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A Novel Method for Relative Quantitation of N-Glycans by Isotopic Labeling Using ^{18}O-Water

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Quantitation is an essential aspect of comprehensive glycomics study. Here, a novel isotopic-labeling method is described for N-glycan quantitation using ^{18}O-water. The incorporation of the ^{18}O-labeling into the reducing end of N-glycans is simply and efficiently achieved during peptide-N4-(N-acetyl-β-glucosaminyl) asparagine amidase F release. This process provides a 2-Da mass difference compared with the N-glycans released in ^{16}O-water. A mathematical calculation method was also developed to determine the ^{18}O/^{16}O ratios from isotopic peaks. Application of this method to several standard glycoprotein mixtures and human serum demonstrated that this method can facilitate the relative quantitation of N-glycans over a linear dynamic range of two orders, with high accuracy and reproducibility.

KEY WORDS: mass spectrometry, tandem mass spectrometry, glycomics, glycoprotein analysis

INTRODUCTION

Glycosylation, a ubiquitous post-translational modification, can significantly affect numerous biological processes, such as protein stability/activity, recognition by antibodies, susceptibility to proteases, and binding specificity.[1] Previous studies indicate that changes in glycosylation are associated with many common human health issues, including cancer, inflammation, neurodegenerative disease, and congenital disorder of glycosylation.[2] To identify disease-related glycosylation changes and discover potentially useful biomarkers,[3] numerous researchers have devoted great effort to characterizing protein glycosylation. Recently, the study of glycomics has undergone a rapid development because of advances in analytical techniques. Mass spectrometry (MS) has become a highly informative analytical tool to provide structure and quantitative measures for glycomics as a result of its high sensitivity, resolution, and mass accuracy.[4–6]

Permethylation of released glycans is a common sample derivatizations before MS analysis, as this procedure offers several benefits for both structural and quantitative analysis. Permethylation converts all of the highly polar -OH, -COOH, and NH- groups into nonpolar derivatives. This change in polarity enables both acidic and neutral glycans to be analyzed in positive-ion mode, leads to more uniform ionization, and also stabilizes sialic acid residues.[7] In addition, permethylated glycans have more predictable fragmentation patterns in MS/MS.[8]

Permethylation can be used to introduce various stable isotopic labels for quantitative glycomics, such as $^{12}CH_3I$/$^{13}CH_3I$, $^{13}CH_3I$/$^{12}CH_2DI$, and others. Alvarez-Manilla et al.[9] reported the $^{12}CH_3I$/$^{13}CH_3I$-labeling method for relative quantitation of glycans. However, this approach may not be appropriate for extremely complex samples, as it increases the spectral complexity.[10] Orlando and coworkers[10] developed a quantitation method by isobaric labeling (QUIBL), which generates isobaric pairs of permethylated glycans with $^{13}CH_3I$/$^{12}CH_2DI$. As the mass difference between those isobaric pairs is so small that it can only be differentiated at high resolution, QUIBL can successfully achieve relative quantitation without causing difficulty in matching the heavy/light pairs. Moreover, it enables the relative quantitation of individual glycans in isomeric mixtures. However, there are several potential issues generated during permethylation that may cause inaccurate quantitation. Namely, samples treated separately may have different yields. Another issue to permethylation is that large errors can be introduced by small variations in labeling efficiency because of the large number of methylation sites on each glycan.[11]

As a common stable isotope, ^{18}O has been used for several different applications. The labeling with ^{18}O has been used for relative proteomic quantitation.[12,13] With glycoproteomics, the ^{18}O-labeling during enzymatic deglycosylation permits sites of N-glycosylation on the protein

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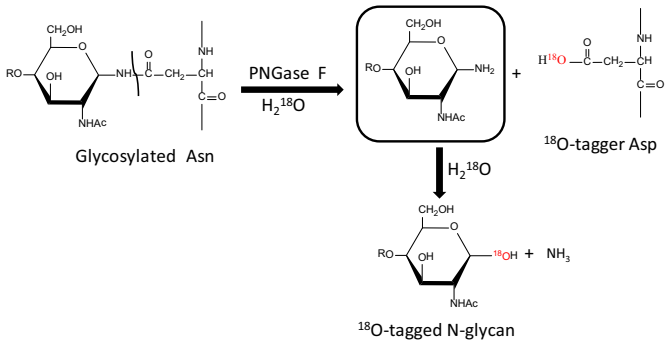

FIGURE 1

^{18}O incorporation during PNGase F deglycosylation. During the initial step of the enzymatic deglycosylation, the C–N bond of glycosylated asparagine (Asn) side-chain is cleaved, releasing a β-glycosylamine, which is not stable and spontaneously hydrolyzes to release ammonia and leave a hydroxyl moiety on the glycan. Asp, Aspartic acid.

to be identified.[14] Furthermore, ^{18}O-labeling of the reducing terminus can aid in the structural analysis of oligosaccharides by breaking the symmetry between the two termini.[15,16]

Here, we describe a simple isotope-labeling procedure to incorporate ^{18}O-labeling into the reducing end of N-glycans during peptide-N4-(N-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F) release. This procedure gives a 2-Da mass shift for the heavily labeled species and can be used for N-glycan relative quantification without altering the normal sample workflow. Furthermore, a new mathematical calculation method is described to determine the ^{18}O/^{16}O ratios from isotopic peaks. Lastly, this method was used in the analysis of several standard glycoproteins and human serum, which demonstrated that this method can facilitate the relative quantitation of N-glycans over a linear dynamic range of two orders, with high accuracy and reproducibility.

MATERIALS AND METHODS

Materials

Bovine fetuin (F) and human serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). ^{18}O-water (97 atom percent) was purchased from Sigma-Aldrich. Trypsin (Tosyl phenylalanyl chloromethyl ketone [TPCK] treated) was purchased from Sigma-Aldrich. PNGase F (glycerol free) was purchased from New England Biolabs (NEB; Ipswich, MA, USA). Iodomethane (CH_3I; reagent plus grade) was purchased from Sigma-Aldrich. Sep-Pak C18 columns were purchased from J. T. Baker (Avantor Performance Materials, Center Valley, PA, USA). All chemicals were of analytical grade.

Protein Digestion

F (200 μg) was dissolved in 200 μL 50 mM ammonium bicarbonate (AmBic), and human serum (200 μL aliquot) was mixed with 200 μL AmBic; then, both samples were heated at 100°C for 5 min to denature the proteins. After

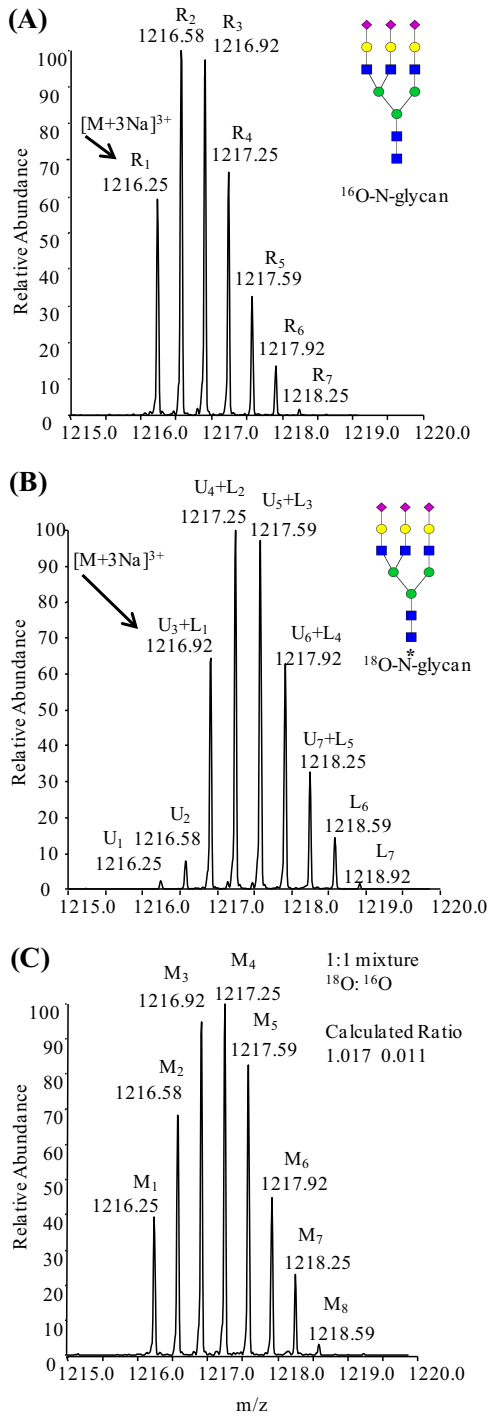

FIGURE 2

FT-ICR spectra of permethylated triantennary glycans from F. (*A*) ^{16}O-N-glycan; (*B*) ^{18}O-labeled N-glycan; (*C*) 1:1 mixture of regular ^{16}O-N-glycan and ^{18}O-labeled N-glycan.

cooling to room temperature, samples were digested at 37°C for 16~18 h with an appropriate amount of trypsin (50 μg trypsin for 1 mg glycoprotein sample).

N-Glycan Release and ^{18}O-Labeling

Each of the samples was divided into two equal aliquots and then dried using a SpeedVac centrifuge. One aliquot was redissolved in 100 μL $H_2{}^{18}O$, and the other aliquot was redissolved in 100 μL $H_2{}^{16}O$. Equal amounts of PNGase F were added into both aliquots (PNGase F from NEB was dried and then redissolved in $H_2{}^{18}O$ or $H_2{}^{16}O$ before use and 20 IUB mU PNGase F for 1 mg glycoprotein. Where 1 U is defined as the amount of enzyme required to remove > 95% of the carbohydrate from 10 μg of denatured RNase B in 1 hour at 37°C in a total reaction volume of 10 μl.). The release of N-glycans was carried out at 37°C for 16~18 h.

N-Glycan Isolation and ^{18}O/^{16}O-Labeling N-Glycan Mixture Preparation

N-Glycans were separated from peptides by reverse-phase liquid chromatography. Each of the PNGase F-digested samples was loaded onto a Sep-Pak C18 column, which had been pre-equilibrated in 5% acetic acid, and the N-glycans were eluted from the column with 4 mL 5% acetic acid. The following ^{18}O-labeled/^{16}O-N-glycan mixtures at various ratios of 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:10 were prepared for N-glycans from the F samples. For N-glycans from the human serum samples, ^{18}O-labeled/^{16}O-N-glycans were mixed at a ratio of 1:1. The solutions of mixture were frozen immediately with dry ice and acetone and lyophilized to dryness.

^{18}O-Labeled/^{16}O-N-Glycan Mixture Permethylation

The N-glycan permethylation for each mixture was performed as described previously.[9] Briefly, each of the dried N-glycan mixtures was stirred to dissolve in 200 μL DMSO and then 200 μL freshly prepared base (200 mg NaOH in 1 mL dry DMSO), and 150 μL dry CH_3I was added. After stirring vigorously for 10 min, followed by sonication for another 10 min, the methylation reaction was stopped by adding 2 mL water. The permethylated N-glycan mixtures were extracted with dichloromethane and dried under N_2 gas.

Permethylated Sample Clean-Up

The permethylated N-glycan mixtures were cleaned by reverse-phase chromatography. Each sample was dissolved in 200 μL 50% methanol and loaded onto a Sep-Pak C18

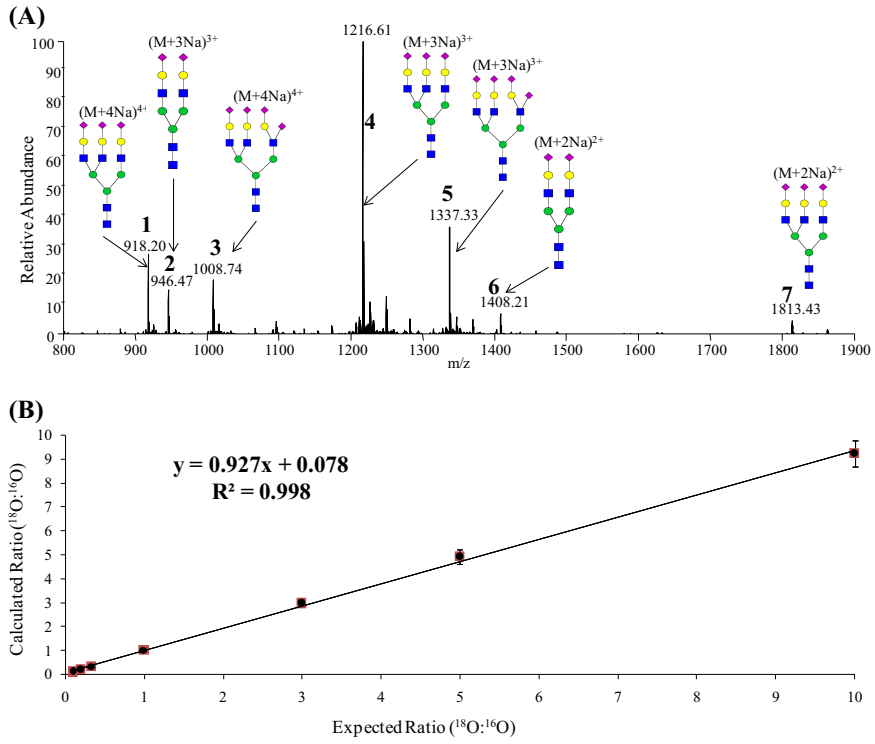

FIGURE 3

(*A*) Full MS spectrum of ^{16}O-N-glycans. (*B*) The experimentally determined ^{18}O/^{16}O ratio is plotted versus those expected from the sample mixture. The ratios of the ^{18}O- to ^{16}O-labeled glycans were measured for the seven major F N-glycan peaks and averaged for a variety of mixtures. The error bar represents the SD among those seven N-glycan peaks. A plot of the expected ratio to that obtained experimentally yielded a straight line (R^2=0.9988).

column, which had been washed with 3 mL methanol and pre-equilibrated in 5% acetic acid. After washing five times with Nanopure H_2O, the permethylated N-glycans were eluted with 2 mL 85% acetonitrile and dried under N_2 gas.

MS Analysis of the Permethylated ^{18}O/^{16}O-Labeling N-Glycan Mixture

The cleaned sample mixtures were dissolved in 100 μL 50% methanol in 1 mM NaOH and analyzed independently in triplicate. The MS analysis was carried out on a hybrid linear ion trap Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (LTQ-FT; Thermo Scientific, Waltham, MA, USA) by direct infusion. Each glycan mixture was infused into the LTQ-FT at a flow rate of 1 μL/min. For relative quantitation of interested N-glycans, the MS scan was performed with a narrow isolation window [10 mass-to-charge ratio (m/z) centered on the analyte ion] by a FT-ICR mass spectrometer at 100,000 resolution.

RESULTS AND DISCUSSION

PNGase F is an amidase that cleaves the glycan-asparagine amino bond of the glycosylated asparagine side-chain, which releases intact N-glycans. During this process, the asparagine residue is converted to aspartic acid, whereas the N-glycans are released initially as a β-glycosylamine. Under the enzymatic deglycosylation condition (pH 7), the β-glycosylamine spontaneously hydrolyzes to a hydroxyl moiety liberating ammonia.[17] Thus, the ^{18}O-labeling can be incorporated into the reducing terminus during the enzymatic release of the N-glycans (Fig. 1)

As the water-^{18}O used to introduce the isotopic label into the reducing terminus is 97% ^{18}O, there is a measurable amount of ^{16}O-labeled glycan. Furthermore, the ^{18}O-label may be lost during the separation step, as $H_2{}^{16}O$ is used to elute N-glycans from the Sep-Pak C18 column, and thus, the ^{18}O-label may exchange with ^{16}O during the ring opening and closing events associated with mutarotation.[18] Both of these factors lead to an appreciable, unlabeled species being present, even in a completely heavy labeled sample.

The incorporation of ^{18}O-labeling gives a 2-Da mass shift in mass (Fig. 2A and B), and thus, several of the peaks from the natural abundance of ^{13}C in the unlabeled species overlap with peaks from the ^{18}O-labeled species. For calculation of the under-labeling (UL) ratio, the percentage (p) of the first two isotopic peaks divided by the summed intensity of all of the isotopic peaks observed solely from the ^{16}O-N-glycan was determined for the ^{16}O-labeled glycans.

$$p = \frac{R1 + R2}{\sum_{i=1}^{n} R_i} \qquad \text{(Equation 1)}$$

$$\mathrm{UL} = \frac{\frac{U1 + U2}{p}}{\sum_{i=1}^{n} Ui + \sum_{i=1}^{n} Li} \qquad \text{(Equation 2)}$$

Here, p is of the summed intensity of the first two isotopic peaks divided by the summed intensity of all of the isotopic peaks; Ri is the intensity of the ith isotopic peak of the ^{16}O-N-glycan; Ui is the intensity of the ith isotopic peak of UL N-glycans; and Li is the intensity of the ith isotopic peak of ^{18}O-labeled N-glycans.

For the ^{16}O and ^{18}O mixture,

$$\begin{cases} \mathrm{X} + \mathrm{Y} = \sum_{i=1}^{n} Mi \\ \mathrm{X} + \mathrm{Y} * \mathrm{UL} = \frac{M1 + M2}{p} \end{cases}$$

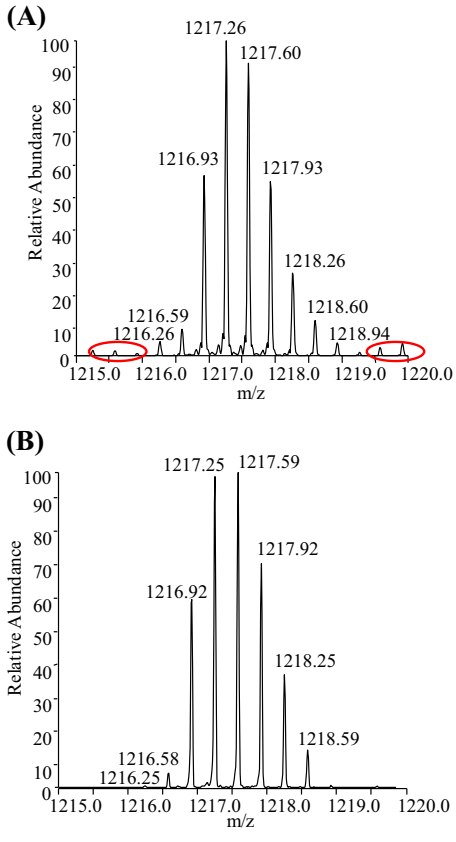

FIGURE 4

Comparison of isolation window size on quantitation. (*A*) Expanded region of spectrum for the triantennary ^{18}O-labeled N-glycan obtained with a full MS scan (500~2000 m/z) and (*B*) that obtained for the same glycan with a narrow isolation window (10 m/z centered on the analyte ion).

So,

$$Y/X = \frac{\sum_{i=1}^{n} Mi - \frac{M1 + M2}{p}}{\frac{M1 + M2}{p} - \sum_{i=1}^{n} Mi * UL} \quad \text{(Equation 3)}$$

Here, X stands for the signal intensity of the ^{16}O-N-glycan; Y stands for the signal intensity of the ^{18}O-N-glycan; M*i* s the relative intensity of the *i*th peak from the ^{16}O- and ^{18}O-N-glycan mixture; and Y/X is the ratio of ^{18}O /^{16}O.

To demonstrate the mathematical calculation of the ^{18}O/^{16}O ratio, one of the triantennary glycans from F was examined at a sample ratio of 1:1. The FT-ICR spectra for a ^{16}O-N-glycan and an ^{18}O-N-glycan are shown in Fig. 2A and B, respectively. With the mathematical calculation method described above, the UL ratio was 0.056 (see Supplemental information for example of mathematical calculation of ^{18}O /^{16}O ratio). The average ratio obtained by applying this method to a standard 1:1 mixture was 1.017 ± 0.011 for the triantennary F glycans.

To evaluate the linearity of response obtained by the ^{18}O-labeling method, seven standard mixtures were prepared by combining F glycans of ^{18}O /^{16}O ratios at 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:10 (Supplemental Fig. 1). For the most abundant peaks from the F sample, the maximum error was <15%. Specifically, when the sample mixture ratio was 1:1, the maximum error was <8% for all of the analyzed N-glycans from F. However, the SD and SE became larger as the ratio increased to 10:1 or decreased to 1:10, resulting from the difficulty associated with differentiating the ^{16}O peaks from those resulting from the labeled ^{18}O-N-glycans. The linearity of response was evaluated with seven peaks from F (Fig. 3A). All seven peaks were measured at each ^{18}O/^{16}O ratio separately. The average of those seven peaks at each dilution was calculated and compared with the theoretical ratio (Fig. 3B).

Effect of Isolation Window Size on Quantitation

The size of the isolation window is expected to have a significant effect on the accuracy of relative quantitation as a result of the automatic gain control used on this instrument, which attempts to fill the trap with the same number of ions in the m/z range selected. Hence, the decrease in the size of the isolation window is expected to increase the number of ions per m/z unit, which in turn, should provide better ion statistics, particularly with low abundance ions. The increased ion density arrives at a cost of increasing the time that ions are permitted to enter the trap. To evaluate the effect of the isolation window size, data were acquired in two different modes: full MS scan mode with a mass range of 500–2000 m/z and with an isolation window of 10 m/z centered on the analyte ion. Figure 4A shows the region of the full MS scan for the triantennary N-glycan from F with ^{18}O-labeling. Figure 4B shows the data acquired with the narrow isolation window for the same N-glycan. The improved ion statistics can readily be observed by the intensity of the background noise (indicated in the ellipses of Fig. 4A) being lower with the narrow isolation window (Fig. 4B). The increased background in the data acquired in full MS mode causes an overestimation of the UL ratio and therefore, leads to errors in the ^{18}O/^{16}O ratios.

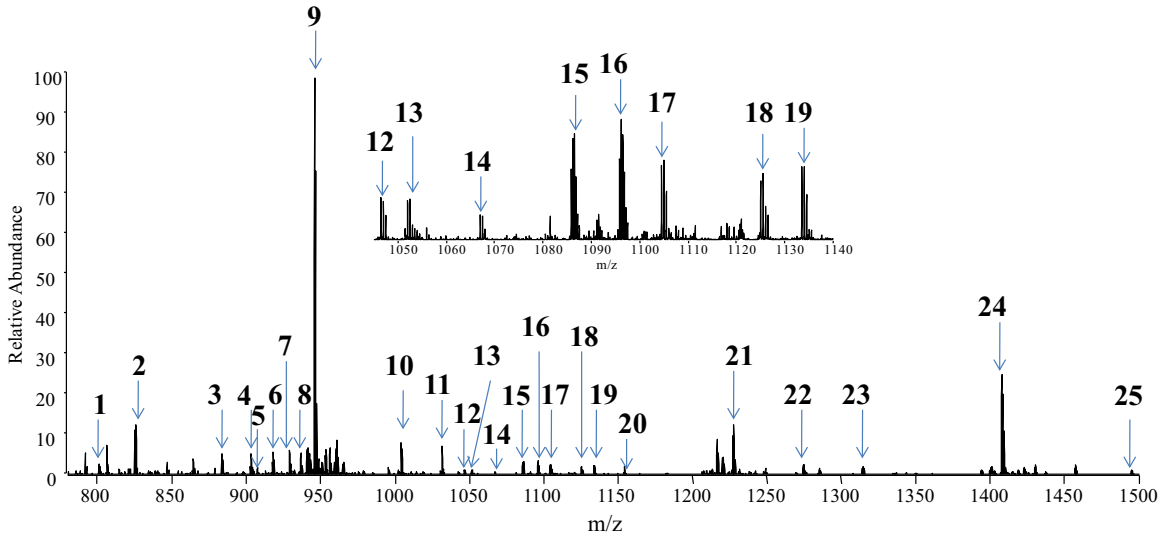

FIGURE 5

Full MS scan of human serum N-glycan mixture (^{18}O/^{16}O=1:1). The 25 labeled peaks corresponded to m/z values of known N-linked glycans and were used for quantitative analysis.

TABLE 1

Quantitation of Human Serum N-Glycans by the ^{18}O-Labeling Method

Peak	Charge state	N-Glycan composition	Calculated ratio (average ± SD)	Expected ratio	Error (%)
1	$[M+2Na]^{2+}$	$HexNAc_2Hex_5$	1.009 ± 0.014	1	0.9
2	$[M+3Na]^{3+}$	$HexNAc_4NeuAc_1Hex_5$	1.066 ± 0.019	1	6.6
3	$[M+3Na]^{3+}$	$Deoxyhex_1HexNAc_4NeuAc_1Hex_5$	1.004 ± 0.019	1	0.4
4	$[M+4Na]^{4+}$	$HexNAc_5NeuAc_3Hex_6$	1.028 ± 0.041	1	2.8
5	$[M+3Na]^{3+}$	$Deoxyhex_2HexNAc_5Hex_5$	1.012 ± 0.006	1	1.2
6	$[M+3Na]^{3+}$	$HexNAc_5NeuAc_1Hex_5$	1.005 ± 0.021	1	0.5
7	$[M+2Na]^{2+}$	$Deoxyhex_1HexNAc_4Hex_3$	1.015 ± 0.021	1	1.5
8	$[M+3Na]^{3+}$	$HexNAc_6Hex_6$	1.005 ± 0.011	1	0.5
9	$[M+3Na]^{3+}$	$HexNAc_4NeuAc_2Hex_5$	0.997 ± 0.007	1	0.3
10	$[M+3Na]^{3+}$	$Deoxyhex_1HexNAc_4NeuAc_2Hex_5$	0.996 ± 0.007	1	0.4
11	$[M+2Na]^{2+}$	$Deoxyhex_1HexNAc_4Hex_4$	1.054 ± 0.015	1	5.4
12	$[M+2Na]^{2+}$	$HexNAc_4Hex_5$	1.075 ± 0.012	1	7.5
13	$[M+2Na]^{2+}$	$Deoxyhex_1HexNAc_5Hex_3$	0.988 ± 0.007	1	1.2
14	$[M+2Na]^{2+}$	$HexNAc_5Hex_4$	1.031 ± 0.011	1	3.1
15	$[M+3Na]^{3+}$	$Deoxyhex_1HexNAc_5NeuAc_2Hex_5$	0.989 ± 0.019	1	1.1
16	$[M+3Na]^{3+}$	$HexNAc_5NeuAc_2Hex_6$	0.959 ± 0.018	1	4.1
17	$[M+2Na]^{2+}$	$HexNAc_3NeuAc_1Hex_5$	0.960 ± 0.008	1	4.0
18	$[M+2Na]^{2+}$	$HexNAc_4NeuAc_1Hex_4$	0.987 ± 0.015	1	1.3
19	$[M+2Na]^{2+}$	$Deoxyhex_1HexNAc_4Hex_5$	1.045 ± 0.021	1	4.5
20	$[M+2Na]^{2+}$	$Deoxyhex_1HexNAc_5Hex_4$	0.963 ± 0.017	1	3.7
21	$[M+3Na]^{3+}$	$HexNAc_4NeuAc_1Hex_5$	1.048 ± 0.002	1	4.8
22	$[M+3Na]^{3+}$	$Deoxyhex_1HexNAc_5NeuAc_3Hex_6$	0.980 ± 0.022	1	2.0
23	$[M+2Na]^{2+}$	$Deoxyhex_1HexNAc_4NeuAc_1Hex_5$	1.040 ± 0.020	1	4.0
24	$[M+2Na]^{2+}$	$HexNAc_4NeuAc_2Hex_5$	1.042 ± 0.004	1	4.2
25	$[M+2Na]^{2+}$	$Deoxyhex_1HexNAc_4NeuAc_2Hex_5$	1.008 ± 0.004	1	0.8

For example, the UL calculated with the full MS scan mode was 0.083 (Fig. 4A), which gave an $^{18}O/^{16}O$ ratio of 1.145 (14.5% error) for a 1:1 sample mixture. The UL calculated for the same glycan with a narrow isolation window was 0.056, which gave an $^{18}O/^{16}O$ ratio of 1.012 (1.2% error) for the same sample mixture. With the 1:10 mixture, the background in full MS scan mode caused an error of 13.19 (31.9%). In comparison, the maximum error was <15% for all data obtained over the two orders of magnitude range evaluated when the instrument was operated with a narrow isolation window. Therefore, data acquired with a narrow isolation window are superior to full scan data for relative quantitation on the LTQ-FT mass spectrometer, and this approach was used for all further quantitative experiments.

^{18}O-Labeling Application to Human Serum N-Glycans

We evaluated the ^{18}O-labeling scheme for relative quantitation with the N-glycans released from human serum. Specifically, the ^{16}O-N-glycans released by PNGase F in regular H_2O and ^{18}O-labeled N-glycans released by PNGase F in ^{18}O-water from the same amount of human serum sample were mixed to give a theoretical ratio of 1:1. The mixture was derivatized by permethylation and then analyzed by an FT-ICR mass spectrometer in triplicate. The full MS spectrum acquired from the mixture of ^{16}O-N-glycans and ^{18}O-labeled N-glycans from human serum at a 1:1 ratio is shown in Fig. 5.

The calculated $^{18}O/^{16}O$ ratios were obtained with the mathematical method described above and shown in Table 1. The comparison of the theoretical ratio with that obtained experimentally reveals that the maximum observed error was 7.5% among the 25 analyzed N-glycan peaks, and the average error was 2.7%. With the incorporation of the ^{18}O isotope during PNGase F release of N-glycans, both isotopic species can be mixed before the derivatization procedure, and thus, both samples will be subjected to identical permethylation conditions. Therefore, the variances caused by different permethylation efficiency can be eliminated, which we expect led to the increased accuracy and precision.

Conclusions

This work presents a novel labeling method for N-glycan quantitation using ^{18}O-water. The ^{18}O-labeling is incorporated efficiently into the reducing end of N-glycans

during PNGase F release without altering the sample workflow. This method can improve quantitation accuracy by eliminating the difference in permethylation efficiency when samples are treated separately in a parallel manner. Also, a new mathematical calculation method for the ^{18}O/^{16}O ratio measurement was developed to resolve the problem caused by the isotopic peak overlapping. This method was applied successfully to quantitate N-glycans released from standard glycoproteins and from human serum. Lastly, the ^{18}O-labeling is expected to be applicable to oligosaccharides, such as O-glycans, as the ^{18}O-labeling could also be incorporated into the reducing end during mutarotation.[18–20]

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