Recently, Goshe et al. (2002) described a technique that used a phosphoprotein isotope-coded affinity tag (PhIAT) in which phosphoserine and phosphothreonine residues were selected and quantified in a manner that was analogous to the capture of cysteine residues in ICAT. To label these residues, O-linked carbohydrates were removed and cysteines were blocked by conversion to cysteic acid. Phosphate groups were removed by β -elimination, and either “heavy” (deuterated) or “light” (non-deuterated) reagents were used to convert the resultant residues to isotopically labeled thiols. The thiols were labeled with an iodoacetamide-based reagent linked to biotin, followed by an avidin-based purification strategy to purify and concentrate phosphorylated peptides. Mass spectrometric analysis determined the relative abundances of phosphorylation states between the samples.

Although these methods of transformation of a phosphorylated residue to a distinctly labeled moiety are still under development, the examples so far show promise for the exploitation of differential isotopic-labeling strategies to concentrate low-abundant protein states from complex

proteomes. Further, such methods can effectively quantify relative protein abundances between cell samples.

6. N-Terminus Labeling

a. Nicotinyl-N-hydroxysuccinimide. Munchbach et al. (2002) described a technique in which the N-terminus, instead of an internal cysteine of a protein, is the site of attachment of a stable isotope-containing tag. Under stringent conditions at pH 5, succinylation occurs specifically at the N-terminus without the modification of other amino groups (e.g., the lysine epsilon amino group) in the sequence (Munchbach et al., 2002). One sample set was labeled with 1-([H_4] nicotinoyloxy)-succinimide ester, and the other with the deuterated 1-([H_4] nicotinoyloxy)-succinimide derivative. When these two samples were pooled and analyzed by MS, doublets with a mass Δ of 4 Da could be seen for each *b*-ion in the series. One of the challenges in quantitation using MS is that the multiple charge states per peptide that are often seen add an additional factor to consider when plotting peak areas for quantitation. Because this method adds this reagent at the N-terminus of the protein, it facilitates the formation of +1 species, therefore, simplifying the mass spectrum and the increasing signal:noise ratio (S:N).

Ji et al. (2000) described similar strategies for the selection of histidine-containing peptides with immobilized metal-affinity chromatography (IMAC), or of glycoproteins with lectin columns, to improve the mass spectrometric dynamic range post-labeling. Peptides were acetylated after digestion at their amino terminus and on any internal lysine residues by reacting with an excess of *N*-acetoxy- d_3 -succinimide and with *N*-acetoxy succinimide for the control sample. Samples were subjected to the above selective chromatographies, and were quantified with ESI-LC-MS/MS.

b. Acylation. Acylation of primary amines with isotopically differentiable reagents, can also be employed for quantitation. For the mass spectrometric quantitation of neuropeptides in mice, Che & Fricker (2002) treated mouse tissues with either the $^1\text{H}_6$ or $^2\text{H}_6$ form of acetic anhydride under conditions that preferentially label primary amines. Anhydrotrypsin agarose was used for the affinity purification of peptides that contained C-terminal basic amino acids. Resultant peptides were quantified by MALDI-TOF and ESI-MS analysis. Advantages of using such a derivatization technique are multiple. For instance, when a peptide is acetylated, the charge state of the peptide in MS is generally reduced. Reduction in charge, in turn, reduces the complexity of peptide sequencing for many peptides. Also, the acetylation modification is commonly listed on search software and facilitates search queries.

Acylation has also been applied to the selection and quantitation of cysteine-containing peptides through covalent chromatography (Wang & Regnier, 2001). In this method, disulfide bridges were disrupted with 2,2'-dipyridyl disulfide, and the proteins were enzymatically digested. Proteins were acylated with succinic anhydride. A thiopropyl sepharose gel was employed to selectively bind cysteine-containing peptides. Cysteine-containing peptides labeled with succinic or deuterated succinic anhydride were subjected to a comparative quantitative analysis.

c. Amidination—quantitation using enhanced signal tags (QUEST). Mass-differentiated amidination of N-terminal and lysine residues for protein quantitation has been described (Beardsley & Reilly, 2003). Instead of employing isotopes for mass differentials, these authors use mass tags that differ by a methyl group. Peptides are either labeled with S-methyl thioacetamidate to form acetamidines (“methyl-coded” peptides), or with S-methyl thio-propionimidate to form propionamidines (“ethyl-coded” peptides). The term QUEST (*Quantitation Using Enhanced Signal Tags*) was coined for this labeling strategy. Amidination is an efficient labeling reaction that can be performed under mild conditions, and has the added advantage of enhancing ion yields for lysine-containing peptides. Further, the mass Δ between the differentially labeled peptides is 14 Da, which avoids quantitation errors because of isobaric interferences that can reduce the effectiveness of other reagents. Thus, this method is an attractive labeling tool for peptides analyzed by MALDI ionization techniques. One drawback of this technique is that it is not yet known how the two reagents elute during reversed phase chromatography. This technique may, therefore, be restricted to in-gel based analyses coupled to MALDI ionization for mass spectrometric analysis.

d. C-terminus labeling. For quantitation of proteins and peptides, Goodlett et al. (2001) labeled the C-terminal carboxylic acid of peptides by esterification with methanol and ($^2\text{H}_3$) methanol. They demonstrated the use of this method for the isotope-ratio quantitation of proteins. This labeling method converted not only the free C-terminus, but also any carboxylic group present on the side-chains of aspartic and glutamic acid, to their corresponding methyl esters. These stable isotope-incorporated samples were mixed, and were subjected to microcapillary high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). The resulting data successfully identified the parent proteins of labeled peptides, and provided the ratios of the proteins in the two original mixtures. Those ratios could be calculated by the normalization of the area under the curve for the identical charge states of d_0 - to d_3 -methylated peptides. Again, the main

advantage of this technique is that it does not rely upon the presence of any specific residue for labeling.

D. Differential Mass Mapping

Differential mass mapping is a technique in which either naturally expressed proteins, or enzymatically or naturally derived peptides, can be analyzed comparatively by MS. Using either MALDI-TOF or a micro-electrospray coupled to a quadrupole ion trap, Q-TOF, or triple quadrupole detector, a mass map can be obtained for each sample to be compared. Either a visual or, preferably, a bioinformatics-based algorithm can be employed to identify differences (e.g., subtractive differences), either by monitoring m/z intensities alone, or by coupling mass intensity data to the chromatographic trace (Falk et al., 1991; Chong et al., 2001; Ball et al., 2002; Lazar et al., 2002).

Chong et al. employed RP-HPLC followed by mass spectrometric analysis to describe the differential screening and mass mapping of proteins from pre-malignant and cancerous cell lines. In the first analysis, they separated the proteins on the RP-HPLC column-collected fractions, and monitored the protein masses by MALDI. Proteins that were differentially expressed between the two cell lines were identified. The RP-HPLC fraction that contained the peak of interest was digested, and tandem MS-based sequencing was used to identify the proteins.

To further explore and quantitate dynamics of site-specific protein phosphorylation, Ruse et al. (2002) employed LS-ESI-MS. They determined the extent of phosphorylation of a protein by comparing the peak area of a specific phosphopeptide to the peak areas of unmodified enzymatically cleaved reference peptides as internal references. In the absence of an internal standard, one must be cautious about relying on a purely mass spectrometric technique because sample complexity and its association with suppressive ionization effects may render quantitation impossible. Suppression effects can be very significant, even with relatively non-complex samples. To address these issues, Bondarenko, Chelius, & Shaler (2002) adopted a tandem strategy. First, they summed the peak areas of identified peptides from one protein. Second, they normalized this sum to the peak area of an internal standard protein digest that was added to each sample at a constant level. Using a digested protein as an internal standard, several representative peptides are spread throughout the chromatographic profile. In this way, it is much less likely that sample complexity and ion suppression will interfere with all quantitative measures.

These techniques are highly effective. However, to succeed in quantitation without using mass tags, separations that minimize those suppressive effects caused by co-elution must be employed.

III. ABSOLUTE QUANTITATION

Although this review focuses mainly on the relative comparison and quantitation of proteins, in many instances it is desirable to quantitate the absolute amount of a given protein (e.g., copies per cell) in a biological sample. Classic analytical strategies may be used to determine the molarity or absolute protein amount in a given sample. These techniques include the addition of an internal standard, or creation of a standard curve based upon one or more peptides derived from the protein of interest. Unlike the comparative quantitation strategies described above, the measurement of exact protein or peptide amounts in a system often involves a qualitative analysis prior to the quantitative analysis, such that the entity to be measured is already well-defined. The following section summarizes some of the techniques used to determine absolute protein or peptide quantity in proteomic studies.

Several groups have described the use of standard curves for the absolute quantitation of proteins and peptides by MS (Desiderio & Zhu, 1998; Oda et al., 1999). Usually, a standard curve is developed with a stable isotope-incorporated peptide. This peptide is used as an internal standard by adding a known quantity to the analytical sample. Thus, the ratio of the synthetic to endogenous peptide is measured, and the absolute amount of this peptide can be calculated. Factors such as choice of instrumentation, particularly ionization instruments, are critical. Although MALDI has been reported as the method of choice, and often gives very good quantification (Oda et al., 1999), factors such as variable crystallization and laser ablation may lead to poor standard curves. Because of this strong technique dependence, such factors must be minimized through a thorough investigation before performing such an analysis.

Overall, the absolute quantitation of MS-based peptide and protein analysis can be achieved, especially as the sensitivity and accuracy of instrumentation continues to improve. In the near future, such methods may supersede immunological quantitation techniques. However, an immunology-based assay such as an enzyme-linked immunosorbant assay (ELISA) or a radioimmunoassay (RIA) (provided an antibody is available) may result in more precise quantitation than MS at very low protein concentrations.

IV. CHOICE OF INSTRUMENTATION

In addition to these considerations, the choice of instrumentation is also extremely important. Applications, as well as budget, are often the key determining factors in purchasing a mass spectrometric instrument. If the main aim of a laboratory is to quantitate, then the following

instrumentation capabilities must be considered: both typical laboratory “work-horses,” such as the quadrupole ion trap and triple quadrupole instruments, are very effective at generating qualitative and quantitative data (Barnidge et al., 2003; Jemal & Ouyang, 2003). In general, ion trap mass spectrometers offer better sensitivity than triple quadrupole instruments when using a full-scan MS/MS operative mode. Therefore, if differential analysis is carried out between two samples that contain a mixture or peptidic species, then ion traps are ideal (Chong et al., 2001; Lazar et al., 2002). However, triple quadrupole instruments offer increased performance over an ion trap when performing single-reaction monitoring experiments, such as when targeting and quantitating a single species, or even a simple mixture of peptides. The triple quadrupole behaves better in this situation because the analyte of interest alone undergoes fragmentation. All other ions are previously excluded from the collision-induced dissociation step, which eliminates interference and suppression effects from other co-eluting species.

In summary, the choice of instrumentation is very important and depends on research goals and needs. Triple quadrupole instruments are superior at performing absolute quantitation or comparative quantitation of non-complex mixtures. However, ion trap mass spectrometers are often the instrument of choice when ICAT-type technologies or differential mass mapping during shotgun type approaches are employed because of their capability of producing reproducible MS/MS spectra.

V. OVERVIEW, DISCUSSION, AND FUTURE DIRECTIONS

The past decade has witnessed the introduction and explosive growth of proteomics. Such growth would have been impossible without the whole-genome mapping of several organisms, which has provided an extensive database of characterized and predicted protein sequences. Now that automation is possible for analysis and sequencing, high-quality, high-throughput proteomics is being rapidly assimilated into most drug discovery efforts (Lill et al., 2001).

This review summarizes a number of quantitation methodologies that have become available to proteomic researchers because of MS-based detection. The accuracy of quantitative measurements depends on the quantitation technique. For accuracy, mass tagging is the technique of choice for the comparison of the relative composition of two or more samples. For the highest level of accuracy, the technique that tags earliest in the analytical process is preferable because unequal sample losses during sample handling will be minimized, and minimal bias will result. When metabolic labeling is not practical, enzymatic or

chemical labeling may be employed. Many techniques are available for enzymatic or chemical labeling. Therefore, an evaluation of each technique with regard to sample set and mass spectrometric instrumentation is important. For instance, when comparing two peptides in a method that introduces only a small Δ (e.g., ^{18}O enzymatic labeling), a mass spectrometer capable of high resolution and mass accuracy should be employed to reduce any spectral, and hence quantitative, ambiguity.

For strategies that employ chemical-labeling reagents, several aspects related to the reagent must be considered:

1. reagent compatibility with buffers and solvents used during analysis;
2. reagent stability under the conditions of preparation and analysis;
3. degree of labeling of all targeted residues; and
4. availability of software capable of handling the methodology for accurate sequence information.

The third point listed above is of particular concern when using a high concentration of unknown proteins. A molar excess and optimal labeling conditions must be applied to eliminate any concern associated with incomplete labeling. Throughout the literature reviewed here, there are no reports of incomplete labeling. Incomplete labeling would skew quantitative analyses and smear mass spectrometric data. Commercially available reagents such as those associated with ICAT are known to label efficiently and completely.

The fourth point listed above is particularly important for high-throughput environments because data analysis may be rate-limiting. For most of the techniques described, including ICAT, acetylation, and enzymatic labeling, search algorithms are now available to accurately identify peptide sequences in the context of chemical modifications.

Recent advancements in comparative quantitative analysis include the introduction of user-defined, data-dependent scanning software to control the mass spectrometer, such as that available with Xcalibur (Thermo Finnigan), where only signals with a pre-defined mass difference are subjected to fragmentation. This strategy is particularly useful for the analysis of complex mass-tagged samples. Because the quality of MS/MS analyses are often limited by the data-collection rate, these software modifications allow valuable CAD time to be spent fragmenting ions that have the correct mass Δ . This improved data quality maximizes the confidence in, and reliability of, the particular quantitation.

Finally, current quantitation strategies employ two mass tags. However, many of these techniques could be adapted to use three or more reagents in parallel so that more than two samples could be compared simultaneously

(e.g., non-malignant, malignant, and metastatic tissues). Because the range of naturally occurring isotopes is limited, such multiplexed approaches may be most feasible with non-isotopic additions such as the addition of one, two, or three methyl groups.

Although proteomic analyses have progressed enormously in the past decade, many unanswered questions remain. As sequencing technology, quantitation technology, and the related software continue to improve, proteomic mass spectroscopy will contribute even more significantly to scientific advances.

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