Quantitative MS for Proteomics: Teaching a New Dog Old Tricks

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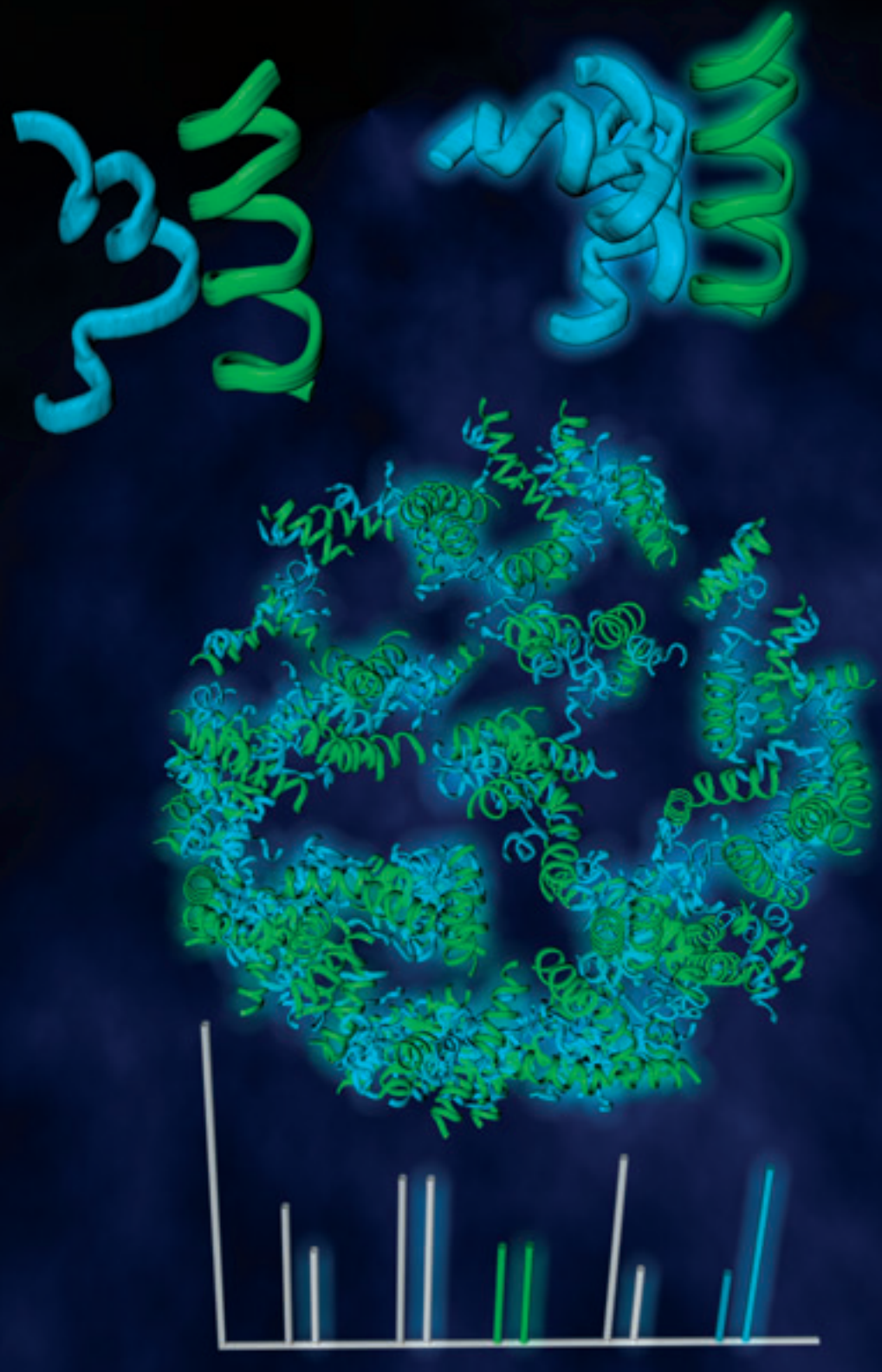

Teaching a New Dog *Old Tricks*

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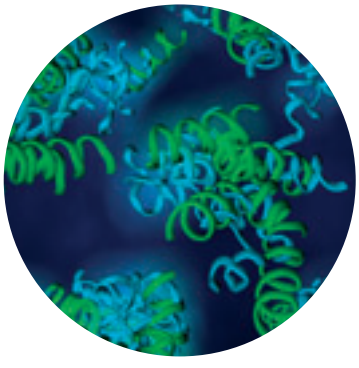


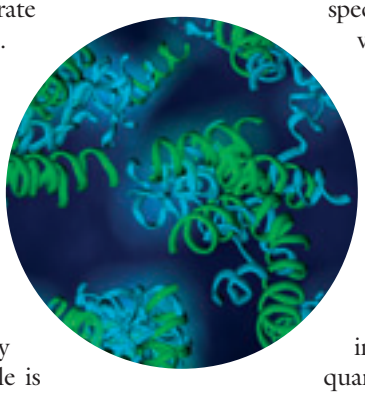
Although these methods can generate an incredible amount of data in a single experiment, we must not forget the fundamentals that ultimately define the fitness, applicability, and robustness of a method.

Unlike the genome, which is static, the proteome is dynamic. Proteins are continually synthesized and degraded to carry out the functions of the cell. They are often covalently modified posttranslationally, and a protein's subcellular localization or association with other proteins may change so that it can function correctly as a part of a larger protein complex. These processes are all dynamic, are often independent of each other, and can be altered by cellular conditions and local microenvironments. Measurements of messenger RNA expression have matured over the past several years; unfortunately, analyzing the RNA transcript does not necessarily describe the resulting complement of proteins, their amounts, or their posttranslational modification at any point in time (*1*, *2*).

Because most biological questions cannot be answered by simply determining whether a given protein is present or absent, proteomics technology must include the ability to measure the amounts of individual proteins. The advantage of MS for quantitation is that the ability to measure masses accurately makes it possible to use stable-isotope-labeled (i.e., ^{2}H, ^{13}C, ^{15}N, or ^{18}O) internal standards. Although MS is one of the premier tools for quantitative measurements, only recently has it been applied to the global quantitation of proteins in a mixture.

In the past decade, developments in electrospray ionization (ESI) and MALDI have enabled the facile and sensitive ionization of peptides and whole proteins for MS analysis (*3*–*7*). However, we are at a challenging point in time: How do we make quantitative measurements of protein abundances and of their modifications and interactions? The most logical approach is to use stable-isotope-labeled analogs of proteins and peptides, but the diversity of proteins to be measured is staggering. How do we prepare these standards for thousands of proteins or tens of thousands of peptides resulting from a protein digest? Quantitative measurement of the proteome of a cell depends upon answering this question.

We will review the current methods and delve into the use of quantitative MS in proteomics research. These methods are complicated and very high tech and can generate an incredible wealth of data in a single experiment. For these reasons, we tend to forget the fundamentals of analytical chemistry that ultimately define the fitness, applicability, and robustness of a given method.



Proteomics methods

Historically, 2-D polyacrylamide gel electrophoresis (2DE) has been used to separate complex protein mixtures and quantify the protein spots (*4*, *8*). The 2DE technique has many strengths, but probably one of the most valuable is that the various protein forms (e.g., phosphorylated, truncated, glycosylated) migrate on the gel in different ways. MS has been used in conjunction with 2DE to qualitatively identify the protein spots that have been excised from the gels. However, 2DE gels cannot be applied globally to the analysis of membrane proteins, their large-scale automation is complex, and their dynamic range is relatively limited. Therefore, alternative, "shotgun" proteomics techniques have been developed.

In these shotgun approaches, an entire unfractionated protein mixture is digested to peptides and separated by LC; spectra are acquired automatically in-line by MS/MS (*3*, *5*, *7*). Handling the mixture as peptides minimizes many of the problems associated with 2DE when analyzing proteins with extreme size, hydrophobicity, or p*I*. Because shotgun systems are based on LC with ESI-MS, they are readily amenable to automation. As the number of proteins in a mixture increases, the number of peptides generated upon digestion dramatically increases, which complicates the LC separation. To handle this complexity, multidimensional separation techniques have been applied (*9*).

In one of the most successful approaches, a biphasic microcapillary column is used that contains strong-cation-exchange and reversed-phase materials in tandem (*10*). In this configuration, increasing levels of salt are used to step-elute peptides from the strong-cation-exchange material onto the reversed-phase material. After each step-elution from the cation-exchange column, a reversed-phase gradient elutes the peptides according to their hydrophobicity into the mass spectrometer. As peptides elute from the reversed-phase column into the mass spectrometer, spectra are acquired of the resulting protonated molecular ions. Product-ion or MS/MS spectra are acquired on each precursor ion to identify the peptide sequence (*3*). MS/MS spectra are obtained automatically because we do not know what peptides will elute or when, or what ions they will produce. Thus, automated "data-dependent analysis" software makes real-time decisions about what type of data the mass spectrometer acquires and how they are obtained (*11*). The analysis enhances the efficiency of the data acquisition and facilitates the online analysis of peptides as they elute.

Because the MS/MS spectrum of a polymer such as a peptide is predictable, sequences from a protein or translated nucleic acid sequence database can be used to generate predicted fragmentation spectra that can be matched against spectra obtained experimentally (*12*, *13*). Matching one or more tandem mass spectra from peptides in the same protein can provide high-confidence protein identification (*14*).

Quantitative mass spec 101

Precision improves as the time spent measuring the analytes of interest increases. In the classical qualitative mode of operation, the mass spectrometer is scanned over a wide range to obtain spectral information for compound identification. However, only a small portion of the total time is spent collecting ions related to a specific analyte. In contrast, quantitation requires precision, and in MS, Poisson statistics dictate that precision improves with the square root of the number of ions measured (*15*). Thus, selected ion monitoring (SIM) and the complementary tandem mass spectrometer technique of selected reaction monitoring (SRM) are used predominantly to measure only specific ions related to the analyte and the internal standard to provide maximum precision. In SIM or SRM, nearly 100% of the collection time can be devoted to measuring preselected analyte ions. The acquisition of a mass spectrum permits the measurement of all ions for qualitative identification. However, quantitative precision suffers when the instrument is scanning, compared with SIM or SRM.

Accurate measurements can only be achieved if an internal standard is used to account for sample-preparation losses. Some of the analyte is commonly lost during sample preparation. To account for these losses, an internal standard is added before sample processing so that it will undergo the same losses. Clearly, the best internal standard, with the least difference in physicochemical properties, is an isotope-substituted molecule. The best isotopic variants increase mass by several daltons to separate the labeled internal standard signal from the naturally occurring isotopic distribution around the ion being measured for the unlabeled analyte ion. For peptides, an increase in mass of >3 Da is needed, and that increase requires the addition of several isotopic labels to the internal standard.

MS precision improves with the use of an internal standard that is as similar chemically to the analyte as possible, that is, an isotope-labeled variant. The measurement of the ion–current ratio between the compound and the internal standard reduces errors and long-term drifts associated with the ion source

and inlet systems because they will experience similar biases. The best (but most expensive) isotopic labels for peptides are ^{15}N, ^{13}C, and ^{18}O because they produce minimal isotope effects. The most commonly used isotopic variants are ^{2}H labels because multiple deuteriums can usually be incorporated relatively easily at a reasonable cost. The only caveat to the use of ^{2}H-labeled internal standards is that deuterium substitution can produce significant physicochemical changes in the interaction of ^{2}H-labeled compounds with other molecules, compared with isotope substitution of heavi[illegible]ganic elements (i.e., C, N, or O).

The net effect is that internal standards with several of their hydrogens replaced with deuterium isotopes will chromatographically elute ahead of their unlabeled counterparts. This shift forward in elution of deuterated internal standards makes it more challenging to identify and integrate the deuterated standard compared with the unlabeled analyte; the advantage obtained with time-dependent noise cancellation is also reduced. Nonetheless, deuterated standards are often the only reasonably affordable choice for labeled standards.

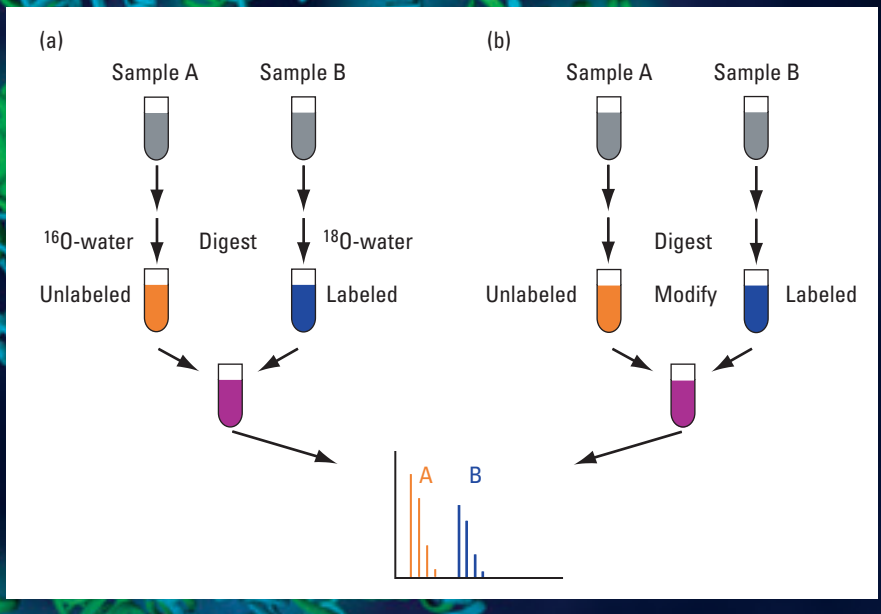


FIGURE 1. Global labeling by modification of peptides at or after protein digestion.

Two different samples are treated by the same process except that sample B is treated with stable-isotope-labeled reagents and sample A is treated with unlabeled reagents. Once each sample has been processed, the samples are combined and the peptides measured by MS. The abundance ratio of each individual peptide is defined by the ion–current ratio of the summation of the isotope peaks corresponding to the labeled peptide B versus the summation of the isotope peaks corresponding to the unlabeled peptide A. (a) ^{18}O is incorporated into each peptide in sample A by digestion of the protein in ^{18}O-water. (b) The digested peptides of sample B are chemically modified with a stable-isotope-labeled reagent, while the digested peptides of sample A are modified with unlabeled reagents.

Accurate measurements require calibration curves from standard samples of analyte plus internal standard. In MS, we measure the ratio of the ion currents produced by the two compounds and not the mole ratio directly. Therefore, quantitative MS measurements are by nature relative. Absolute amounts of an analyte are determined by calibrating the instrument response with samples containing a "known" mole ratio of the analyte and its labeled internal standard. The relationship of a measured ion–current ratio to a mole ratio is

$$R = R_b + k(n_a/n_b) \qquad (1)$$

in which R is the measured ion–current ratio for the two isotopic species, a (unlabeled analyte) and b (labeled internal standard); n_a and n_b are the amounts in moles; and k is the mass spectrometer response factor that relates the measured ion–current ratio to the mole ratio of the two species measured. Ideally, $k = 1$, that is, the mass spectrometer produces a 1:1 change in ion–current ratio with a change in the unlabeled-to-labeled mole ratio. R_b is the ion–current ratio measured by the mass spectrometer when the internal standard is measured by itself; it represents any unlabeled signal in the labeled standard.

We are assuming that a significant enough increase in mass occurs in the labeled standard that the unlabeled material does not produce a significant signal at the ion measured for the labeled compound. If it does, a nonlinear equation must be applied (*16*, *17*). Equation 1 can be rearranged to $n_a/n_b = (R - R_b)/k$ to measure an unknown mole ratio as a function of a measured ion–current ratio. When $R_b << R$, this relationship simplifies to $n_a/n_b = R/k$. The accuracy of any quantitative MS measurement relies heavily on the assessment of k, which corrects for systematic errors between the response of the unlabeled analyte and that of the labeled internal standard. Unpredictable systematic errors can produce an altered ion-intensity response ($k \neq 1$) between a sample and a stable-isotope-labeled internal standard (*18–20*).

If two samples contain different amounts of analyte n_{a1} and n_{a2} but equal amounts of internal standard n_b and the samples' ion–current ratios are measured by the mass spectrometer as R_1 and R_2, respectively, then we can relate sample 1 to sample 2 by

$$\frac{(R_1 - R_b)/k}{(R_2 - R_b)/k} = \frac{n_{a1}/n_b}{n_{a2}/n_b} \qquad (2)$$

which reduces to

$$(R_1 - R_b)/(R_2 - R_b) = n_{a1}/n_{a2} \qquad (3)$$

The value of this approach is that characterization [illegible]e labeled internal standard is not necessary; it just needs to be added to the samples in equal amounts. Even if the MS responses of the unlabeled and labeled analytes are not equal ($k \neq 1$), any inaccuracies will cancel in the same manner as k does from Equation 2. The drawback is that the measurement of the amount of analyte in sample 1 is now only relative to the amount in sample 2. In proteomics, we rarely have standard samples of proteins to make absolute measurements, and almost all quantitative proteomics measurements are relative or comparative measurements.

To make proteomics MS methods quantitative, we need an isotope-labeled internal standard for every peptide or protein be-

cause ionization efficiencies and other variables differ among peptides. Four general schemes have been developed that use stable isotope labels either inherent in peptides or attached to peptides after protein digestion. However, none of these schemes makes absolute measurements of amounts of proteins because we generally do not have individual proteins available to prepare standard curves for measurement. The field of quantitative proteomics has been restricted to comparing the amounts of individual proteins in one sample with those in another.

Globally adding labels after protein digestion

The most common strategy to incorporate stable isotope labels is to modify peptides from one sample with a reagent containing only naturally abundant, stable isotopes and to modify a second sample of peptides with an identical reagent in which several of the atoms have been enriched in heavy isotopes (*21–23*). After modification, the samples are recombined and measured to obtain the ratio of unlabeled versus labeled for each individual peptide (Figure 1). These peptide ratios provide a measure of the relative amounts of individual proteins between the two samples. The global labeling scheme can be divided into two approaches: digestion of proteins in ^{18}O-labeled and unlabeled water and modification of peptides post-digestion with labeled and unlabeled reagents.

Several groups have digested protein samples in ^{18}O-water to incorporate ^{18}O into each peptide (Figure 1a; *24–26*). Both oxygens of the carboxyl terminus exchange when trypsin is the digestion enzyme, and a 4-Da increase occurs that is sufficient for ratios of most mixtures of labeled and unlabeled peptides to be resolved and measured (*27*). The incorporation of the ^{18}O is robust enough that samples may be prepared and analyzed with minimal back-exchange of ^{18}O after the ^{18}O- and ^{16}O-prepared samples are combined. However, because all commercially available sources provide ^{18}O-water that is <100% enriched, care must be taken to account for incomplete enrichment in the digested peptides.

Johnson and Muddiman have carefully considered the production of labeled peptides with only one C-terminal peptide carboxyl ^{18}O when 95% ^{18}O-water is used (*27*). Stewart and coworkers considered the variable incorporation of two ^{18}O atoms with trypsin during cleavage (*28*). These groups defined the limits of this problem and suggested approaches to minimize errors. Fenselau's group has also studied the use of alternative cleavage enzymes other than trypsin (*29*, *30*). For example, endopeptidase Glu-C also incorporates two ^{18}O atoms during cleavage (*29*).

In a variation of this approach, protein samples are first digested in unlabeled water and dried. One sample is incubated with unlabeled water while the other is incubated in ^{18}O-water still in the presence of the cleavage enzyme to catalyze complete exchange of the C-terminal carboxyl oxygens. The samples are then recombined and measured. This scheme effectively uncouples protein digestion from incorporation of ^{18}O (*30*).

An alternative global labeling approach is to modify either the N- or C-terminus of every peptide after digestion (Figure 1b). Regnier and Chakraborty described several approaches for the acetylation of the N-terminus (*31*). One protein-digest sample is treated with an unlabeled reagent, and another protein-digest sample is treated with an ^{2}H-labeled reagent to produce peptides that have an acetylated N-terminus. The peptides containing the ^{2}H-acetyl will have a mass increase of 3 Da over those acetylated with the unlabeled reagent. Zhang et al. promote a similar approach with an ^{2}H-propionyl reagent that produces a more substantial 5-Da increase (*32*). In this case, C-terminal lysine amino acid residues were first guanidinated to improve the MALDI-TOF response of these peptides and to ensure that the propionylation only added a single propionyl group per peptide on the N-terminal amino group.

A downside of derivatization reactions that target the N-terminus is that this modification may re-

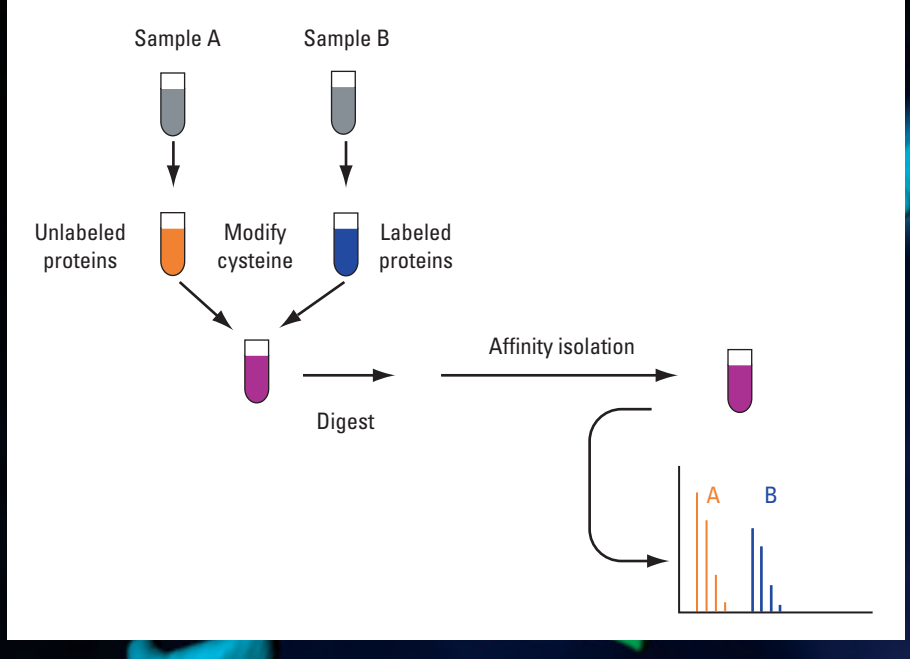
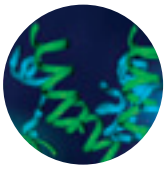
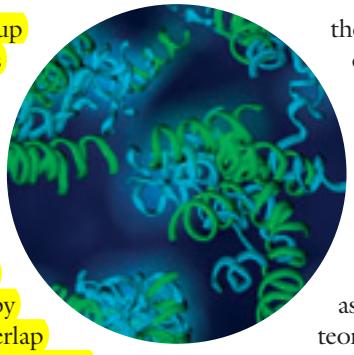


FIGURE 2. Chemical modification of proteins with an ICAT.

The protein from sample B is treated with a stable-isotope-labeled ICAT reagent, and sample A is treated with an unlabeled ICAT reagent. The tags bind only to cysteine residues. After processing, the protein samples are combined and digested; the cysteine-containing peptides are sequestered by affinity isolation and measured by MS. The ratio of peptide abundance is defined from the ion–current ratio, as in Figure 1.



duce ionization of the N-terminal amino group and hinder sensitivity. An obvious alternative is to label the C-terminus. Goodlett et al. have made methyl esters of peptides to incorporate either an unlabeled or an ^{2}H-labeled methyl per carboxylic acid for a mass difference of 3 Da (*33*). A drawback of the methyl ester scheme is that a peptide that contains no glutamate or aspartate residues will result in labeled and unlabeled mixtures that are only separated by 3 Da. A shift this small can produce isotopic overlap between the unlabeled and labeled peptides; however, longer-chain esters with more ^{2}H could easily be used.



Adding labels before digestion

This scheme is based on the isotope-coded affinity tag (ICAT) method described by Gygi et al. (*21*; Figure 2), which has two substantial differences from the global labeling approach. First, modification is performed at the level of the protein, not after digestion of the protein to peptides. Second, this approach by definition is directed to specific residues in a protein—in this case, the cysteines. Using iodoacetamide or other alkylating reagents to alkylate cysteine thiols is an old technique in protein chemistry and proteomics that prevents reduced cysteine thiols from forming oxidized disulfide bonds (*22*).

The original ICAT reagent contained a linker that was labeled with eight deuterium atoms; it was used to alkylate cysteine residues in place of a more traditional alkylating reagent (*21*). The deuterated reagent has since been replaced by an improved proprietary reagent that contains nine ^{13}C atoms (*34*, *35*). A drawback to the original ICAT approach was that the addition of eight deuterium atoms to the linker produced a substantial isotopic chromatographic fractionation effect of the labeled and unlabeled peptides and thus required additional care in the measurement of the ICAT-labeled peptides (*22*, *23*).

Both the ^{2}H- and ^{13}C-labeled ICAT contain a biotin tail connected via the linker to a thiol reactive moiety, and the ^{13}C reagent contains a cleavable linker to remove the affinity tag after enrichment. The digested peptides are passed over an avidin column to enrich for the biotin–cysteine-containing peptides. Next, the biotin-tagged peptides are released from the column and measured by MS. A comparative measurement between two samples is made by preparing one protein sample with an unlabeled ICAT reagent and the other with an $^{2}H_{8}$- or $^{13}C_{9}$-labeled reagent (Figure 2). The ICAT-treated unlabeled and labeled samples are combined before digestion and measurement. This approach provides more than adequate mass separation (8–9 Da) between unlabeled and labeled peptides and substantially reduces the number of peptides to be measured by enriching for those containing cysteines (*7*).

The method is not global, in that only cysteine-containing peptides are measured. Depending on the organism being studied, a substantial fraction of the proteins may not contain any cysteines and may go unmeasured and unidentified in any shotgun measurement of proteins in a system (*22*). Nevertheless, even with the deficiencies of these reagents and their high cost, the ICAT approach is commonly applied and is one of the most publicized of the quantitative proteomics methods.

Metabolic labeling of proteins in vivo

An alternative approach is to grow proteins already labeled. Oda et al. grew *Saccharomyces cerevisiae* cells in media enriched in ^{15}N (>96%) as the only nitrogen source for a quantitative proteomics application (*36*). These cells labeled with ^{15}N-proteins were then mixed with a second pool of cells grown in media containing naturally abundant nitrogen. The value of labeling the proteins in the entire set of cells is that labeled and unlabeled cells can be mixed together before the introduction of any variability that will occur during the sample preparation process. The other schemes introduce the label later in the process.

In contrast to the other two schemes in which a defined modification is made and a defined change in mass is introduced to every peptide that has been modified and labeled, in this approach, the number of biosynthetic-labeled atoms incorporated into peptides will vary from peptide to peptide. Thus, for every peptide measured during an LC/MS analysis, the number of labels present must be determined before the ratio of the amount of unlabeled to labeled peptide can be calculated. This difference in mass among peptides has the added benefit of providing an independent confirmation of the peptide sequence identified by the database search. The expected difference in the average mass between the labeled and unlabeled peptides can be readily calculated from the peptide sequence and the ^{15}N atomic enrichment of the material used to label the protein (*20*).

Mammalian cells need a more complicated culture in which to grow, and they require media that contain amino acids and other factors. Thus, the stable-isotope-enriched atoms must be added to the cells as labeled amino acids to produce adequately labeled protein. Conrads et al. extended this approach to mammalian cells by using commercially available media containing ^{15}N-labeled amino acids obtained from hydrolyzed ^{15}N algae protein (*37*). Ong et al. grew mammalian cells with media to which $^{2}H_{3}$-leucine had been added to label all leucines in the protein (*38*). Proteins from cells grown with $^{2}H_{3}$-leucine were then compared with proteins from cells grown in media containing unlabeled leucine, to define changes in cell state between the two systems. A variety of ^{2}H- and ^{13}C-labeled amino acids have been used to label specific amino acids in cells grown in culture (*38*, *39*).

These uses of labeled protein have been restricted to cell culture systems, but ultimately proteomics methods should be extended to tissue and even to entire multicellular organisms. Krijgsveld et al. labeled nematodes and fruit flies by feeding them ^{15}N-enriched bacteria and yeast, respectively (*40*). After two generations, the worms and flies were completely labeled and could be used as internal standards for quantitative proteomics. Wu et al. have demonstrated that it is feasible to grow a rat entirely labeled with ^{15}N (*41*). A recently weaned rat pup was fed ^{15}N-protein and allowed to grow until adulthood; by

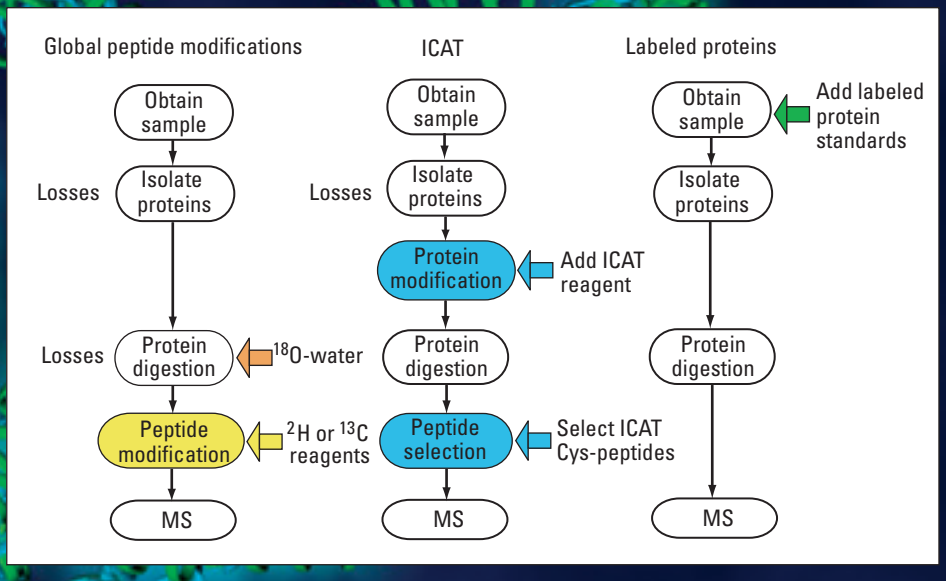


FIGURE 3. Steps where potential losses of sample could occur before introduction of the label.

then, its protein was highly labeled (≥90%). The rat was then sacrificed and its organs and tissues saved as labeled protein sources to serve as internal standards. Although the initial investment in growing an ^{15}N-labeled animal is expensive, many experiments can be performed from the tissues because only a very small amount of labeled protein is required for a given experiment. This approach is fully applicable to shotgun proteomics experiments and is actually less expensive per experiment to perform than conventional modification methods outlined in the other two approaches.

Quantitation of specific peptides

In this scheme, a specific protein is targeted and one or more peptides are identified from that protein for measurement. The peptides are then synthesized with labeled amino acids and are added to a protein sample so that the peptides and, therefore, the target protein can be quantified (*42*). This scheme is limited to specific known proteins for which appropriate labeled and unlabeled peptides are synthesized and provides best results when combined with SRM-based data acquisition approaches. SRM can be used to improve the precision and dynamic range of the measured peptides under any of the other labeling schemes, but this approach is ideally suited to directed MS analysis because the internal-standard labeling itself is limited to selected peptides.

Accuracy and precision

Let us now put into perspective the points made under "Quantitative mass spec 101" with the various quantitative proteomics schemes. The first point describes the need to maximize the time spent measuring the analyte ions to maximize precision, but proteomics requires both identification and quantitation of peptides. Spectrum acquisition takes time, which limits the time that can be spent collecting specific ions of interest. Acquisition takes even longer when data-dependent MS/MS spectra are obtained. All quantitative shotgun proteomics methods will have limited precision because much of the measurement time is spent acquiring spectra for peptide identification versus optimizing the collection of ions for quantitation.

The second point states that the primary reason for using isotope-labeled internal standards is to improve accuracy by accounting for losses in the sample preparation process. Figure 3 diagrams the sample preparation processes for three of the schemes and indicates where unaccounted-for losses could occur. The post-digestion scheme introduces the internal standard at or after protein digestion. Any loss of protein that occurs from the protein isolation step to the digestion step will go unmeasured, thereby reducing accuracy. In comparative proteomics, the hope is that sample losses will have been equal in both samples before they are combined and measured. If this assumption is true, then no loss in accuracy will occur. However, precision will diminish because sample-preparation losses for the two samples will introduce a variable amount of random error, depending on the analytical method.

The ICAT scheme adds the label before protein digestion, thereby eliminating the potential for inaccuracy caused by digestion and peptide processing. However, this method cannot account for differences in cell lysis and protein isolation. The in vivo labeling method best accounts for sample-preparation and protein-isolation problems because the internal standard is added directly in the form of labeled tissues or cells of the same type as those being collected. The internal standard (as labeled protein in cells or tissues) undergoes identical sample preparation to the unlabeled sample protein. In addition, this method subjects the sample to the least number of required manipulations during sample processing (Figure 3). The expectation is that this approach would provide the best overall precision and accuracy.

Another reason for using isotope-labeled internal-standard peptides is that the labeled and unlabeled peptides are measured at the same instant, and time-dependent noise will affect both species equally and cancel. This advantage is greatly reduced, however, when the internal standard contains multiple deuterium atoms (e.g., with the original deuterated ICAT reagent; *22*). Fractionation and early LC elution of the ^{2}H-labeled peptides may negate the advantage of reducing the noise associated with drift in the MS measurement. A similar problem occurs to a lesser degree when global labeling of peptides is performed with other deuterated modifiers. In contrast, peptides labeled with heavier isotopes (e.g., ^{13}C, ^{15}N, or ^{18}O) produce minimal and usually insignificant chromatographic fractionation, even when multiple labels are introduced (*41*).

We have already discussed the fact that all quantitative MS measurements are relative and are only absolute if we have known protein standards for calibration curves. Most quantitative proteomics studies have limited their focus to the simple comparative case of sample A (e.g., treated cells) versus sample B (e.g., untreated cells). However, if an adequate supply of labeled internal-standard pro-

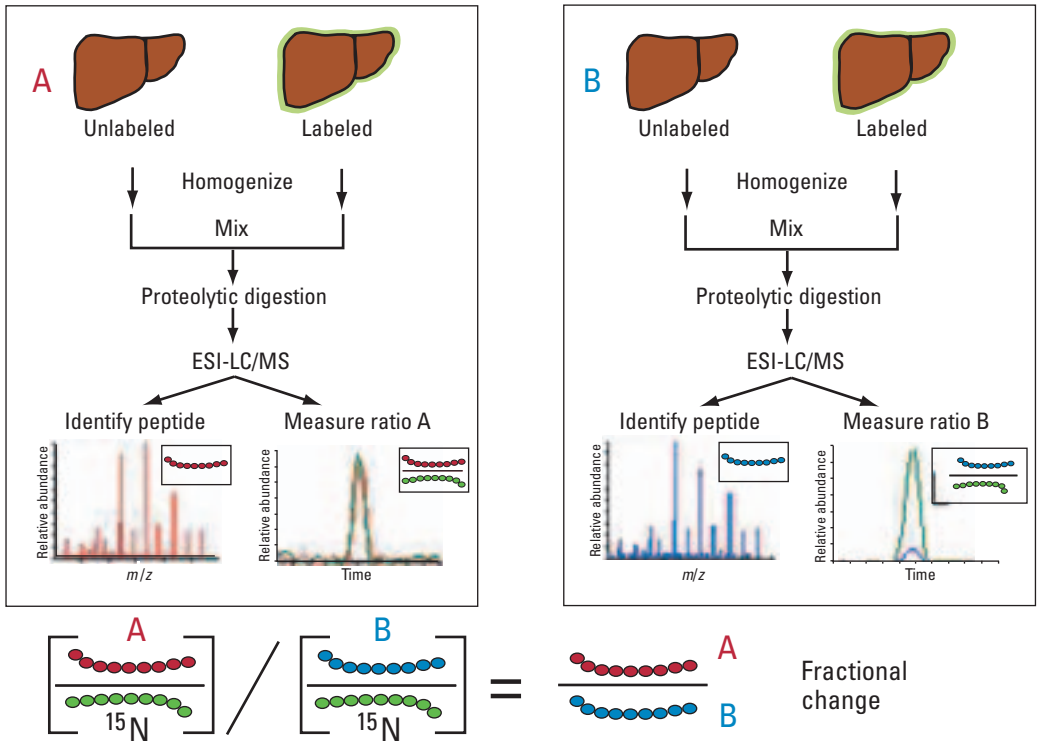


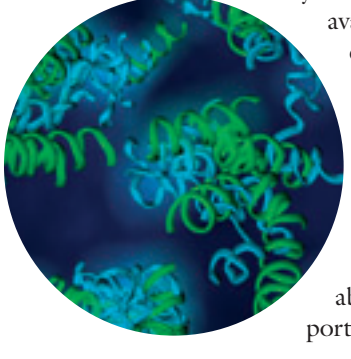
FIGURE 4. Biosynthetically prepared ^{15}N-protein standard is used to quantitate unlabeled protein under different experimental conditions.

Livers of control rats (A) and rats administered with cycloheximide (B) are independently mixed with a known amount of liver taken from an ^{15}N-labeled rat liver. The ratio of protein in A versus B is compared with the measured ratio of unlabeled to ^{15}N-labeled protein. (Adapted from Ref. 41.)

tein exists, we can measure the relative amounts of protein among many different samples (A, B, C, etc.) versus the labeled internal-standard protein sample on the basis of Equations 2 and 3. Here, the labeled protein is added to every sample, and this makes multiple measurements of a specific state (*43*) or multiple states possible. Figure 4 illustrates this approach, which allows us to obtain replicate samples and compare multiple, different states (*41*). Replicate measurements that use a ratio of two ratios improve the accuracy of the quantitative data by minimizing systematic errors (*20*, *41*) and facilitate assessment of the variance from different parts of the process.

Unlike microarray analyses, in which dozens of conditions are often studied simultaneously, repeated measurements and measurements of multiple states from quantitative proteomics experiments have rarely been reported. A 2-D chromatographic run that measures thousands of peptides by LC/MS/MS may take a day to complete; replicate determinations for a single sample may take a week. This bottleneck will be very difficult to remove. An alternative approach is to obtain results for multiple peptides per protein in the same LC/MS run. In theory, every labeled peptide pair from the same protein should give the same ratio as that indicated by Equation 1 for any of the schemes outlined. The variance in the measured ratios of peptides from a common protein will then reflect the error in data for that protein.

Although measuring multiple peptides per protein will provide an assessment of uncertainty, it is rarely reported in the literature. Papers assessing figures of significance concerning a new method will often report data for multiple peptides for a limited number of proteins that have usually been prepared from commercially available standards (*21*, *44*). However, most reports that compare the abundance ratios of proteins in two samples (e.g., treated and untreated cells) include impressive tables listing many proteins and their abundance ratios but do not provide any level of uncertainty about the abundance-ratio values. These reports often extrapolate conclusions from protein ratios that exceed an empirically derived threshold instead of an objectively determined statistical significance. The use of the ratio magnitude as a measure of significance is unacceptable because ratios that deviate the most from unity will have the greatest relative error (*15*). Because multiple (i.e., >2) peptides are measured for most proteins, a standard error can be computed for most protein abundance ratios and levels of significance can be calculated. With the approach in which ^{15}N-labeled protein was added to samples of untreated and treated liver protein, we were able to calculate uncertainties for several hundred proteins. In many cases, >10 peptides were identified and quantified per protein (*41*).



Although replicate values of individual samples are preferred for determining error and the significance of observed changes in protein abundances, the time required for a single measurement often places practical limits against doing so. Assessing variance by multiple peptides per protein in a single run is a viable alternative that approximates the same result. Unfortunately, very few quantitative studies in proteomics provide any figures of uncertainty based upon either replicate measurements of samples or measurements of multiple peptides per protein.

The continuous advances in MS instrumentation have been important in improving the number of peptides in a protein digestion mixture that can be measured. These advances have significantly increased both the speed at which data are acquired and the fraction of time spent measuring peptide ion intensities. In addition, several recent computer programs have been described for the analysis of quantitative proteomics data (*20*, *45*, *46*). Although a majority of these programs are restricted to pep-

tides with fixed m/z differences between them (e.g., ICAT), only one is readily compatible with ^{15}N metabolically labeled proteomics data and, even more importantly, can account for any atomic enrichment of the labeled peptide (*20*). As the amount of data collected increases, it becomes more and more important that these programs be able to handle peak detection and integration, background subtraction, and outlier omission without subjective human intervention. Furthermore, quantitative measures of data quality should be incorporated into these algorithms for the objective analysis and comparison of large data sets and to ensure the integrity of data transfer between laboratories.

Conclusions

Quantitative proteomics methods have evolved through the use of stable-isotope-labeled internal standards. The variety of approaches for introducing these standards include chemical modification of peptides post-digestion, the addition of commercialized reagents before digestion, and the use of specifically grown cells and even whole animals in which all proteins have been labeled biosynthetically. The need to both identify and quantify thousands of proteins in shotgun proteomics experiments will always limit the ultimate precision that can be obtained in quantitation. Novel mass spectrometer designs and improved data acquisition and processing approaches continue to raise the quality of our quantitative measurements. If this article has a take-home message, it is that the traditional approaches to quantitative MS data evaluation and validation should not be forgotten.

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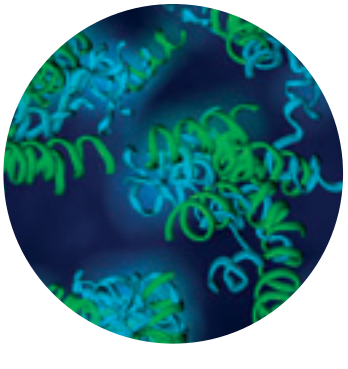

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