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^{18}O Labeling over a Coffee Break: A Rapid Strategy for Quantitative Proteomics

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Abstract

Proteomics-based quantification methods for differential protein expression measurements are among the most important and challenging techniques in the field of mass spectrometry. Though numerous quantification methods have been established, no method meets all the demands for measuring accurate protein expression levels. Of the various relative quantification methods by isotopic labeling, ^{18}O labeling method has been shown to be simple, specific, cost-effective and applicable to a wide range of analyses. However, some researchers refrain from using the method due to long incubation periods required during the labeling process. To address this problem, we demonstrate a method by which the labeling procedure can be completed in 15 min. We digested and labeled samples using immobilized trypsin on micro-spin columns to speed up the enzyme-mediated oxygen substitution, thereby completing the labeling process within 15 min with high labeling efficiency. We demonstrate the efficiency and accuracy of the method using a four protein mixture and whole cell lysate from rat vascular endothelial cells.

Keywords

^{18}O labeling; proteomics; mass spectrometry; relative quantification; ZoomQuant; trypsin spin column

Introduction

Proteomics, the study of the cellular proteome, is an important and fast emerging field.^{1,2} Recent advances in the field of mass spectrometry (MS) and the development of efficient separation techniques have revolutionized the study of proteins at the systems level. After the initial protein identification and characterization, a remaining challenge in proteomics is the quantification of the proteins identified. Various methods for quantification by MS have been developed recently, each having its own merits and shortcomings.^{3–5} Relative quantification methods like differential gel electrophoresis⁶ (DIGE), stable-isotope labeling techniques like isotope-coded affinity tag⁷ (ICAT), stable isotope labeling with amino acids in cell culture^{8,9} (SILAC), isobaric tags for relative and absolute quantification^{10,11} (iTRAQ) and other mass tagging methods are well-established. However, DIGE is not optimal for membrane proteins, and allows analysis of only limited number of proteins. Other labeling methods require expensive reagents and complex experimental approaches. ICAT is specific to cysteine containing peptides only, SILAC to cell cultures only. Furthermore, not all the peptides generated are ionized under MS conditions, limiting the usefulness of methods that label specific residues. A technology that alters all the peptides that are detected by mass

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spectrometry would be the best approach for relative quantification studies. Thus, enzymatic labeling of peptides using heavy water (H_2^{18}O) has been proposed to be a method of choice over chemical tagging methods, as it is highly specific and is almost universally applicable.^{12–19} Proteolytic digestion exchanges the oxygen atoms from the carboxyl group with one or two ^{18}O atoms leading to the mass difference of 2 or 4 Da.

Though this ^{18}O labeling method has been established as an efficient method of relative quantification, it has limitations. Most concerns focus on the time-consuming labeling step, the back-exchange of ^{18}O with ^{16}O , and the difficulty in measuring the labeled peptides due to lack of efficient computational tools. Some of these limitations have been addressed in previous studies.^{16,20,21} In this study, we focus on addressing the time required for the experimental procedure. We demonstrate an increased reaction rate for labeling tryptic peptides and show that it can be done within 15 min. The method is demonstrated to be fast and equally efficient compared to the conventional long incubation methods.²² With this increased proteolysis rate, same-day results can be obtained using ^{18}O labeling, accelerating proteomics research.

Experimental Methods

Materials and Reagents

All chemicals were purchased from Sigma-Aldrich Corporation, St. Louis, MO, unless specified otherwise. Dithiothreitol (DTT) and micro BCA assay kits were purchased from Pierce, Rockford, IL. The trypsin spin columns were purchased from Sigma-Aldrich, St. Louis, MO. The mass spectrometry-grade trypsin gold used was obtained from Promega, Madison, WI. Methanol, acetonitrile, and water were HPLC grade solvents from Burdick & Jackson, Muskegon, MI. ^{18}O water (95%) was procured from Cambridge Isotope Laboratories, Inc., Andover, MA.

Instrumentation

Nano-HPLC-MS experiments were performed on an LTQ mass spectrometer (Thermo Electron) coupled with a Surveyor HPLC system (Thermo Electron) equipped with an autosampler. The instrument was interfaced with a capillary column (100×0.1 mm), in-house packed with $5 \mu\text{m}$ C_{18} RP particles (Luna C_{18} , Phenomenex). The fused silica capillaries (Polymicro Technologies, AZ) for the columns were pulled by a micropipette puller P-2000 (Sutter Instrument Company, CA) and packed with C_{18} resin using a bomb-loader.

Trypsin Digestion and ^{18}O Labeling

A mixture of four proteins (10 pmol each of bovine serum albumin (BSA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α -lactalbumin (LALBA), myoglobin (MYG)) or rat vascular endothelial cell lysate (50 μg of total protein as measured by micro BCA assay) was reduced and alkylated with DTT and iodoacetamide (IAA) at 10 mM and 55 mM concentration, respectively. The denatured proteins were digested in-solution by the addition of trypsin (1:50 protease/protein) and incubation of the reaction mixture overnight at 37 °C. The ^{18}O labeling was carried out during in-solution digestion wherein all the buffers were made in ^{18}O water (95%) instead of the normal ^{16}O water. The tryptic peptides were cleaned using C_{18} zip tips before mass spectral analysis.

Fast ^{18}O Labeling Using Trypsin Spin Columns

A mixture of four proteins (10 pmol each of bovine albumin, α -lactalbumin, glyceraldehyde-3-phosphate, myoglobin) or rat vascular endothelial cell lysate (50 μg total of protein as measured by micro BCA assay) was reduced and alkylated with DTT and IAA at 10 mM and 55 mM

concentration, respectively. The trypsin spin column (Sigma-Aldrich) was equilibrated with reaction buffer (100 mM ammonium bicarbonate) and milliQ water by spinning at 1000g for 2 min in the respective solutions. The denatured protein mix was dissolved in 100 μ L of reaction buffer and was loaded onto the trypsin spin column. The column was spun at <200g for 30 s to load the sample solution onto the column bed. Incubation was carried out for 15 min at room temperature. The column was then spun again at 1000g to collect the tryptic peptides. The same procedure was followed for labeling the peptides, but all buffer solutions used were made with ^{18}O water. The labeled and unlabeled peptides were mixed in 1:1 ratio before the analysis by mass spectrometry.

Nano-LC-ESI Mass Spectrometry

The protein digest was analyzed using an ion trap LTQ mass spectrometer interfaced with a nano-LC system. The samples were loaded through an autosampler onto a C_{18} capillary column. The solvents A and B used for chromatographic separation of peptides were 5% acetonitrile in 0.1% formic acid and 95% acetonitrile in 0.1% formic acid, respectively. The peptides injected onto the microcapillary column were resolved at the rate of 200 nL/min, by the following gradient conditions: 0–30 min 0–5% B, 30–180 min 5–35% B, 180–240 min 35–65% B, 240–250 min 65–100% B; 100% B was held for 10 min, then switched to 100% A and held for another 40 min.

The ions eluted from the column were electrosprayed at a voltage of 1.8 kV. The capillary voltage was 45 V and the temperature was kept at 200 $^{\circ}\text{C}$. No auxiliary or sheath gas was used. Helium was used in the trap, which was also used as a collision gas for fragmentation of ions. The AGC target values for the ion trap were set at 3×10^4 ions in MS scan mode, 1×10^4 ions in MS/MS mode and 3×10^3 in Zoom Scan mode. Data was acquired in the triple play data dependent mode, with a full scan spectrum (400–2000 m/z) followed by zoom scan and a MS/MS scan of the most abundant peak from the full scan spectrum. Dynamic exclusion was enabled for 30 s. The chromatographic and mass spectral functions were controlled by the Xcalibur data system (ThermoFinnigan, Palo Alto, CA).

The MS data obtained were searched using the SEQUEST²³ algorithm against Uniprot Rodent database v49.1. The search was limited to only tryptic peptides, and identifications were filtered from the search results using the Eptomize program.²⁴ Eptomize reads all the SEQUEST.out files in a directory, filters the files based on user-defined levels of Xcorr, and outputs the proteins identified. The Xcorr versus charge state filter used was set to Xcorr values of 1.8, 2.5 and 3.0 for charge states +1, +2 and +3, respectively. These filter values are similar to others previously reported for SEQUEST analyses.^{25,26} Protein hits that passed the filter were annotated using the generic GO slim. All proteins were identified by two or more peptides, and those identified with a single peptide were included in the analysis only if identified in two or more scans. Finally, the peptides listed were manually verified for correct identification by comparing the experimental spectra with the theoretical *b* and *y* ion spectra.

Quantification of the ^{18}O Labeled Peptides

The labeled peptides were quantified with the computational tool ZoomQuant (version 1.4)^{22,24} that analyzes the mass spectra of ^{18}O labeled peptides from ion trap instruments and determines relative abundance ratios between two samples. The tool takes the SEQUEST results file and the .zcn file extracting the zoom scans from raw data obtained by the MS, and compares the ratios of the peptides labeled. It also generates a summary report of the relative abundance of the peptides identified in the two samples. The detailed description of ratios and labeling efficiency calculation by ZoomQuant is reported in a previous work by our group.²⁴

Results

Comparison of Spin Column versus In-Solution ^{18}O Labeling Methods

Three sets of protein mixture (BSA, GAPDH, LALBA, MYG) samples were prepared such that one was digested using trypsin spin columns for 15 min (experiment 1), the other two sets digested in-solution using trypsin gold for 15 min (experiment 2) and overnight (experiment 3) (Figure 1). Each set of samples included two aliquots of equal concentration of proteins, one aliquot being ^{18}O labeled, and the other ^{16}O labeled (unlabeled). All three sets of samples were mixed in 1:1 ratio and analyzed using nano-HPLC-mass spectrometry in a triple play data-dependent mode. Proteins were identified using SEQUEST algorithm and quantified for ^{18}O incorporation using ZoomQuant as described. The results show that maximum ^{18}O incorporation is observed in samples labeled using trypsin spin columns within 15 min only, which is similar to the incorporation observed in samples digested according to standard protocol (overnight in-solution), as measured by $^{18}\text{O}/^{16}\text{O}$ ratios and the efficiency of labeling (Table 1). The ratio of labeled to unlabeled peptide is calculated as $R = (A_1 + A_2)/A_0$; and the efficiency of labeling is a measure of ^{18}O incorporation that is calculated by the equation $E = A_2/(A_1 + A_2)$, as measured by ZoomQuant,²⁴ where A_0 is the peak area of the unlabeled species, A_1 is the area of the peak with partial labeling due to the exchange of only one of the carboxyl oxygen atoms and A_2 is the peak area of the fully labeled species due to exchange of both the carboxyl oxygen atoms with ^{18}O at the C-terminus of the tryptic peptide.

As can be seen in Table 1, the enzyme-mediated ^{18}O substitution is highest in experiments 1 and 3 with an efficiency ranging from 50–60%, whereas in experiment 2 (short digestion in-solution), ^{18}O incorporation is not complete as observed from the $^{18}\text{O}/^{16}\text{O}$ ratios and labeling efficiency. As a result, the number of peptides identified was almost equal in experiments 1 and 3, but less in experiment 2 due to incomplete digestion (data not shown).

The ratios of peptides obtained from each of the four proteins in experiment 1 using trypsin spin columns are listed in Table 2. The ^{18}O labeled protein mix was added in equal amount to the ^{16}O tryptic digest (unlabeled sample), and hence, the ratio is expected to be 1.0. It shows (Table 2) that the ratios are close to 1 for almost all the peptides identified from each of the four proteins. All ratios and the efficiency of labeling shown are an average of ratios obtained for each of the peptides in multiple scans.

For a better evaluation of the results, a paired *t* test was performed to verify the statistical significance of the difference in the $^{18}\text{O}/^{16}\text{O}$ ratios and the labeling efficiency in the three experiments with spin-columns for 15 min, in-solution digestion for 15 min and overnight incubation. No significant difference was detected between the $^{18}\text{O}/^{16}\text{O}$ ratios obtained from experiments using spin columns and in-solution overnight incubation methods ($P = 0.53$), and the labeling efficiency for the two approaches is only marginally significantly different ($P = 0.05$). However, both the ratios and labeling efficiency are significantly different when compared with the in-solution digestion method with short incubation period ($P < 10^{-5}$).

Applicability to Complex Protein Mixtures: Analysis of Vascular Endothelial Cell Lysate

After the successful initial experiments with a simple four protein mixture, we demonstrated the applicability of the method to a complex protein lysate. In this study, we used whole cell lysate of rat vascular endothelial cells. Approximately 50 μg of the whole cell lysate was digested and analyzed. Three aliquots of cell sample were digested, one using trypsin spin columns and the other two sets digested in-solution for 15 min and overnight. The ^{18}O labeled sample was mixed in equal amounts with unlabeled sample and analyzed by nano-LC tandem mass spectrometry.

The total mean ratio of $^{18}\text{O}/^{16}\text{O}$ for a 1:1 mixture of peptides that are labeled by enzyme-mediated ^{18}O incorporation using trypsin spin columns was 1.04, whereas in-solution digestion for the same time resulted in a mean ratio of 0.56 only. The efficiency of labeling as measured by ZoomQuant is 0.44 and 0.19, respectively, for these two experiments. The total mean of the peptide ratios for the experiment carried out by the traditional overnight in-solution trypsin digestion method was 0.96 with an efficiency of 0.42. This is indistinguishable from the results obtained using the spin columns. A peptide (LGD-LYEEEMR) from vimentin is shown in Figure 2 as an example for the comparison of the ratios obtained in all three experiments. The $^{18}\text{O}/^{16}\text{O}$ ratios of all the peptides obtained in all three experiments are plotted (Figure 3). In experiments carried out with trypsin spin columns and by traditional overnight in-solution digestion methods, the ratios are centered at 1.0, whereas those obtained from 15 min in-solution digestion method fall on the lower end of the ratios (<1). This clearly demonstrates that the fast ^{18}O labeling method by trypsin spin columns can be used for differential protein expression studies using complex protein mixtures with results comparable to traditional overnight protocols.

As observed in the four protein mixture experiments, there is no significant difference between the $^{18}\text{O}/^{16}\text{O}$ ratios obtained from experiments using spin columns and in-solution overnight incubation methods ($P < 0.52$) when compared with the ratios obtained for the same set of peptides by both the methods. To further evaluate the method of labeling using spin columns, the standard deviation values are calculated between the ratios obtained by spin column labeling and in-solution overnight labeling methods. In general, both the labeling methods show 20% standard deviation between the ratios obtained by two consecutive experiments using the same methodology. It has been observed that only 14% of the peptides are observed to fall above the standard deviation (ratio $\geq (\pm 0.2)$) and no more than 4% of the peptides fall above 2 times the standard deviation (ratio $\geq (\pm 0.4)$) illustrating that the efficiency of fast labeling method is comparable to the overnight labeling method. To further dissect the similarity of results across a wide range of $^{18}\text{O}/^{16}\text{O}$ ratios, we compared the observed ratios in the two experiments for significantly up- and down-regulated proteins. SPARC precursor protein is found to be up-regulated more than 2 times as observed by both the methods with ratios 2.32 ± 0.17 and 2.41 ± 0.08 , respectively (Figure 4). The protein thioredoxin is down-regulated as shown by the ratios 0.61 ± 0.06 and 0.58 ± 0.08 for the two methods using spin columns for 15 min and overnight in-solution labeling, respectively (Figure 4).

Discussion

Several methods of relative quantification have been established for differential protein expression analysis. Of these, ^{18}O labeling is a relatively inexpensive and simple method for the quantification of proteins. Though the method has limitations such as the time-consuming labeling step, the back-exchange of ^{18}O with ^{16}O , and the difficulty in measuring the labeled peptides due to lack of computational tools, the method is ideal for differential protein expression studies due to its applicability to a wide range of experiments including global analysis studies. Computational tools have been recently developed by our group,²⁴ and the other two issues are remedied by the current studies, as discussed below.

In this study, we focused on decreasing the time required to label peptides with high efficiency. We demonstrate that the peptides can be labeled within 15 min with similar labeling efficiency as the well-established traditional long incubation method for ^{18}O labeling. The ultramicro spin columns (Sigma-Aldrich Co.) contain highly purified, TPCK-treated porcine trypsin immobilized on a spherical $20\ \mu\text{m}$ silica support, chemically modified to minimize nonspecific adsorption. By accelerating the protein digestion, the immobilized trypsin improves the two atom ^{18}O incorporation and the labeling efficiency by increasing the molar ratio of protease-to-substrate.

Besides efficient and fast labeling, there are other advantages of using trypsin spin columns for enzyme-mediated ^{18}O labeling. In general, the protease concentration is increased during enzymatic digestion resulting in increased labeling efficiency. However, the excess of enzyme can initiate back-exchange of the ^{18}O with ^{16}O atoms, another limitation in the labeling method. To avoid back-exchange in the reaction, the excess protease should either be inactivated or removed from the reaction mixture. Traditionally, after enzymatic digestion, the activity of trypsin is reduced by the addition of organic acids like formic acid or trifluoroacetic acid. However, this is challenging as excess addition of acid can initiate acid hydrolysis which further leads to ^{18}O replacement. Consequently, a method of removal of trypsin from the reaction mixture would be helpful to stop the undesired back-exchange. Immobilized trypsin can be easily removed from the peptide mixture after digesting the sample. Trypsin immobilized on beads can be separated from the sample mixture after digestion by either centrifugation or by filtration as demonstrated by Sevnisky et. al.²⁰ However, the use of immobilized trypsin beads adds an extra step to the experimental procedure for sample separation from the trypsin and, hence, leads to increased variability of the results. Spin columns are advantageous due to easy elution of peptides without any further extra steps in experimental protocol. The sample is digested on immobilized trypsin in a column with septa to hold the column bed. The tryptic digest is eluted into a clean tube without any contamination from active trypsin into the eluent. We observed no change in the $^{18}\text{O}/^{16}\text{O}$ ratios for up to 12 h after elution and only a 20% of reduction in the ratios is observed even after 24 h for the samples that are left in the autosampler overnight at 8 °C (data not shown).

The results obtained in our study show that the method using trypsin spin columns for ^{18}O labeling is highly efficient and comparable to the established methods of labeling. In addition, it is very fast. We successfully achieved two atom ^{18}O incorporation using trypsin spin columns for labeling with minimal back-exchange. In combination with our specifically developed software tools, this quick and efficient labeling approach overcomes the cumbersome long incubation times and undesired back exchange, resulting in a rapid, efficient and reliable proteomic quantification methodology.

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Figure 1.
Workflow of the ^{18}O labeling experiments carried out by different methods using trypsin spin columns and in-solution digestion/labeling.

Figure 2.

A vimentin peptide (LGDLYEEEMR) identified and quantified by three different experiments: (1) using trypsin spin columns for 15 min, (2) in-solution digestion for 15 min and (3) overnight in-solution digestion. The left panel (a) depicts the zoom scan spectra obtained from raw MS files and the right panel (b) shows the quantified spectra from ZoomQuant. The peptide represented is +2 charged; and the color-shading shows the areas of isotope peaks as determined by ZoomQuant.

Figure 3.

Histogram depicting $^{18}\text{O}/^{16}\text{O}$ ratios obtained from the experiments carried out using the rat vascular endothelial cell lysate by using ultramicro spin columns for 15 min (SC-15 min), in-solution digestion for 15 min (IS-15 min) and in-solution digestion overnight (IS-O/N). The labeled samples are mixed with equal amounts of unlabeled sample before mass spectral analysis.

Figure 4. $^{18}\text{O}/^{16}\text{O}$ ratios calculated by ZoomQuant software representing the peptide TFDSSCHFFATK from SPARC protein labeled using (1a) spin columns for 15 min and (1b) in-solution overnight; and the peptide EAFQEALAAAGDK from Thioredoxin labeled using (2a) spin columns for 15 min and (2b) overnight in-solution method.

Table 1

Ratios of ¹⁸O/¹⁶O Peptides Obtained from Experiments Using Ultra-Micro Spin Columns and Trypsin Gold for 15 min and Overnight Incubation

protein ^a	spin column (15 min incubation)		in-solution (15 min incubation)		in-solution (overnight incubation)	
	ratio	efficiency	ratio	efficiency	ratio	efficiency
BSA	1.10 ± 0.2	0.56	0.55 ± 0.3	0.20	1.12 ± 0.3	0.48
GAPDH	1.18 ± 0.2	0.61	0.47 ± 0.2	0.18	1.02 ± 0.3	0.51
LALBA	1.08 ± 0.3	0.53	0.45 ± 0.3	0.12	1.17 ± 0.2	0.50
MYG	0.99 ± 0.3	0.59	0.91 ± 0.1	0.45	1.23 ± 0.3	0.61

^aBSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LALBA, α-lactalbumin; MYG, myoglobin.

Table 2

The $^{18}\text{O}/^{16}\text{O}$ Ratios of the Tryptic Peptides Obtained from the Four Protein Mixtures Digested Using Ultra-Micro Spin Columns for 15 minutes^a

peptide sequence	ratio	efficiency
Serum Albumin		
CCTESLVNR	1.15	0.66
GLVLIAFSQYLQQCPFDEHVK	1.00	0.34
KVPQVSTPTLVEVSR	1.14	0.55
LGEYGFQNALIVR	1.22	0.60
RPCFSALTPDETYVPK	1.50	0.58
SLHTLFGDELCK	1.04	0.55
YICDNQDTISSK	1.18	0.54
HLVDEPQNLIK	0.99	0.59
VPQVSTPTLVEVSR	0.80	0.37
LVNELTEFAK	0.86	0.36
LKPDPTLTCDEFK	1.39	0.57
RHPEYAVSVLLR	0.89	0.35
<i>Protein Mean</i>	1.10	0.56
<i>Standard Deviation</i>	0.20	0.11
α-Lactalbumin		
ALCSEKLDQWLCEK	1.27	0.59
DDQNPSSDICNISCDK	1.12	0.47
VGINYWLAHK	0.99	0.56
LDQWLCEK	1.04	0.56
<i>Protein Mean</i>	1.18	0.61
<i>Standard Deviation</i>	0.28	0.14
Myoglobin		
GLSDGEWQQVLNVWGK	1.19	0.56
HGTVVLTALGGILK	1.06	0.70
VEADIAGHGQEVLR	0.79	0.45
YLEFISDAIIHVLHSK	0.87	0.49
<i>Protein Mean</i>	0.99	0.59
<i>Standard Deviation</i>	0.25	0.14
Glyceraldehyde-3-phosphate		
GAAQNIIPASTGAAK	1.28	0.75
AITIFQER	0.78	0.41
LISWYDNEFGYSNR	0.90	0.76
VPTPNVSVVDLTCR	1.08	0.61
<i>Protein Mean</i>	1.18	0.61
<i>Standard Deviation</i>	0.23	0.17

^aThe tryptic peptides are mixed 1:1 with unlabeled sample and analyzed by triple play data dependent mode using nano-LC mass spectrometer.