IDAWG: Metabolic incorporation of stable isotope labels for quantitative glycomics of cultured cells

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Abstract

Robust quantification is an essential component of comparative –omic strategies. In this regard, glycomics lags behind proteomics. Although various isotope-tagging and direct quantification methods have recently enhanced comparative glycan analysis, a cell culture labeling strategy, that could provide for glycomics the advantages that SILAC provides for proteomics, has not been described. Here we report the development of IDAWG, **I**sotopic **D**etection of **A**minosugars **W**ith **G**lutamine, for the incorporation of differential mass tags into the glycans of cultured cells. In this method, culture media containing amide-^{15}N-Gln is used to metabolically label cellular aminosugars with heavy nitrogen. Because the amide side chain of Gln is the sole source of nitrogen for the biosynthesis of GlcNAc, GalNAc, and sialic acid, we demonstrate that culturing mouse embryonic stems cells for 72 hours in the presence of amide-^{15}N-Gln media results in nearly complete incorporation of ^{15}N into N-linked and O-linked glycans. The isotopically heavy monosaccharide residues provide additional information for interpreting glycan fragmentation and also allow quantification in both full MS and MS/MS modes. Thus, IDAWG is a simple to implement, yet powerful quantitative tool for the glycomics toolbox.

INTRODUCTION

A range of strategies have been developed for the high throughput quantitative/comparative analysis of large scale systems to enable the expression levels of various molecules to be compared between different biological states. In the –omics fields, the techniques used in the field of proteomics are probably the most developed, and can be broadly subdivided into two general schemes – those that involve the use of labels and those methods that are label free. In the label free approaches, various aspects of the peptides/proteins such as normalized ion intensities, spectral counts, mass, scan number and signal intensity, and accurate mass plus

retention time have been successfully used to assign protein expression levels for comparative investigations [1–4]. However, the ionization efficiency of each analyte depends on numerous factors, such as molecular mass, proton/cation affinity, surface activity, the presence of other compounds which compete with or interfere with the ionization of the analyte, etc., and thus ion intensities do not directly correlate with concentration. In addition, instrument response can vary over time, so that the direct comparison of data from two or more analyses can yield dramatically different results. Using relative quantification as an alternative strategy overcomes these issues by simultaneously analyzing pairs of isotopically labeled populations. The heavy and light labeled peptide pairs are analyzed under exactly the same conditions, allowing a direct comparison of relative abundances for that peptide. Relative quantification between the isotopically labeled populations is performed by taking a ratio of the area or the intensity of the light and heavy monoisotopic peaks.

Numerous strategies have been developed for introduction of a stable isotope into populations of proteins [5–12]. For example, isotope-coded affinity tags (ICAT) chemically target specific amino acids, typically cysteine, in the peptide sequence for differential labeling [5]. Other chemical approaches target other functional groups of the polypeptides [6, 8–12]. Stable isotopes can also be introduced into biological systems through metabolic labeling. For instance, stable isotope labeling with amino acids in cell culture (SILAC) provides a simple and straightforward method for the incorporation of an isotopic label into proteins prior to MS based proteomics [7]. In a SILAC experiment, two cell populations are grown in culture media that are identical except that one of them contains a "light" and the other a "heavy" form of particular amino acids (^{12}C and ^{13}C labeled lysine and arginine for example). The labeled analogs of amino acids are supplied to cells in culture instead of the natural amino acids, and it becomes incorporated into all newly synthesized proteins. After a number of cell divisions, each instance of the particular amino acids is replaced by its isotope labeled analog. An advantage of this approach, over the chemical tagging approaches, is that the cells are mixed together immediately after cell lysis. Thus, proteins from both cell types are subjected to the exact same experimental conditions during sample handling, digestion, purification, etc., eliminating the differential losses that can occur when the samples are treated separately in a parallel manner. For this reason, SILAC is often considered the "gold standard" for quantitative proteomic analyses [13].

The field of comparative glycomics is not as mature as proteomics; however several of the quantitative proteomic tools have been adapted for glycomic analysis. For instance, total ion mapping (TIM) is a label free method that determines the prevalence, percent of an individual glycan to the total of all glycans in the sample, based upon the sum of the fragment ion intensities, and is in some ways similar to the label free methods used in proteomics [14]. Isotopic labeling by chemical modification has also been developed by a number of groups. For N-linked glycans and free oligosaccharides, several isotope containing tags have been derived to label the reducing terminus [15–18]. O-linked glycans are usually released from the protein backbone via reductive β-elimination, and thus are not amenable to these approaches. However, a quantitative method relying on β-elimination to introduce a mass label into the glycan has been developed [19]. Another proposed method for comparative isotopic labeling of oligosaccharides relies on heavy methyl iodide ($^{13}CH_3$, $^{12}CDH_2$, $^{12}CHD_2$, and/or $^{12}CD_3$) vs. light methyl iodide ($^{12}CH_3$) labeling during

standard permethylation, which is a commonly used derivatization procedure prior to MS analysis of both N-linked and O-linked glycans [14, 20, 21]. In addition, an isobaric labeling strategy using permethylation with $^{13}CH_3$ and $^{12}CDH_2$ has been developed for both N- and O-linked glycans, and is particularly useful for the quantification of individual glycans present in isomeric mixtures [22, 23]. All of these *in vitro* approaches are useful tools for glycomics.

Here, we describe an *in vivo* labeling strategy for glycomic studies. This methodology termed IDAWG, **I**sotopic **D**etection of **A**minosugars **W**ith **G**lutamine, relies on the hexosamine biosynthetic pathway that uses the side-chain of glutamine as its sole donor source of nitrogen for aminosugars in the production of sugar nucleotides. Thus, introduction of glutamine with a ^{15}N labeled side-chain (amide-^{15}N-Gln) into Gln-free media allows for all aminosugars, including GlcNAc, GalNAc, and sialic acids, to become labeled with ^{15}N. Hence, the mass of N- and O-linked glycans, glycolipids, and extracellular matrix polysaccharides, should all be increased by +1 Dalton per aminosugar. We demonstrate the utility of this approach by the analysis of both N-linked and O-linked glycans released from proteins of murine embryonic stem cells grown in both the light and amide-^{15}N-Gln. The success of these experiments leads us to predict that the IDAWG technology will be useful for a variety of future comparative glycomic studies in cell culture.

EXPERIMENTAL PROCEDURES

Cell culture

The R1 line of murine embryonic stem cells (mESCs) were cultured essentially as previously described [24]. The mESC culture media was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Commonwealth Serum Laboratories), 2 mM L-glutamine (either ^{14}N or amide-^{15}N), 0.1 mM 2-mercaptoethanol, and 1000U/ml recombinant murine leukemia inhibitory factor (LIF) (ESGRO, Chemicon International). Amide-^{15}N-Gln (98% purity) was purchased from Cambridge Isotopes Inc (Andover, MA). The mESCs were cultured at 37°C under 10% CO_2. The media was changed daily for 3 days allowing for approximately 6 doublings from the originally plated 2×10^5 cells, after which the ESCs ($\sim 1 \times 10^7$) were collected by dissociation buffer and scraping, placed into a 15 ml conical tube, and pelleted at $1,000 \times G$. The cells were washed 3 times in ice cold phosphate buffered saline (PBS) followed by centrifugation at $1000 \times G$ after each wash. All supernatant was removed from the tube and the cell pellets were stored at -80 °C until analysis.

ES cell lysis, delipidation, glycan release, and permethylation

The isolation of permethylated N- and O-linked permethylated glycans was essentially as previously described [14, 22, 23, 25]. Briefly, cells were resuspended in water and lysed by sonication. Delipidation was carried out using organic extraction in chloroform:methanol:water. The resulting proteins were subjected to trypsin digestion and glycans were released by PNGaseF treatment and β-elimination for N- and O-linked glycans, respectively. Glycans enriched by their inability to tightly bind a reverse-phase

solid support were permethylated with methyl iodide and cleaned up by reverse-phase enrichment and dried down.

MS analysis of the permethylated glycans

The glycans were analyzed as previously described [14, 22, 23, 25] on either a linear ion trap mass spectrometer (LTQ, ThermoFisher) or on a hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT, ThermoFisher) or on a hybrid linear ion tap Fourier transform orbitrap mass spectrometer (LTQ-Orbitrap, ThermoFisher). Briefly, permethylated glycans were dissolved in a total of 50 μL of sample solution: 15 μL of 100% methanol followed by the addition of 35 μL of 1 mM NaOH in 50% methanol and infused directly into the mass spectrometer using a nanospray ion source with a fused-silica emitter (360 × 75 × 30 μm, SilicaTip™, New Objective) at 2.0 kV capillary voltage, 200 °C capillary temperature, and a syringe flow rate of 0.4 μL/min. Full ITMS (Ion trap mass spectrometry) spectra in positive ion and profile mode were collected at 400–2000 m/z for 30 sec with 5 microscans and 150 maximum injection time (ms). The centroid MS/MS spectra following collision-induced dissociation (CID) were obtained from 400 to 2000 m/z at 34% and 28% normalized collision energy for *N-* and *O*-linked glycans respectively, 0.25 activation Q, and 30.0 ms activation time by total ion mapping (TIM). Parent mass step size and isolation width were set at 2.0 m/z and 2.8 m/z respectively for automated MS/MS spectra with TIM scans. MS^n experiments were manually carried out in profile mode. Glycan precursor ions were isolated for MS^n using an isolation width of 2.5 m/z. For FTICR and Orbitrap analysis, an isolation width of 10 or 8–9 m/z at 100,000 resolution was used respectively. For the quantification experiment, a 1:1 mixture of heavy and light permethylated samples was initially generated based off equal starting proteins amount and slightly adjusted (less than 20% addition of light) to normalize quantification of the disialyated Tn antigen to the theoretical 1:1 ratio. Manual interpretation of all glycan structures was carried out relying on in-house fragmentation rules and glycoworkbench (http://www.dkfz-heidelberg.de/spec/ EUROCarbDB/GlycoWorkbench/). Essentials of Glycobiology symbolic drawing conventions are used for all glycan structures [26]. Underincorporation and ratios are calculated as described in the supplemental and are similar to those used for other stable isotope labeling in cell culture approaches.

RESULTS

Mechanism for Isotope Incorporation into Glycoconjugates

The hexosamine biosynthetic pathway converts the glycolysis intermediate fructose-6-phosphate into UDP-GlcNAc ([27], Fig. 1A). The first and rate-limiting step in this pathway converts fructose-6-phosphate to glucosamine-6-phosphate with the concomitant conversion of Gln to Glu [28]. Thus, the side-chain amide of Gln is the source of nitrogen in the production of UDP-GlcNAc. The other major aminosugar-containing sugar nucleotides, UDP-GalNAc and CMP-sialic acid, are biosynthesized from UDP-GlcNAc[29, 30]. Thus, all GlcNAc, GalNAc, and sialic acid containing molecules are targets for isotopic labeling by supplementation of cell culture media with amide-^{15}N-Gln (Fig. 1).

Initial experiments were performed to evaluate the possibility of using metabolic labeling as a method to incorporate a stable isotope into the glycans of cells grown in culture. In these experiments, R1 murine embryonic stem cells (mESCs) were grown using standard conditions. Cell culture media is typically supplied without glutamine, as this amino acid decomposes to glutamic acid and ammonia under aqueous conditions. This forumulation simplifies the IDAWG labeling approach, because there is no need to specifically deplete the media of Gln. Either amide-^{15}N-Gln or the ^{14}N-version was used to supplement the media at the standard concentration (2mM). This straightforward substitution is the only alteration to the normal cell culturing procedure needed for IDAWG. Thus, we labeled murine ES cells for three days with heavy or with light Gln and then isolated both N- and O-linked glycans from proteins for mass spectrometry-based analysis (Fig. 1B). Labeling for four days did not improve labeling efficiency, however IDAWG analysis of certain differentiated cell types has shown the need for prolonged labeling up to one week for efficient incorporation (data not shown).

^{15}N Incorporation into N-linked Glycans

The incorporation of ^{15}N into the N-linked glycans of mESCs was investigated by comparing the full spectra of permethylated N-linked glycans, following release from cells grown in either amide-^{14}N-Gln or amide-^{15}N-Gln (Fig. 2). Both samples were subjected to total ion mapping analysis and MS^n to confirm structural assignments as necessary. The abundant ions observed in these spectra are predominantly high mannose glycans (Man$_{5-9}$ GlcNAc$_2$) that are either singly or doubly charged (structures 1–3, 6, 12–13, and 15 from Fig. 2). Comparison of these two spectra reveals that the high mannose glycan ions obtained from the cells grown in the amide-^{15}N-Gln containing media are increased by 1 m/z unit for the doubly charged ions and 2 m/z units for the singly charged ions, within the mass tolerance of the linear ion trap,. This is the expected result provided that ^{15}N has been incorporated into the two core GlcNAcs, which are the only nitrogens present in high mannose glycans. Also contained within the full spectra are ions representing complex glycans containing additional GlcNAc (structures 4, 5, 7, 8, 10, 11, and 14 from Fig. 2) as well as sialic acid as Neu5Ac (structure 11 from Fig. 2) residues. These glycans are all shifted in the amide-^{15}N-Gln samples by the appropriate mass for ^{15}N-incorporation into GlcNAc and Neu5Ac.

For calculation of labeling efficiencies, the samples were analyzed on a hybrid LTQ-FT instrument. Closer inspection of the doubly charged molecular ion, $[M+2Na]^{2+}$, resulting from the GlcNAc$_2$Man$_7$ glycan (structure 2 of Fig. 2), which appears at 1005.5 and 1006.5 m/z units from glycans grown in the ^{14}N and amide-^{15}N Gln media, respectively, is shown in Fig. 3. In these spectra, the most intense ion appears at the monoisotopic mass calculated with either ^{14}N for light media or at two ^{15}N for heavy media, demonstrating that this glycan has ^{15}N incorporated into both possible sites. Ions are present that correspond to under labeling, i.e., the incorporation of zero and one ^{15}N, however the dominant species is fully labeled. Zoom scans were performed on several structures containing theoretically 2–5 ^{15}N-residues. The ratios of the intensities of the different isotopes in the ^{15}N spectrum indicate an average 95.8% incorporation of ^{15}N into N-linked glycans (Table 1), which is very similar to the extent of ^{15}N in the glutamine used for this experiment (98%). Thus, ^{15}N from

amide-^{15}N-Gln becomes efficiently and extensively incorporated into amino sugars of N-linked glycans.

MS/MS spectra resulting from the fragmentation of N-linked glycans display patterns consistent with aminosugar labeling. For example, fragmentation profiles for a heavy- and a light-labeled, core fucosylated, fully sialylated, biantennary N-linked glycan are consistent with ^{15}N-incorporation into Neu5Ac as well as core and antennary GlcNAc residues (Fig. 4). Therefore, IDAWG labeling can aid in the assignment of structures based on the identification of fragments that include isotopically tagged monosaccharide residues.

^{15}N Incorporation into and Quantification of O-linked Glycans

To study the incorporation of ^{15}N into the O-linked glycans of mESCs, the full spectra of the permethylated O-linked glycans were compared, following release from cells grown in either ^{14}N-Gln or amide-^{15}N-Gln. Both samples were subjected to total ion mapping analysis and MS^n when necessary to confirm the structures. To demonstrate the incorporation of ^{15}N into all 3 aminosugars contained in O-glycans, MS/MS spectra from heavy- and light-labeled samples are shown for three different O-linked glycan types (Fig. 5): an O-Man initiated tetrasaccharide containing GlcNAc and Neu5Ac, an O-GalNAc initiated structure containing a branching GlcNAc (core 2), and an O-GalNAc initiated structure containing two Neu5Ac residues (disialyl core 1). The isolation m/z (parent ion mass) and fragmentation profiles of all 3 O-linked structures are consistent with efficient ^{15}N-incorporation into all GlcNAc, GalNAc, and Neu5Ac monosaccharides. As can be seen from the fragmentation patterns, the reducing end GalNAc is isotopically labeled in the heavy samples as are the GlcNAc and NeuAc in the extended chains.

Furthermore, we mixed equal amounts of a heavy and light labeled pools of O-glycans from mESCs based off generating a 1:1 ratio for the disialyated Tn antigen and then quantified the eight other most abundant O-glycans (Fig.6, Table 2). We observed an average ratio of 0.94:1 (1:1 theoretical) with a standard deviation of 0.08. The results of figures 5 and 6 highlight that ^{15}N is incorporated into all 3 aminosugars of O-Glycans and that simultaneous analysis and fragmentation of light and heavy structures should aid greatly in glycan assignment and quantification. Furthermore, as shown in table 2 and illustrated for two structures in figure 6, robust quantification is achievable.

DISCUSSION

Herein we have described a simple to implement but powerful isotopic labeling strategy for glycans in cell culture systems. The IDAWG methodology relies on the fact that the amide side chain of Gln is the sole nitrogen donor for UDP-GlcNAc via the hexosamine biosynthetic pathway and that UDP-GlcNAc is the substrate for the biosynthesis of UDP-GalNAc and CMP-Neu5Ac (or its glycolylated counterpart, CMP-Neu5Gc). Using mESCs we demonstrate that replacement of the normal Gln supplement with amide-^{15}N-Gln allows for nearly complete isotope labeling of GlcNAc, GalNAc, and Neu5Ac residues of N-linked and O-linked glycans within a 72 hour labeling period. This technique should provide for robust quantification of glycan expression differences following their release from proteins and lipids isolated from differentially labeled samples.

A significant advantage of the IDAWG labeling strategy, similar to SILAC for proteomics, is that differentially labeled cells can be mixed together at the beginning of the analytic procedure, minimizing the contribution of handling and work-up to overall variability. Furthermore, the expected mass differences between the heavy- and lightlabeled forms of even the most complex glycans is under 10 mass units for their singly charged ions. For most glycan structures this difference is below 5 mass units. Therefore, fragmentation of heavy- and light-labeled glycans can be monitored and quantified simultaneously within the same isolation window, further reducing another source of analytic variablity. Such relative quanitification of glycan prevalence using MS/MS or perhaps MS^n, in which signal-to-noise is greatly increased, also provides a significant gain in sensitivity for characterizing alterations in minor glycans. In our quantitative experiment for O-glycans, we observed no more than 20% deviation from expected, so that induced reproducible changes of less than 2-fold should be readily quantifiable.

The IDAWG technique provides a labeling strategy that should also be amenable for characterizing glycopeptides directly and we are currently exploring this possibility by examining the dynamic O-GlcNAc modification [31] on proteins isolated from light and heavy Gln-labeled cell culture systems. While shotgun proteomic experiments performed on peptides obtained from the ^{15}N labeled samples identified peptides that incorporated amide-^{15}N-Gln, no other heavy amino acids were observed (data not shown). This observation that ^{15}N-Gln also is incorporated into proteins, without migration of the heavy nitrogen label into other amino acids, provides a SILAC-like labeling strategy that could be used alone or in combination with heavy Arg/Lys SILAC [7] to quantify glycans, proteins, and glycoproteins/glycopeptides all from the same set of samples.

Finally, the metabolic labeling of glycans using IDAWG provides new opportunities for assessing the dynamics of glycan turnover during the course of any cellular behavior that can be induced or sustained in culture. By completely labeling cells with heavy Gln and then replacing the media supplement with light Gln, the synthesis and degration of any aminosugar-containing glycans can be evaluated. Previously, glycan turnover studies required incorporation of radioactive monosaccharide and extensive subsequent fractionation to identify specific changes in glycan expression. Generally, the radiotracer techniques allowed for very sensitive detection of glycan classes, but lacked the resolution to follow individual glycan structures or subsets of biosynthetically-related species. The stable isotope incorporation method reported here weds the analytic advantages of high-resolution mass spectrometry to the biological necessity of understanding the dynamics of glycan turnover. Sharp focus on individual glycans or on glycan subsets may reveal that turnover reports cellular differentiation or metabolic state in a more informative manner than is possible when simple glycan prevalence is sampled at a biological endpoint. Thus, we believe that IDAWG is a powerful new quantitative tool for exploring the biological role of glycans, glycoproteins, and glycolipids in cell culture systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A.

IDAWG: Isotopic Detection of Aminosugars with Glutamine

Glc
↓
Glc-6-P
↓
Fru-6-P → GlcN-6-P → GlcN-1-P → GlcNAc-1-P → UDP-GlcNAc
↓
GLN GLU → Multiple Other AAs

NH4+

GLYCOCONJUGATE
↓

+1 DALTON

B.

Light Medium   Heavy Medium

Amide-14N L-glutamine   Amide-15N L-glutamine
"Gln-14"   "GLN-15"

Harvest & Combine
↓ Homogenization and delipidation
Mixed Protein Powder
↓ Tryptic digestion
Peptide Mixture

C18 reverse phase   β-elimination
PNGase F digestion

N-linked Glycan Mixture   O-linked Glycan Mixture

C18 reverse phase   Deionization
Permethylation   Permethylation

Permethylated Glycans   Permethylated Glycans

Mass Spectrometry

Light   Heavy
Gln-14   GLN-15

Quantification by Full MS   Characterization by MS/MS

Figure 1. IDAWG Labeling Theory and Strategy

A. A schematic of the hexosamine biosynthetic pathway that converts the glycolysis intermediate fructose-6-phosphate (Fru-6-P) to UDP-GlcNAc showing the addition of nitrogen into the pathway from the amide side-chain of Gln. UDP-GlcNAc goes on to generate UDP-GalNAc and CMP-Neu5Ac so that the aminosugars GlcNAc (blue square), GalNAc (yellow square), or Neu5Ac (purple diamond) in glycoconjugates will have an additional +1 dalton to their mass if amide-^{15}N-Gln is used as the nitrogen donor. Glc, glucose; GlcN, glucosamine. B. Schematic of the IDAWG in vivo cell culture labeling

strategy for comparative glycomics. Amide-^{15}N-Gln or Gln is added to the media to label cultured cells. Upon harvesting cells can be combined, then N-linked and O-linked glycans isolated from digested proteins for permethylation and tandem mass spectrometry analysis.
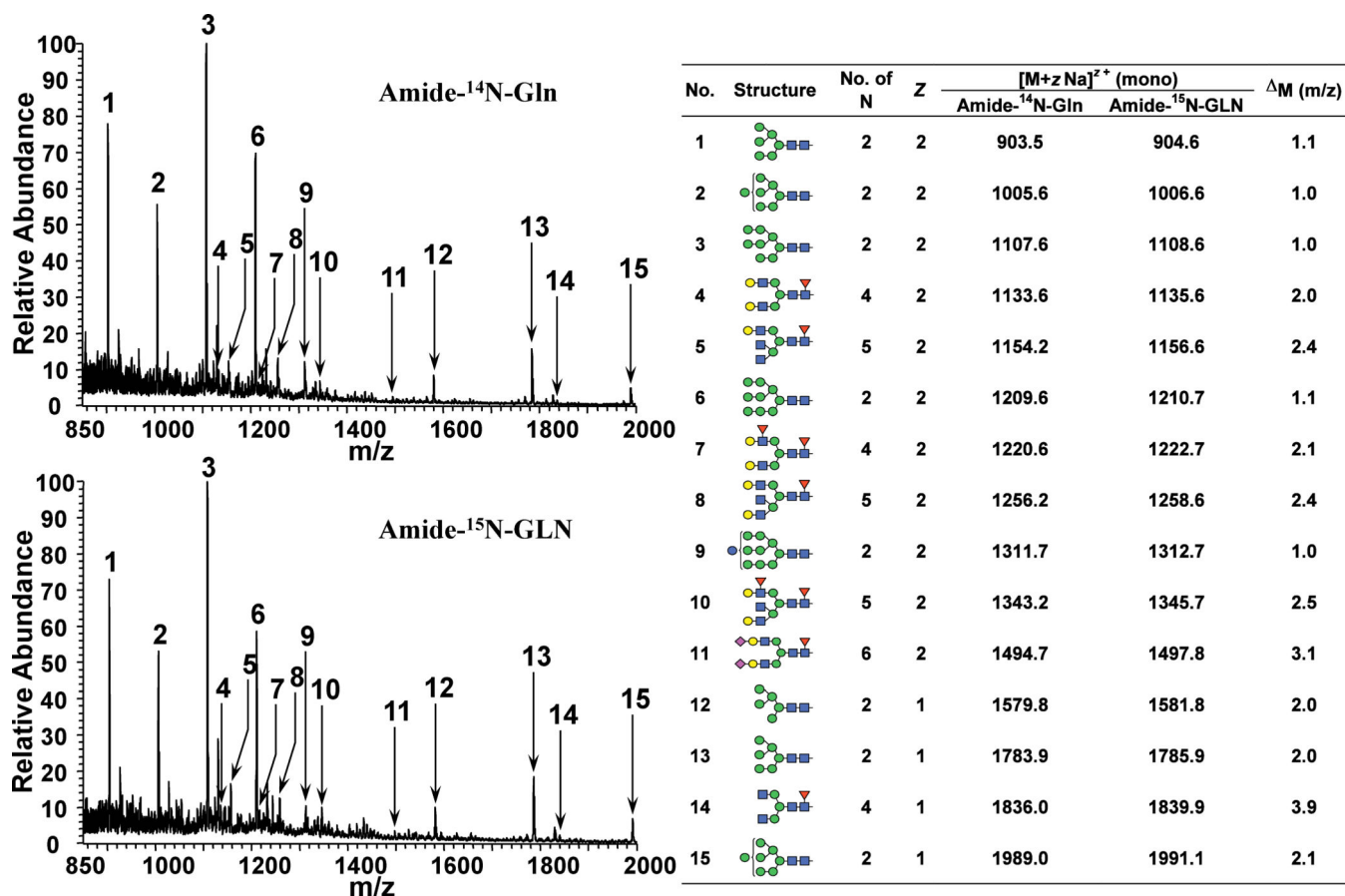
Figure 2. Isotopically Labeled N-linked Glycans
Full spectra from 850–2000 m/z of the permethylated N-linked glycans released from cells grown in either ^{14}N-Gln or amide-^{15}N-Gln analyzed on a linear ion trap (LTQ). Major glycans identified by total ion mapping and MSn are shown along with charge state (Z), number of nitrogens, and observed shift in mass between the samples.
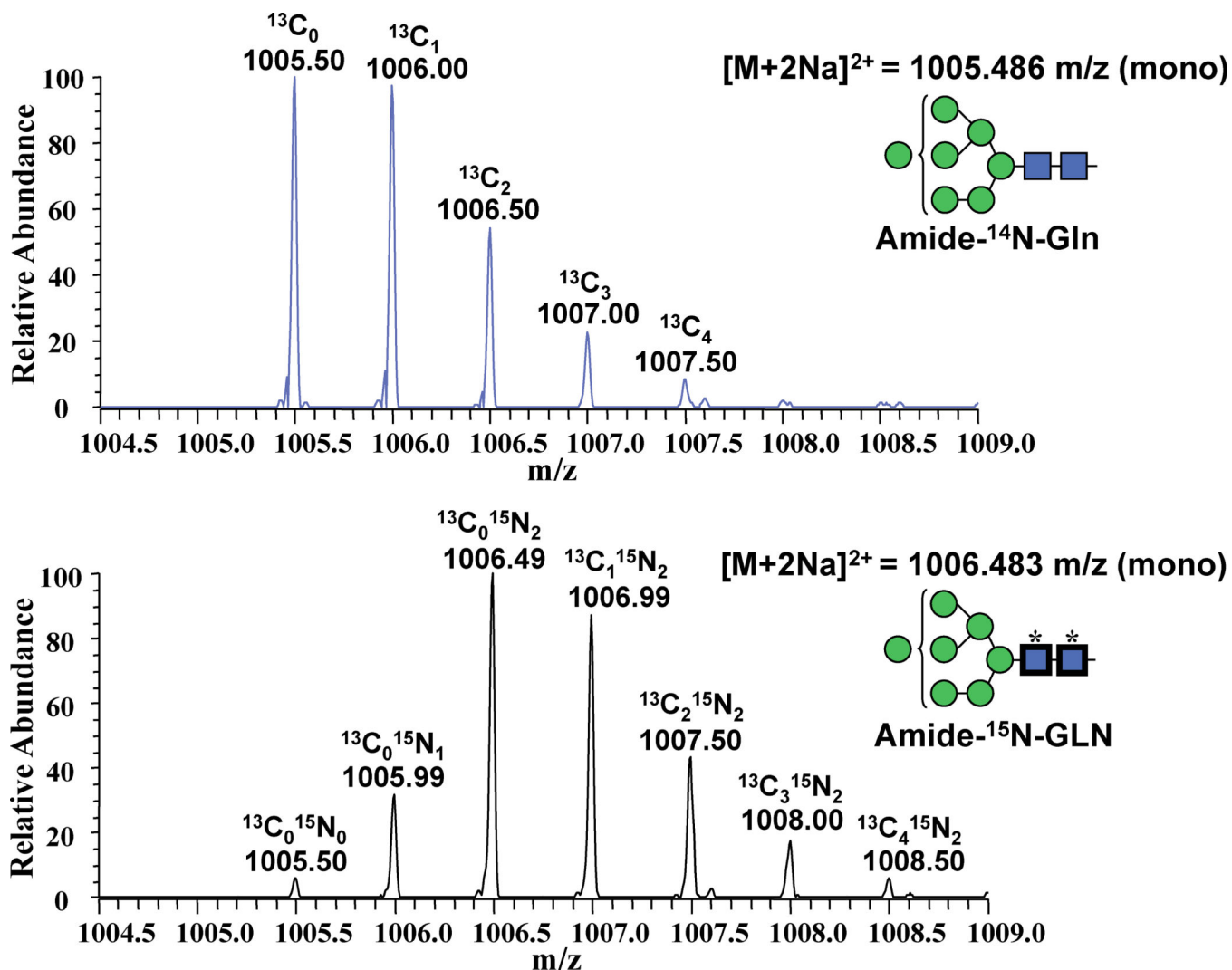
Figure 3. Isotope Pattern of Light and Heavy Man$_7$GlcNAc$_2$
Zoom scans from a FTICR (LTQ-FT) show the expected mass shift of 2 daltons for the core GlcNAc residues that are outlined with bold with an asterisk in the amide-^{15}N-Gln labeled sample (bottom spectrum). Note the under incorporation in the heavy spectrum which allowed us to calculate the labeling efficiency in Table 1.
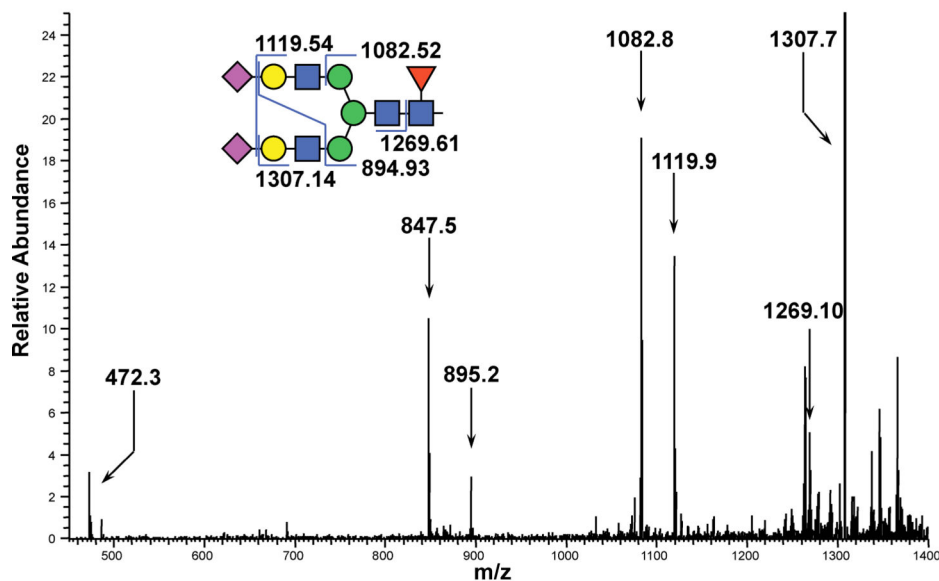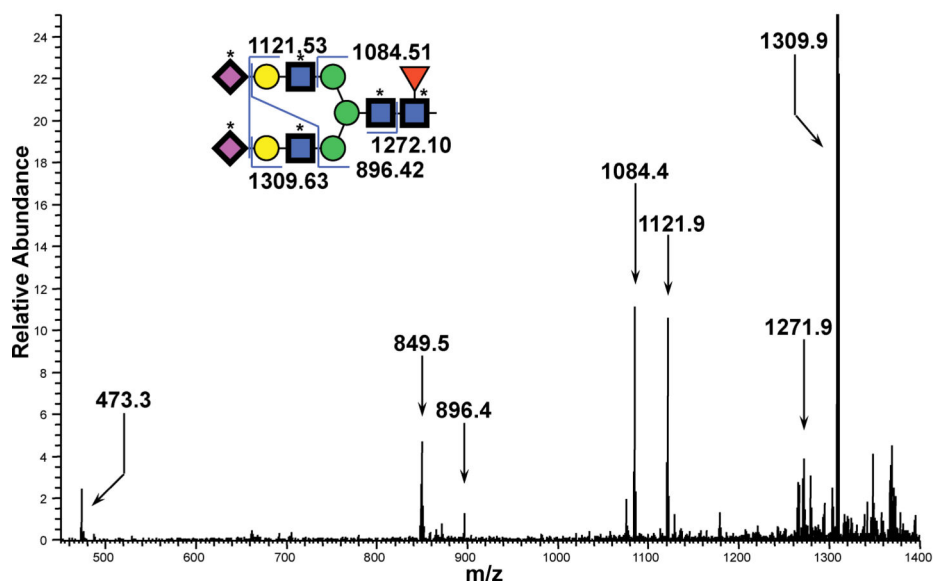
Figure 4. Fragmentation of a Light and Heavy Labeled Complex N-linked Glycan
A. MS/MS fragmentation spectrum of a permethylated, core fucosylated, fully sialyated biantenneary N-linked glycan from mouse embryonic stem cells. B. MS/MS fragmentation spectrum of the same structure as in A. from the amide-^{15}N-Gln labeled sample. Note the m/z shift of ~3 Da on the LTQ spectrum for the [M+2Na]2+ parent ion and the appropriate shift in m/z for the fragments corresponding to labeling of all 4 GlcNAc residues as well as the 2 Neu5Ac monosaccharides. Theoretical fragmentation is shown for all structures.

Theoretical internal fragmentation, such as the internal Gal-GlcNAc at ~472/473 m/z, are not illustrated on the cartoon for clarity.
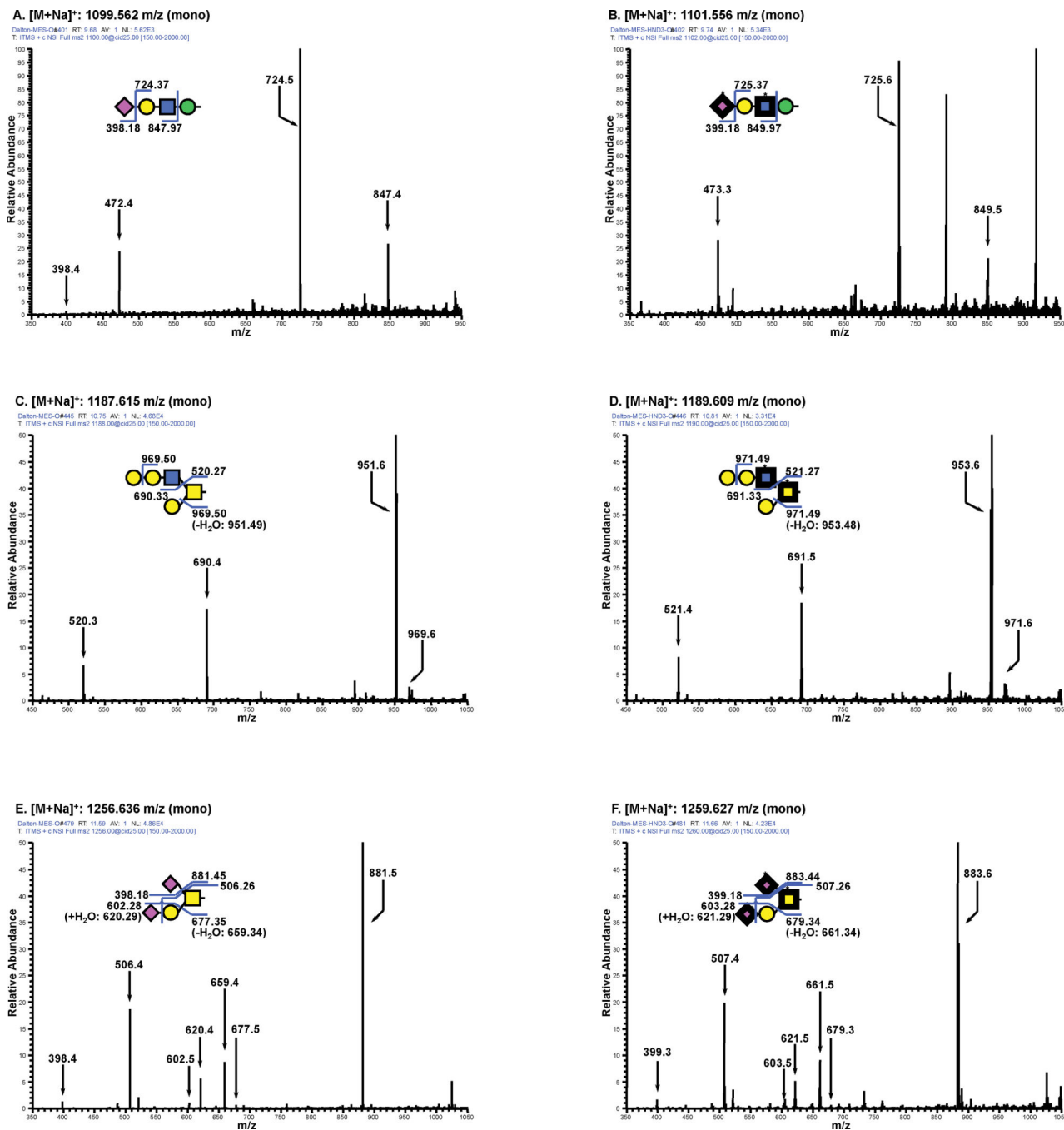
Figure 5. Light and Heavy Labeling of O-linked Glycans
A. and B. display spectra of the MS/MS fragmentation of an O-Man initiated structure from light and heavy, respectively, labeled cells showing incorporation of ^{15}N into both the GlcNAc and NeuAc residue. Note that B. also trapped another glycan structure not shown. C. and D. display spectra of the MS/MS fragmentation of a core 2 O-GalNAc initiated structure from light and heavy, respectively, labeled cells showing incorporation of ^{15}N into both the GalNAc and GlcNAc residue. E. and F. display spectra of the MS/MS fragmentation of the disiayl T-antigen from light and heavy, respectively, labeled cells

showing incorporation of ^{15}N into GalNAc and both NeuAc residues. Theoretical fragments are shown for all structures that were acquired on the LTQ.
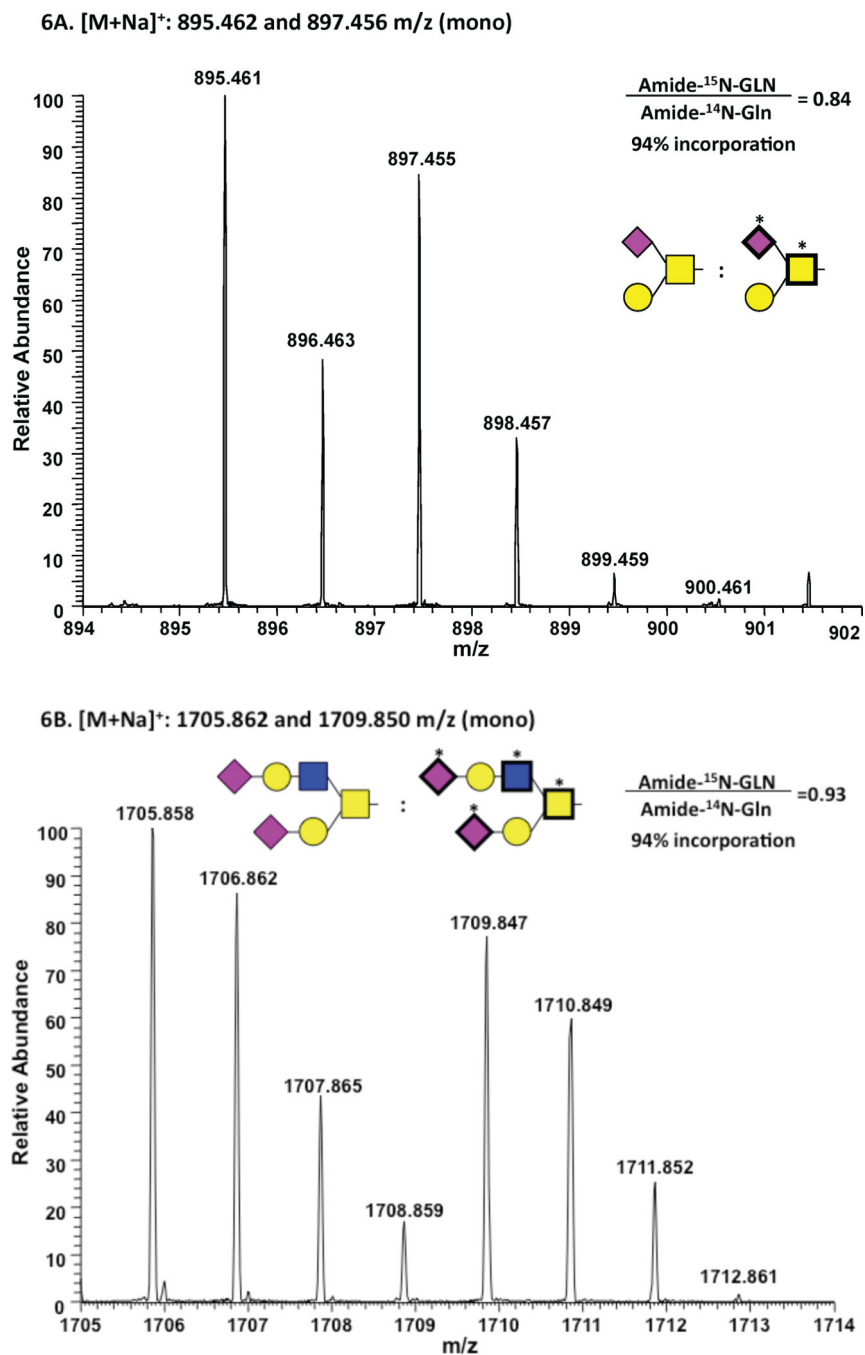
Figure 6. Quantitative Analysis of O-linked Glycans
A mixture of O-linked glycans from a 1:1 mixture of light/heavy labeled mESCs were mixed together and analyzed on an LTQ-Orbitrap. A. Quantification of the monosialyated T-antigen in a 8 m/z window (2 nitrogens). B. Quantification of a disialyated core 2 structure in a 9 m/z window (4 nitrogens).

Table 1

IDAWG labeling efficiencies

| No | N-linked glycan composition | Structure | Number of nitrogens | Z | [M+2Na]$^{2+}$ | | Labeling efficiency (%) |
					m/z (mono)	M (*m/z*)	
1	(Man)4+(Man)3(GlcNAc)2		2	2	1005.48	1.0	95
2	(Man)5+(Man)3(GlcNAc)2		2	2	1107.53	1.0	96
3	(Gal)2(GlcNAc)2(Fuc)1+(Man)3(GlcNAc)2		4	2	1133.55	2.0	96
4	(Gal)2(GlcNAc)2(Fuc)2+(Man)3(GlcNAc)2		4	2	1220.60	2.0	95
5	(Gal)1(GlcNAc)3(Fuc)1+(Man)3(GlcNAc)2		5	2	1154.07	2.5	97

Table 2

Quantifying O-linked Glycans

No.	Glycan Structure	[M+Na]+ (mono)		Ratio
		Amide-^{14}N-Gln	Amide-^{15}N-GLN	
1		534.29	535.29	0.88
2		895.46	897.47	0.84
3		1157.6	1159.61	0.93
4		1344.69	1347.7	0.97
5		1432.74	1435.75	1.03
6		1518.78	1521.78	0.85
7		1705.86	1709.87	0.93
8		1793.91	1797.93	1.08