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Measurement of the Isotope Enrichment of Stable Isotope-Labeled Proteins Using High-Resolution Mass Spectra of Peptides

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Stable isotope-enriched molecules are used as internal standards and as tracers of in vivo substrate metabolism. The accurate conversion of measured ratios in the mass spectrometer to mole ratios is complicated because a polyatomic molecule containing enriched atoms will result in a combinatorial distribution of isotopomers depending on the enrichment and number of "labeled" atoms. This effect could potentially cause a large error in the mole ratio measurement depending on which isotope peak or peaks were used to determine the ratio. We report a computational method that predicts isotope distributions over a range of enrichments and compares the predicted distributions to experimental peptide isotope distributions obtained by Fourier transform ion cyclotron resonance mass spectrometry. Our approach is accurate with measured enrichments within 1.5% of expected isotope distributions. The method is also precise with 4.9, 2.0, and 0.8% relative standard deviations for peptides containing 59, 79, and 99 atom % excess ^{15}N, respectively. The approach is automated making isotope enrichment calculations possible for thousands of peptides in a single μLC−FTICR-MS experiment.

The use of internal standards has become common practice for high-precision quantitative analysis of biomolecules in complex mixtures. Internal standards that mimic the sample analyte being quantified are added to a mixture to account for losses during sample preparation and to minimize errors associated with the analytical measurement. Mass spectrometry enables the use of stable isotope-labeled analogues as an internal standard, which is usually the best internal standard with the least difference in physicochemical properties between the sample and the standard. To facilitate quantitative proteomics measurements, technologies have been developed that incorporate stable isotope-enriched atoms (usually ^{2}H, ^{13}C, ^{15}N, or ^{18}O) into proteins using in vivo metabolic labeling,[1−3] chemical derivatization,[4−6] or enzymatic catalysis.[7−9] A protein sample containing only natural-abundance isotopes is then mixed with the internal standard containing proteins enriched in "heavy" stable isotope atoms. The mixed protein mixture is then digested to peptides, and the mole ratio between the labeled and unlabeled proteins is estimated from the ion current ratios of the labeled and unlabeled peptides measured by mass spectrometry.

Unfortunately, the accurate conversion of mass spectrometry measured ion current ratios to mole ratios is complicated because a polyatomic molecule containing multiple enriched atoms will be composed of differentially enriched isotopomers. The molar distribution of labeled isotopomers will depend on (1) the elemental composition of the molecule, (2) the number of labeled atoms, and (3) the enrichment of those individual labeled atoms. Figure 1 illustrates this effect of two different ^{15}N isotope enrichments on the overlapping distribution of labeled isotopomers. Thus, for a single peptide sequence, the mass spectrometer response will be distributed between different isotopomers at different m/z "peaks" of the isotope distribution. Depending on the enrichment of the labeled peptide and the m/z range used to derive the labeled peptide intensity, the fractional abundance of peptide intensity will likely be estimated incorrectly.

Traditional quantitative mass spectrometry-based analyses address this problem by creating a standard curve for each analyte measured. Using a series of standards where the ion current ratios are measured for a range of known mole ratios, the contribution of unlabeled material in the labeled sample can be adjusted and the different fractional contributions of a given isotope peak to

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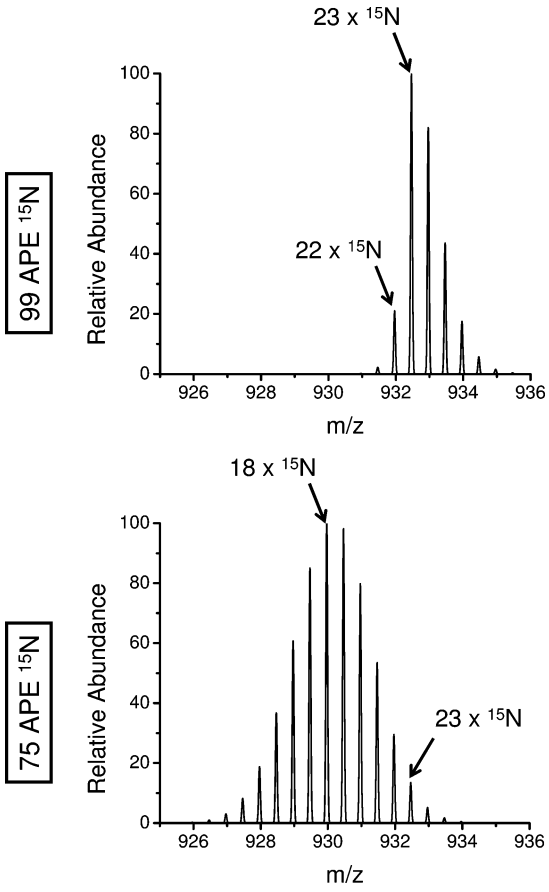

Figure 1. Theoretically calculated isotope distributions to illustrate the effect of the enrichment of labeled atoms on the overall distribution of labeled isotopomers. The two panels are the predicted isotope distributions for the doubly charged peptide SIVPSGASTGVHEALEMR containing 23 nitrogen atoms at both 99 and 75 atom % excess (APE) ^{15}N, respectively. Whereas the most abundant isotope peak is at the *m*/*z* expected for an isotopomer containing 23 ^{15}N atoms, there is still substantial contribution to the total isotope distribution from an isotopomer containing only 22 ^{15}N atoms. In contrast, at 75 APE, the intensity of the isotope peak at $[M + 23]^{2+}$ is only 13.5% of the isotope peak at $[M + 18]^{2+}$; where M is the mass of the monoisotopic mass of the unlabeled peptide.

the overall isotopomer response can be corrected based on the slope and the intercept determined from the standard curve. Unfortunately, in proteomics it is not possible or practical to run standards for every peptide in an unknown mixture. Thus, most proteomics methods assume that the measured response between a peptide containing only natural-abundance isotopes and the same peptide enriched with labeled atoms will be 1:1.

Because we are unable to run standards for every measured peptide ratio, we previously reported two simple approaches to minimize systematic errors and maximize the quantitative accuracy of proteomics experiments. When using a standard curve, the slope of the known mole ratios versus their respective measured ion current ratios is used to correct for any nonunity response (i.e., slope $\neq 1$) between an unlabeled and stable isotope-enriched analyte. The same correction can be achieved using a ratio of two ratios, where both ratios are measured relative to the same stable isotope-labeled standard.[3,10,11] Because both ratios will have the same systematic error, the combination of both ratios will result in a correction analogous to dividing a ratio by the slope of a standard curve. A similar but different correction can be accomplished by performing a reciprocal labeling experiment as described by Oda et al.[1]

The second approach to maximize the accuracy of different fractional contributions of a single isotope peak toward the total isotope distribution is to use the entire isotope distribution in the calculation of the unlabeled to labeled ion current ratio. We have shown that when mass chromatograms are produced from a *m*/*z* range spanning the entire isotope distribution, as opposed to a single isotope peak, the molar response approximates unity regardless of the enrichment of the atoms the labeled peptide is composed.[10] This approach, although powerful, requires the accurate knowledge of the elemental composition, number of enriched atoms, and their respective enrichment. Using the accurate enrichment of the atoms in the labeled peptide, we can calculate the *m*/*z* range spanning the entire isotope distribution to extract ion chromatograms for quantitative analysis. Under conditions where the organism's protein can be completely equilibrated with the precursor it is synthesized from, the isotope enrichment may be approximated using the known enrichment of the atoms in the media or diet. However, in cases where the protein is not equilibrated completely or different cell and tissue types may have different isotope enrichments,[3] this value needs to be calculated on a protein by protein basis. Furthermore, whereas summing the *m*/*z* range that spans the entire isotope distribution is a simple and reasonable solution using a low-resolution mass spectrometer that cannot resolve the individual isotope peaks of a multiply charged precursor,[10] this solution is impractical for high-resolution data where individual multiply charged isotope peaks are well resolved. However, accurate knowledge of the isotope enrichment of the labeled peptide facilitates the a priori calculation of a correction factor between the unlabeled and labeled peptide regardless of the *m*/*z* range used to estimate the mole ratio.

In this report, we describe a high-throughput and robust approach for the measurement of protein isotope enrichment using high-resolution mass spectra of peptides. We demonstrate this approach using Fourier transform mass spectrometry; however, these analyses can be conducted with any mass spectrometer capable of resolving the isotope distribution of a peptide. The accuracy and precision of our method is validated with proteins of known enrichment and with selected ion monitoring gas/chromatography mass spectrometry (SIM-GC/MS) measurements of the amino acids from the acid hydrolysis of the protein mixtures.

METHODS

Sample Preparation. (1) Yeast. Yeast (*Saccharomyces cerevisiae* S288C) were cultured in ^{15}N-enriched minimal media (YNB/5% glucose/0.5% $(NH_4)_2SO_4$) with four distinct enrichments of ^{15}N (0, 59.6, 79.7, and 99.0 APE) and grown to log phase (1.0 OD_{600}/mL) at 30 °C. Cells were lysed and proteins from each sample were resolved by 2D gel electrophoresis. Identical gel spots from each of the four ^{15}N-enriched samples were excised for in-gel

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protease digestions. Briefly, each gel slice was washed in 100 mM NH_4HCO_3 for 20 min. Proteins were reduced by incubation in 25 mM dithiothreitol/100 mM NH_4HCO_3 for 15 min at 60 °C. After being cooled to room temperature, proteins were alkylated by incubation in 75 mM iodoacetamide/100 mM NH_4HCO_3 for 15 min in the dark. Gel slices were then washed with 50% acetonitrile/100 mM NH_4HCO_3 for 20 min, and each slice was cut into 1-mm^3 pieces and dehydrated in 100% acetonitrile for 15 min. After removing the solvent, the gel pieces were dried and rehydrated in 0.5 μg of modified trypsin (Promega) in 25 mM NH_4HCO_3. Additional buffer without enzyme was added to cover gel pieces, and the samples were incubated at 37 °C for 12 h. Peptides were extracted from the gel pieces with 100 μL of 60% acetonitrile/0.1% trifluoroacetic acid for 20 min at 28 °C on a Thermomixer (Eppendorf) set at 1400 rpm. The peptide extracts were dried using a Speed-Vac and resuspended in 20 μL of 5% formic acid immediately prior to analysis by μLC−MS/MS using a reversed-phase chromatography column (100-μm-i.d. chromatography column packed with 12-cm, 5-μm Aqua) placed in-line with an LTQ-FT mass spectrometer (ThermoElectron). A single Fourier transform mass spectrum was acquired in the ICR in parallel with five low-resolution MS/MS spectrum in the linear ion trap.

(2) Rat Tissue Samples. Three-week-old male Sprague−Dawley rats were fed a specialized diet consisting of a protein-free rodent diet (Harlan Teklad, TD93328) supplemented with >99% ^{15}N-labeled *Spirulina platensis* (2 parts Harlan diet/1 part algae by mass). This mixture was offered to the rats for 30−45 min every 6 h (6 a.m., 12 p.m., 6 p.m., 12 a.m.) for 44 days. Rats were then sacrificed and the livers collected. The liver tissue was sliced into ~1−2-mm-thick slices, frozen in liquid N_2, and stored at −80 °C. Frozen labeled rat livers were homogenized using a Dounce homogenizer, and proteins were precipitated using MeOH/$CHCl_3$. Precipitated protein samples (25 μg) were then resolved on a 10% polyacrylamide gel. Proteins were stained using Coomasie blue, and protein bands were excised for in-gel protease digestions and measured by μLC−MS/MS as described above. All animal procedures were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, Washington, DC).

Gas Chromatography/Mass Spectrometry. The supernatants were collected from yeast lysis, and tissue homogenates and protein concentration was determined using the Lowry H protein assay (Bio-Rad, Hercules, CA). Protein (1 mg) was precipitated from the supernatant using MeOH/$CHCl_3$.[12] The protein pellet was hydrolyzed by adding 1 mL of 6 M HCl, capping the vials, and heating at 110 °C for 24 h. The samples were dried under N_2 gas and then reconstituted in 1 mL of 1 M acetic acid. Each sample was vortexed and poured directly onto a disposable column containing 0.5 mL of washed cation-exchange resin (AG 50W-X8 100−200 mesh, hydrogen form, Bio-Rad Laboratories, Richmond, CA). The cation resin was washed twice with 5 mL of distilled water, and the bound material (including the amino acids) was eluted with 2 mL of 3 M NH_4OH into clean conical-bottom reaction vials. The vials were dried under N_2, and the resulting amino acids were derivatized to form the *tert*-butyldimethylsilyl (tBDMS) derivatives as described previously.[13] All GC/MS measurements were made on a Hewlett-Packard model 5971A gas chromatograph−mass spectrometer (Palo Alto, CA) using electron ionization at 70 eV. The tBDMS-derivatized amino acids were separated on a DB-1 30 m × 0.25 mm × 0.25 μm film capillary column (J&W Scientific, Palo Alto, CA). The $[M - \textit{tert}\text{-butyl}]^+$ fragment ion was monitored for the unlabeled and ^{15}N-enriched isotopomers of alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartate/asparginine, and glutamate/glutamine. Likewise, the $[M - COOtBDMS]^+$ fragment ion was used for the measurement of methionine and tyrosine. The integrated areas of the unlabeled and ^{15}N-labeled extracted ion chromatograms were used to calculate the ^{15}N/unlabeled amino acid mole ratio using a least-squares analysis of the overlapping mass spectra.[14] The resulting mole ratio (R) was converted to an enrichment (E) using the equation

$$E = \frac{R}{1+R} \times 100 \tag{1}$$

Calculation of Protein Enrichment from the Isotope Distribution of Peptides. For each peptide sequence identified by tandem mass spectrometry, a selected narrow m/z range of the ultrahigh-resolution precursor ion survey scan was extracted from the Xcaliber RAW file and written to an ASCII-based file with an *.ape extension. The selected m/z range spanned the minimum m/z expected from the unlabeled peptide isotope distribution (i.e., the monoisotopic mass) to the maximum m/z of the 100% enriched isotope distribution. A computer program called The Atomizer, interated through and predicted the isotope distribution at all ^{15}N isotope enrichments (0.1 APE intervals) for a selected peptide sequence (Figure 2). The predicted isotope distribution was based on all natural-abundance isotopes with the exception of selected atoms for each amino acid defined by the user to be considered "enriched". Importantly, a biologically relevant $^{13}C/^{12}C$ isotope ratio of 1.096% was used instead of the more carbonate specific ratio of 1.112%[15] in the calculation of each peptide isotope distribution. Each predicted isotope distribution was then correlated against the measured isotope distribution. Based on analyses by Senko et al.,[16] only isotope peaks of >20% relative abundance were used in correlation analysis. The Pearson correlation coefficient (r) was used to estimate the predicted isotope distribution that is most representative of the experimentally measured isotope distribution. The definition is given by the equation

$$r = \frac{\sum X_i Y_i - \frac{\sum X_i \sum Y_i}{N}}{\sqrt{\left(\sum X_i^2 - \frac{(\sum X_i^2)}{N}\right)\left(\sum Y_i^2 - \frac{(\sum Y_i^2)}{N}\right)}} \tag{2}$$

where X_i and Y_i are intensities from the experimental and

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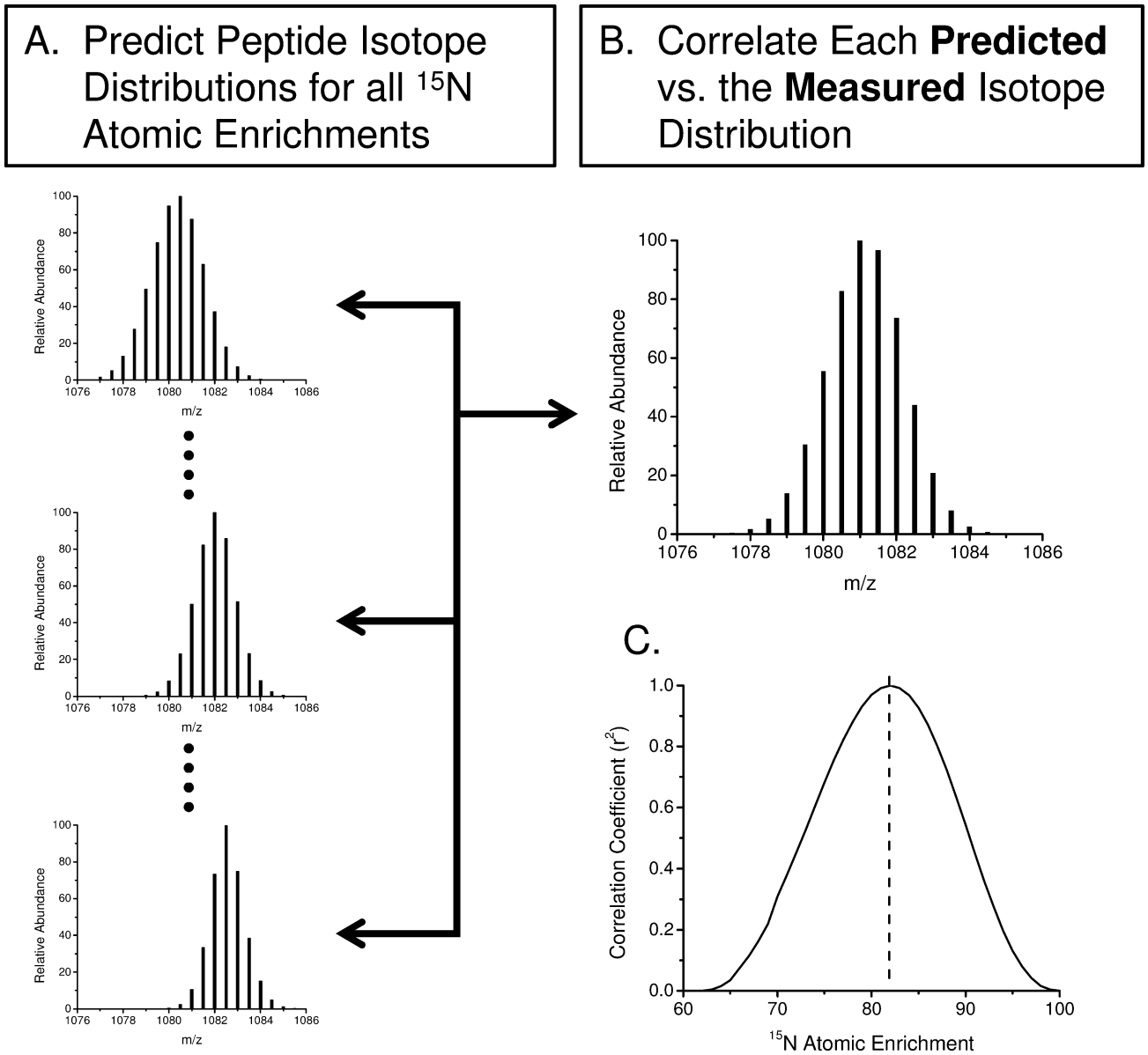

Figure 2. Scheme for the calculation of ^{15}N isotope enrichment from the polyatomic isotope distribution of a peptide. (A) The isotope distribution is predicted iteratively for all ^{15}N enrichments. (B) The predicted isotope distribution is correlated against the measured isotope distribution to find the best match. (C) The correlation coefficient between the predicted distribution and the measured distribution is used to determine the isotope enrichment of the peptide (dashed line).

predicted isotope distributions at $m/z = i$ and N is the number of pairs of observations. The relative intensities for each peak in the predicted isotope distribution was calculated as described by Kubinyi,[17] and the exact m/z of each peak of the isotope distribution was calculated using the probability-weighted sum of all the individual components contributing to each isotope peak as reported by Rockwood et al.[18] A binary executable of the described computer program for Microsoft Windows is available from the authors upon request.

RESULTS

Yeast cells were grown in the presence of ^{15}N-enriched ammonium salts as the only source of nitrogen. Protein from these samples should have equilibrated completely with the enriched $(^{15}NH_4)_2SO_4$ of the growth media because yeast were cultured for greater than 10 doublings. These samples were used as the "known standards" to validate the accuracy of the described approach in the context of a complex mixture.

Three different yeast cultures, each grown in media containing different enrichments of $(^{15}NH_4)_2SO_4$, were lysed and proteins separated by two-dimensional gel electrophoresis. The same four spots were excised from each gel (total of 12 spots), digested with trypsin, and analyzed by microcapillary liquid chromatography mass spectrometry using a hybrid Fourier transform mass spectrometer. The peptides were identified from their respective low-resolution tandem mass spectra and the ^{15}N isotope enrichment was calculated from the high-resolution mass spectrum obtained in the ion cyclotron mass analyzer. Three of the four

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Table 1. Measurement of Protein Isotope Enrichment in Peptides of Yeast Proteins by High-Resolution Mass Spectrometry

gene	spot	enrichment[a] sample 1	sample 2	sample 3
PMI40	1	59.2 ± 0.9 (4)	78.7 ± 1.5 (10)	98.7 ± 0.7 (13)
ENO1	1	57.8 ± 2.8 (8)	77.7 ± 2.4 (14)	99.0 ± 1.5 (26)
ENO2	1	58.7 ± 1.5 (12)	80.1 ± 1.5 (14)	99.2 ± 0.3 (19)
PDC1	1	60.7 ± 2.1 (2)	77.7 ± 1.6 (7)	99.2 ± 0.9 (3)
PGK1	2	56.0 ± 5.8 (2)	79.3 ± na (1)	99.2 ± 0.5 (16)
ENO1	2	58.2 ± 3.4 (13)	78.5 ± 1.4 (18)	99.1 ± .0.5 (24)
ENO2	2	58.5 ± 3.7 (12)	79.1 ± 1.9 (16)	99.1 ± 0.4 (20)
ASC1	3	58.6 ± 1.7 (16)	78.7 ± 1.0 (14)	99.2 ± 0.3 (23)
ADH1	3	61.5 ± 0.1 (2)	79.8 ± 1.3 (3)	99.5 ± 0.3 (5)
YHR029C	3	57.7 ± 6.0 (3)	80.3 ± 0.3 (2)	99.3 ± 0.2 (7)
EFB1	4	59.5 ± 2.8 (9)	79.8 ± 0.5 (6)	99.1 ± 0.8 (15)
weighted average:		58.6 ± 2.9	78.8 ± 1.6	99.1 ± 0.8

[a] Enrichment is expressed as atom percent excess ± standard deviation. The number of peptides per protein loci is shown in parentheses.

Table 2. SIM-GC/MS Measurement of Amino Acid Enrichments from Protein Obtained from Yeast Grown under Different Enrichments of Nitrogen Salts

amino acid	enrichment (MPE) sample 1	sample 2	sample 3
Ala	59.11	78.88	98.74
Gly	58.95	78.37	97.55
Val	59.06	78.86	98.75
Leu	58.91	78.74	98.74
Ile	58.98	78.81	98.68
Pro	59.30	78.88	98.30
Met	59.26	78.93	na[a]
Ser	58.89	78.65	98.57
Thr	59.15	78.97	98.79
Phe	59.33	79.09	98.32
Asx	58.81	78.57	98.82
Glx	58.67	78.53	98.83
Tyr	58.91	78.79	97.81
average	59.03 ± 0.20	78.77 ± 0.20	98.49 ± 0.42

[a] na, the signal-to-noise rato of the extracted ion chromatogram of the $[M - COOtBDMS]^+$ fragment of the derivatized methionine was too low to integrate accurately.

2D gel spots identified peptides mapping to multiple protein loci in all three cultures (Table 1). This identification of multiple proteins from single 2D gel spots is common and consistent with previous results.[19,20]

A summary of the enrichment calculations of peptides from yeast proteins in each respective "spot" is presented in Table 1. The average weighted ^{15}N isotope enrichments (as APE ± SD) measured by Fourier transform mass spectrometry was 58.6 ± 2.9, 78.8 ± 1.6, and 99.1 ± 0.8 in the cultures grown in media expecting to contain 60.2, 80.1, and 100 mole % excess $(^{15}NH_4)_2SO_4$, respectively. Although these samples were used in these experiments as "known standards", the expected enrichment of the material in the growth media is estimated by the mole ratio of ^{15}N-enriched/unlabeled salts weighed on an analytical balance during the media preparation and based on the assumption that the chemical and isotopic purity provided by the manufacturer of the components used to derive the standards is accurate. Therefore, although the mole ratio of the ^{15}N-enriched/unlabeled salts is known, the $^{15}N/^{14}N$ isotope ratio is an approximation based on the isotopic purity of the material supplied by the manufacturer—which is always <100%. This difference between the predicted and measured enrichment highlights a limitation of any method that requires a known standard for absolute quantitation. Whereas all traditional quantitative mass spectrometry methods are relative and ultimately limited by the quality of the standards that they are calibrated against, our approach is capable of absolute isotope enrichment measurements without standards.

To confirm the accuracy of our measurements, each protein sample was also hydrolyzed to amino acids prior to separation by gel electrophoresis and the respective ^{15}N amino acid enrichments measured by SIM-GC/MS. The data for the measurement of ^{15}N enrichments for 13 different amino acids by SIM-GC/MS is shown in Table 2. These specific amino acids were chosen for measurement because they each contain only a single nitrogen atom—simplifying the extrapolation of the measured molar labeled amino acid enrichment to the ^{15}N isotope enrichment. The average enrichments (±SD) for these amino acids were 59.0 ± 0.2, 78.8 ± 0.2, and 98.5 ± 0.4 APE for the samples grown in 60.1, 80.2, and 100 MPE $(^{15}NH_4)_2SO_4$ media, respectively. The enrichment measured by GC/MS in the first two samples, 60.1 and 80.2 MPE $(^{15}NH_4)_2SO_4$, were indistinguishable from the respective enrichments measured by FTMS. Interestingly, the ^{15}N enrichment measured by FTMS of the peptides (99.1 ± 0.8) was significantly higher than when determined from the amino acids from the same sample measured by GC/MS (p value <0.001).

In comparing the results obtained between the two approaches, the differences in the most enriched sample, 98.5 ± 0.4 APE by GC/MS versus 99.1 ± 0.8 APE by FTMS, reflects fundamental differences in how the two approaches measure the enrichment. While the FTMS approach associates a shift in shape and average m/z of the peptide isotope distribution with best-fit ^{15}N enrichment, the SIM-GC/MS approach measures the magnitude of the ion current intensity ratio between the unlabeled and ^{15}N amino acid. The average mole ratio between the ^{15}N-enriched and unlabeled amino acids for the 98.5 APE measurement was 65.7:1 (see eq 1). However, for a sample containing 99.1 APE ^{15}N, the mole ratio would have been a much greater, 110.1:1. Because both the lower enrichment measurements between the GC/MS and FTMS were indistinguishable and the magnitude of the observed ratio measurements for the most enriched sample was high, it is likely that the GC/MS measurement underestimated the actual enrichment and the ratio (at this intensity) was beginning to exceed the linear working range of the detector. Thus for molecules with high isotope enrichment, the FTMS would provide greater enrichment accuracies relative to alternative strategies that are forced to make large ion current ratio measurements.

An example of the measurement of ^{15}N-enriched yeast peptides is shown in Figure 3. In Figure 3A, the experimentally measured isotope distribution is shown for the $[M + 2H]^{2+}$ isotope distribution for the peptide sequence, AVDDFLLSLDGTANK, obtained

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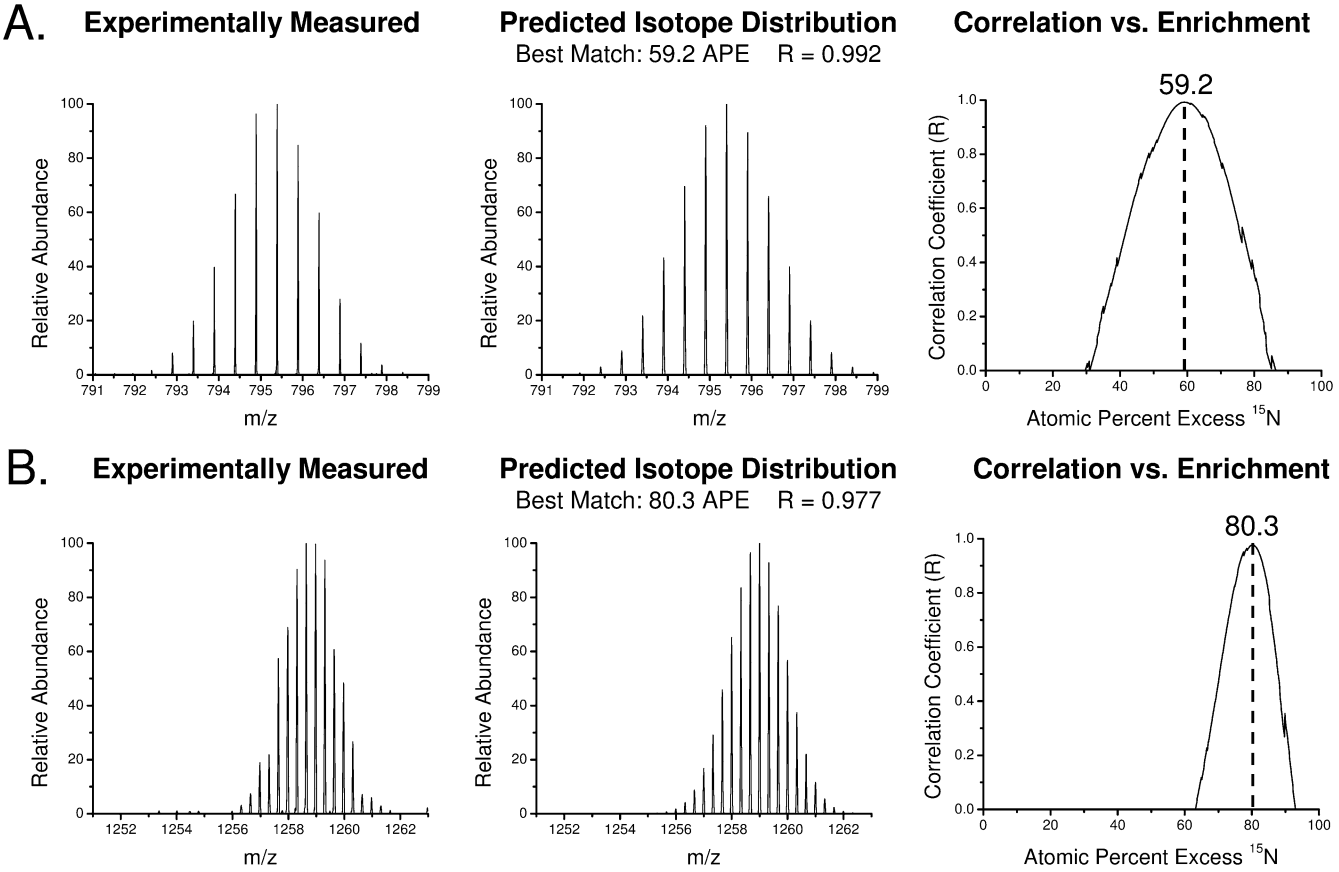

Figure 3. Comparison between the experimentally measured and predicted isotope distributions of two different yeast peptides. (A) Measurement of the ^{15}N isotope enrichment for the $[M + 2H]^{2+}$ isotope distribution of the peptide AVDDFLLSLDGTANK. The predicted isotope distribution with the highest correlation to the experimentally measured isotope distribution was from 59.2 APE ^{15}N. (B) Measurement of the enrichment for the $[M + 3H]^{3+}$ isotope distribution of the peptide with the sequence TSPYVLPVPFLNVLNGGSHAGGALALQEFMIAPTGAK.

Table 3. Measurement of Isotope Enrichment in Rat Liver Proteins

locus ID	description	enrichment APE ± SD	N^a
gi|16758804|ref|NP_446374.1|	acetyl-coenzyme A carboxylase β	93.8 ± 1.6	2
gi|8394158|ref|NP_059028.1|	fatty acid synthase	92.9 ± 2.6	11
gi|11968144|ref|NP_071992.1|	10-formyltetrahydrofolate dehydrogenase	92.1 ± 1.8	4
gi|13928936|ref|NP_113863.1|	α-actinin 4	89.1 ± na[b]	1
gi|17865351|ref|NP_446316.1|	valosin-containing protein	91.2 ± 4.6	3
gi|11560087|ref|NP_071604.1|	liver glycogen phosphorylase	89.6 ± 1.2	4
gi|20806145|ref|NP_620802.1|	dimethylglycine dehydrogenase precursor	89.5 ± na	1
gi|8393296|ref|NP_058941.1|	eukaryotic translation elongation factor 2	92.5 ± 3.8	4
gi|6755863|ref|NP_035761.1|	tumor rejection antigen gp96	91.5 ± 0.7	3
gi|8659555|ref|NP_002188.1|	aconitase 1	88.1 ± na	1
gi|8393186|ref|NP_058768.1|	carboamyl-phosphate synthetase 1	91.0 ± 3.0	6
gi|7657431|ref|NP_055205.1|	EBNA-2 coactivator	93.4 ± 2.3	2
gi|8392839|ref|NP_058683.1|	ATP citrate lyase	93.1 ± 0.6	2
weighted average		91.8 ± 2.1	44

[a] Number of peptides used in the calculation of the protein atom percent excess (APE). [b] na, not applicable.

from yeast containing 60.1 MPE $(^{15}NH_4)_2SO_4$ in the growth media. The plot of the correlation versus the enrichment of the predicted isotope distribution reaches a maximum at $R = 0.992$ for the isotope distribution predicted to contain 59.2 APE ^{15}N. The returned isotope enrichment is accurate and deviates from the "known" ammonium sulfate enrichment of the growth medium by <1.5%. Though there are slight visual deviations between the predicted and measured isotope distributions in Figure 3, the isotope distribution at 59.2 APE provides a better match than any other predicted distribution at 0.1% enrichment intervals.

Figure 3B shows the calculation of the isotope enrichment from the $[M + 3H]^{3+}$ isotope distribution of the peptide TSPYVLPVPFLNVLNGGSHAGGALALQEFMIAPTGAK. This spectrum was obtained from a tryptic digest of yeast grown in media containing 79.9 APE $(^{15}NH_4)_2SO_4$. The isotope distribution predicted from an 80.3 APE ^{15}N provided the best correlation with the measured isotope distribution ($R = 0.977$) and deviates from the actual enrichment of the nitrogen source of the growth media by less than 1%. This peptide contains a large number of enriched atoms (43 nitrogen atoms) in addition to a high background of natural-

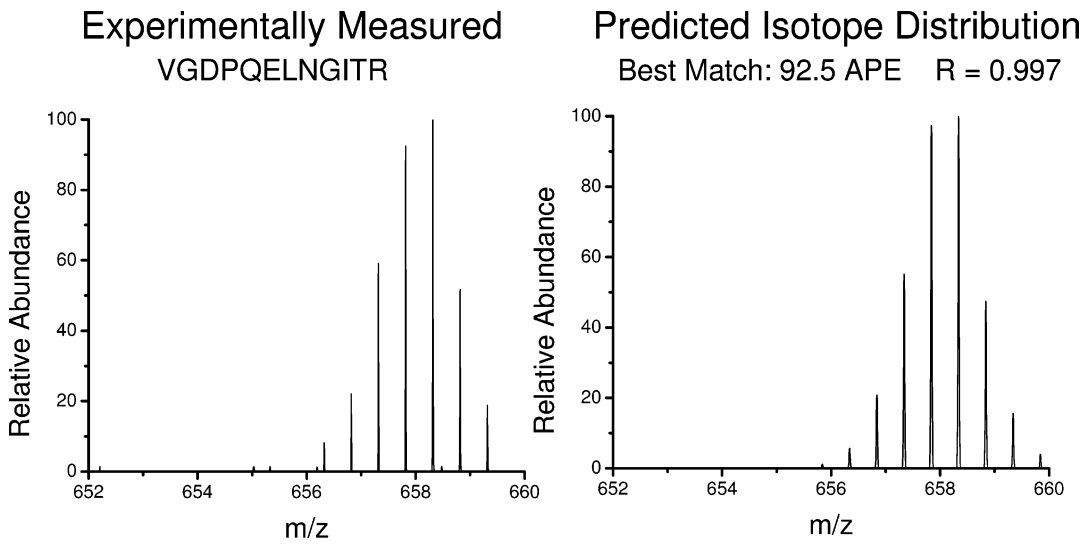

Figure 4. Measurement of the ^{15}N isotope enrichment of a peptide from rat liver fatty acid synthase. The prediction of the isotope distribution with nitrogens containing 92.5 APE ^{15}N provided the best correlation with the experimentally measured isotope distribution ($R = 0.997$).

abundance heavy isotopes. Regardless of the isotope composition of the peptide, the true isotope enrichment can be accurately derived using the described approach.

To demonstrate that these measurements are not constrained to single-cell organisms with well-defined growth conditions, we also measured the enrichment of several proteins from rat liver after 44 days on a diet consisting of ^{15}N-enriched algae as the only source of nitrogen. Because of the long labeling period, we expected that all of the proteins would have an isotope enrichment that was reflective of the enrichment of the precursor pool they were synthesized from. Furthermore, because of the high homogeneity of the tissue studied, this precursor pool enrichment would be indistinguishable for all proteins.

A total of 44 peptide isotope distributions from 13 different liver proteins were measured using a hybrid ion trap Fourier transform mass spectrometer (Table 3). The weighted average enrichment measured from these liver proteins was 91.8 ± 2.1 APE. This weighted average ^{15}N enrichment was not different from the average enrichment obtained by measuring the average ^{15}N enrichment of the respective amino acids by SIM-GC/MS (91.1 ± 2.1 APE ^{15}N). An example of the comparison between an experimentally measured and predicted isotope distribution by FTMS is shown in Figure 4 for the peptide VGDPQELNGITR unique to the protein fatty acid synthase. Our data correlate well with predicted isotope distributions, and the accuracy and precision of these data suggest that this approach is capable of measuring the isotope enrichment of proteins without prior prefractionation using a combination of microcapillary separation of peptides interfaced with a high-resolution mass spectrometer.

DISCUSSION

With the increased application of in vivo metabolic labeling as an approach for creating stable isotope-enriched internal standards for quantitative proteomics, we face an increased need for an accurate and robust approach for characterizing the isotopic labeling of these protein standards. Even alternative strategies that use enzymatic catalysis and chemical derivatization require accurate knowledge of the isotope enrichment of the labeled species incorporated into protein to correctly derive the true mole ratio from a series of mass spectrometry-measured ion current ratios.[8,9,21] Because of the complexity of many samples studied, we need an approach that is capable of measuring different enrichments of labeled atoms in different proteins without prior prefractionation. Furthermore, because of the heterogeneity of many systems being studied, an ideal approach would incorporate the measurement of the isotopic enrichment of the labeled protein(s) while simultaneously measuring the quantitative mole ratio between the unlabeled and the enriched standard—resulting in an isotope enrichment measurement for each protein. The approach described here measures the enrichment of proteins using the isotope distribution of peptides unique to the respective protein sequence. By measuring the enrichment of the peptide instead of the amino acids, the qualitative identity of the protein is maintained and, thus, is compatible with unfractionated mixtures.

Accuracy and Precision of This Approach Relative to GC/MS. Though the measurement of isotope enrichment of individual proteins on the peptide level eliminates the need for prior protein purification, there are tradeoffs in comparison to the measurement of enrichment on the amino acid level. The most obvious tradeoff appears to be a sacrifice in precision. Each isotope enrichment measurement was less precise by LC−FTMS of the peptides when compared to SIM-GC/MS of amino acids. Also, the FTMS precision was poorer for enrichments from the broad isotope distributions as opposed to those of compact isotope distributions (Table 1). Although the average mass of the isotope distribution is correlated linearly with the ^{15}N enrichment,[22] the width of the isotope distribution is broadest near 50 APE and narrowest at 0 and 100 APE. Assuming Gaussian-distributed, signal-independent noise,[23] the precision for the estimate of the weighted average of the isotope distribution will be proportional to the width or spread of the isotope distribution—supporting our observation of greater precision in the samples with more compact isotope distributions.

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In contrast to SIM-GC/MS of amino acids where the greatest precision will be observed for ion current ratios near 1:1 (near 50 APE),[13] our approach for the measurement of APE by FTMS of peptides will have greater noise near 50 APE compared to all other enrichments.

To improve the precision, the number of ions at the frequencies defining the isotope distribution needs to be increased.[24] In our experiments, the FTMS enrichment was calculated from a single scan with a target of 400 000 ions spanning a 400−1400 m/z range. Depending on the complexity of the number of components eluting from the chromatography column at any one point in time, the number of ions defining a low-abundance isotope distribution may be limited to only be a few hundred. To improve the precision of the isotope distribution measurement, the ion cyclotron cell can be filled with only a narrow mass range spanning the respective isotope distribution. Using the selected ion monitoring feature of the LTQ-FT, a stored waveform inverse Fourier transform isolation can be applied to selectively trap only a narrow mass range in the ion cyclotron. The isolation of ions from a narrow mass range will increase the number of ions for the isotope distribution of interest without altering the m/z from space charge effects.[25]

The ability to obtain accurate enrichment measurements without standards reduces the dependence on standards to calibrate the isotope ratio measurement response. In a field like proteomics, a standard of known purity or composition for all species is impossible and impractical to obtain. Furthermore, because our method uses only isotope peaks that are >20% relative abundance, the measured intensity range between any number of signals is greatly reduced without reducing the range of enrichment measurements. This narrow intensity range is preferable compared to traditional isotope ratio measurements where extremes in enrichment (either low or high) result in a large ion current ratio measurement, increase the relative Poisson's distributed noise,[13] and may exceed the linear dynamic range of the detector.

Application of Isotope Enrichment Measurements in Quantitative Proteomics. Knowing the enrichment of the labeled atoms of an internal standard is essential to determine the mole ratio between an unlabeled peptide and its respective labeled internal standard.[3,10] In cases where the labeling time is long and the sample is homogeneous, the manufacturer's isotopic purity assessment or the use of SIM-GC/MS to measure the isotope enrichment of amino acids is an acceptable measure of enrichment for all proteins. However, in heterogeneous tissue samples (e.g., brain tissue) where there are numerous different cell types with different average turnover rates or if the labeling period is short and the proteins have not had sufficient time to equilibrate with the intracellular precursor pool—these assumptions are likely invalid. The approach described in this article can be implemented into quantitative analysis software so that corrections can be achieved on a protein-by-protein basis instead of the naïve application of a single global correction factor.

Alternative Applications of the Measurement of Protein Specific Isotope Enrichment in Mixtures. Although we have emphasized the measurement of isotope enrichment on improving the mole ratio measurement for proteomics, our methodology has applicability beyond that of relative abundance measurements in proteomics. An obvious extension of this approach can be used for the assessment of intracellular precursor pools for substrate kinetic measurements. The yeast experiments in this report were grown purposely in altered ammonium sulfate enrichments to produce precursor amino acids for protein synthesis with "known" enrichment. Similarly, because the isotope enrichment of a protein will reflect the isotope enrichment of the amino acids it was synthesized from,[26,27] this method can be combined with proteins of known localization and used as a proxy measure of intracellular amino acid enrichment sample not readily accessible by biopsy in multicellular organisms.

An additional application of our software is toward characterizing the hydrogen/deuterium exchange in experiments probing protein structure. When incubated in the presence of D_2O, protein amide bonds will exchange hydrogen atoms of natural abundance with the enriched deuteriums they are incubated in. The rate of exchange is dependent on the accessibility of the respective amide bonds to the deuterium of the solution.[22,28] Mass spectrometry is used to characterize the rate of deuterium exchange of selected peptides in different regions of the protein to probe questions of protein structure.[29] The deuterium enrichment is normally characterized in peptides produced from the digestion of proteins using pepsin by taking the centroid of the isotope distribution and determining the shift in m/z compared to an unenriched peptide. Our approach, which matches a predicted isotope distribution at all isotope enrichments to the experimentally measured isotope distribution, could also be used for the measurement of peptide deuterium exchange without modification.

New proteomics methods for the quantitation of proteins in complex methods are continually being developed. The key to these new methods, as with any quantitative mass spectrometry assay, is the accuracy and precision with which the measurement is made. To ensure the accuracy of any mole ratio measurement between an unlabeled and labeled molecule, it is essential to consider the enrichment of the atoms of which the labeled species is composed.

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