Tools for glycomics: relative quantitation of glycans by isotopic permethylation using $^{13}\text{CH}_3\text{I}$

Gerardo Alvarez-Manilla[1,2], Nicole Lynn Warren[2], Trina Abney[2], James Atwood III[2], Parastoo Azadi[2], Will S York[2], Michael Pierce[2], and Ron Orlando[2]

[2]Complex Carbohydrate Research Center, University of Georgia, 315 Riverbend Road, Athens, GA 30602

Analysis of oligosaccharides by mass spectrometry (MS) has enabled the investigation of the glycan repertoire of organisms with high resolution and sensitivity. It is difficult, however, to correlate the expression of glycosyltransferases with the glycan structures present in a particular cell type or tissue because the use of MS for quantitative purposes has significant limitations. For this reason, in order to develop a technique that would allow relative glycan quantification by MS analysis between two samples, a procedure was developed for the isotopic labeling of oligosaccharides with ^{13}C-labeled methyl iodide using standard permethylation conditions. Separate aliquots of oligosaccharides from human milk were labeled with ^{12}C or ^{13}C methyl iodide; the labeled and non-labeled glycans were mixed in known proportions, and the mixtures analyzed by MS. Results indicated that the isotopic labeling described here was capable of providing relative quantitative data with a dynamic range of at least two orders of magnitude, adequate linearity, and reproducibility with a coefficient of variation that was 13% on average. This procedure was used to analyze N-linked glycans released from various mixtures of glycoproteins, such as α-1 acid glycoprotein, human transferrin, and bovine fetuin, using MS techniques that included matrix assisted laser desorption ionization-time of flight MS and electrospray ionization with ion cyclotron resonance-Fourier transformation MS. The measured $^{12}\text{C}:^{13}\text{C}$ ratios from mixtures of glycans permethylated with either $^{12}\text{CH}_3\text{I}$ or $^{13}\text{CH}_3\text{I}$ were consistent with the theoretical proportions. This technique is an effective procedure for relative quantitative glycan analysis by MS.

Key words: oligosaccharides/MALDI-TOF MS/isotopic labeling/permethylation

Introduction

Glycoprotein glycans are essential for and regulate many physiological processes during development and disease progression (Hakomori 1989; Muramatsu 1993; Olden 1993;

Fukuda 1996). Identifying specific glycan structures, deciphering the proteins that express each glycan, and understanding in detail how these structures change, e.g., as cells differentiate or as tumor cells progress, are components of the emerging field of glycoproteomics. A large number of proteins are involved in regulating glycan expression and function, including glycosyltransferases, glycosidases, other enzymes involved in sugar nucleotide metabolism and transport, as well as carbohydrate-binding proteins known as lectins (Coutinho et al. 2003; Breton et al. 2006). The genes that encode many of these enzymes have been isolated, expressed, and characterized extensively by functional studies, including generating null mice (Taniguchi et al. 2002). It is estimated that the murine glycome, for example, encodes over 650 genes (Lowe and Marth 2003). A major challenge, therefore, is to determine how glycan structures change during progression, how transcripts of genes in the glycome change as cells initiate differentiation programs, and then to synthesize an understanding of how transcript changes can be used to identify and predict changes in glycan expression. A sensitive, quantitative technique for glycoprotein glycan analysis, therefore, is a critical component, just as quantitative methods have had a significant impact on comparative proteomics.

The requirements of a methodology for quantitative glycan expression comparison are its capability of detecting even subtle changes in structure with high sensitivity and high resolution. Mass spectrometric techniques based on electrospray ionization-mass spectrometry (ESI-MS) or matrix assisted laser desorption ionization with time of flight detection (MALDI-TOF-MS) have found important applications in high-throughput proteomic analyses due to substantial improvements in the instrumentation and the development of computer algorithms that allow the analysis of large amounts of data (Mann et al. 2001). Studies have shown that it is often possible to detect glycans released from glycoproteins using similar MS techniques without derivatization (Keck et al. 2005). However, the composition of native oligosaccharides are varied due to the presence of N-acetylated and acidic residues, and this variation affects the ionization capabilities of the oligosaccharides, sometimes preventing them from being detected by MS. For this reason, derivatization of oligosaccharides by chemical means such as permethylation is often performed before MS analysis, because this chemical modification stabilizes the sialic acid residues in acidic oligosaccharides. Additionally, the addition of methyl groups to an oligosaccharide population also enables them to become more uniformly ionized by reducing the variation of the chemical properties among the glycans, caused by the presence of highly polar $-$OH and $-$NH$_2$ groups in the molecule; therefore, facilitating relative quantitation of the individual

[1]To whom correspondence should be addressed. E-mail: alvarez@uga.edu

oligosaccharides in a mixture. Methylated glycans ionize more efficiently than their native counterparts, and due to their hydrophobic nature, are easily separated from salts and other impurities that may affect the MS analysis (Dell et al. 1994; Kang et al. 2005). Additionally, the fragmentation of methylated glycans is more predictable than that of their native counterparts, leading to accurate structural assignments when MS/MS analysis is performed (Dell et al. 1994; Viseux et al. 1997; Reinhold and Sheeley, 1998; Sheeley and Reinhold, 1998; Morelle et al. 2004; Mechref et al. 2006; Wuhrer and Deelder, 2006).

Several MS procedures, Including MALDI-TOF-MS and ESI-MS have been used successfully to analyze methylated glycans with high sensitivity and resolution (Dell et al. 1994; Reinhold and Sheeley 1998; Sheeley and Reinhold 1998). A significant drawback of these techniques, however, is their restricted ability to provide quantitative information due to differences in ionization efficiencies that still remain among the various components present in the sample even after methylation (Chen and He 2005), as well as the possible presence of different amounts of interfering ions among the samples. Although there are some reports on the use of MALDI-TOF MS for quantitative studies of oligosaccharides and protein glycosylation (Pitt and Gorman 1997), there are still important limitations on the use of MS for quantitative purposes.

In the field of proteomics, one means of obtaining quantitative proteomic data from mass spectrometric analyses is through the incorporation of specific isotopic label into a population of molecules, either by metabolic incorporation or chemical derivatization (Goshe and Smith 2003; Tao and Aebersold 2003). In this approach, the sample containing the "heavy" isotope is mixed with that of the "light" isotope, followed by MS analysis of the resulting mixture. The mass analyzer resolves the isotopically labeled species, permitting their relative abundances to be determined from the ratio of the light- and heavy-labeled molecular ions. Numerous isotopic labeling procedures have been established for the study of protein mixtures and are widely used in high throughput proteomic studies (Goshe and Smith 2003).

The use of isotopic labels for the quantitative analysis of glycans offers promise for the detection and measurement of changes in the abundance of specific oligosaccharide structures present in complex glycoprotein mixtures such as those obtained from cells or tissues. To demonstrate this approach, we investigated the use of [^{13}C] methyl iodide (^{13}CH$_3$-I) as an isotopic label in permethylation reactions for oligosaccharide analysis by MALDI-TOF MS. Various neutral human milk oligosaccharides, as well as N-linked glycans released from several purified glycoproteins were permethylated, either with ^{13}CH$_3$-I or with ^{12}CH$_3$-I, different proportions of isotopically labeled and nonlabeled glycans were mixed, and each mixture analyzed by MALDI-TOF MS or ESI-MS on a hybrid linear ion trap Fourier transform mass spectrometer (LTQ-FT). Quantitative data were obtained from the peak heights of the different derivatives and analyzed for linearity and reproducibility. The results obtained demonstrated that isotopic labeling with ^{13}CH$_3$-I provides a linear response, with good reproducibility. This technique of labeling oligosaccharides with ^{13}CH$_3$I during permethylation offers the potential for rapid and accurate detection of the relative

difference in the abundance of glycan structures during the analysis of glycans from distinct populations such as those from non-diseased and diseased cells or cells at different stages of differentiation.

Results

Isotopic labeling of oligosaccharides with ^{13}CH$_3$I

Exhaustive permethylation of oligosaccharides was used to improve their signals by mass spectrometric analyses. When ^{13}C-labeled methyl iodide was utilized instead of the ^{12}C-labeled reagent, a mass increase directly proportional to the amount of available —OH groups in the oligosaccharide should be detected by the mass analyzer. Therefore, the larger the size of a glycan, the larger mass difference between the ^{12}C- and the ^{13}C-labeled isotopic pair. The isotopic labeling procedure used in this study was initially tested with an oligosaccharide fraction purified from human milk. This fraction contained a mixture of two isomeric tetrasaccharides, (Gal$_2$-GlcNAc-Glc), three isomeric fucopentasaccharides, (Gal$_2$-Fuc-GlcNAc-Glc), and at least two isomeric di-fucohexasaccharides, (Gal$_2$-Fuc$_2$-GlcNAc-Glc) (Kobata et al. 1978). Figure 1 shows a MALDI-TOF MS spectra obtained from a mixture of the ^{12}C- and ^{13}C-labeled permethylated human milk oligosaccharides described earlier that were mixed in equal amounts before MS analysis. Signals at mass-to-charge ratio (m/z) 926.3, m/z 1100.3, and 1274.4 in the spectrum correspond to the monoisotopic masses of the sodium adduct ions of the ^{12}C permethylated oligosaccharides, whereas the masses of their respective ^{13}C-labeled isotopic
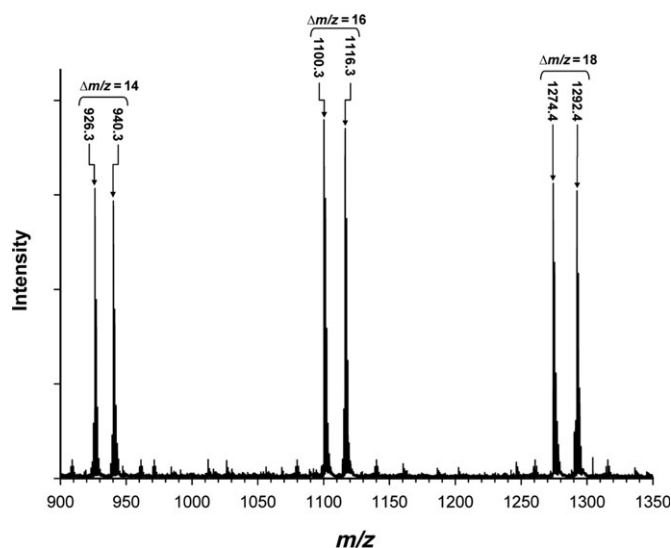

Fig. 1. MALDI-TOF MS spectrum of ^{12}C- and ^{13}C-labeled neutral oligosaccharides from human milk. Equal amounts of oligosaccharides fractions were derivatized with ^{12}C or ^{13}C methyl iodide and mixed before analysis by MALDI-TOF-MS. The isotopic pairs in the mixture can be observed, and the monoisotopic masses of each labeled oligosaccharide in the isotopic pair as well as the mass differences between the components are indicated. The oligosaccharides in the fraction and the m/z values of their respective ^{12}C:^{13}C methylated isotopic pairs are a mixture of tetrasaccharides, Gal$_2$-GlcNAc-Glc, (m/z 926.3:940.3), a mixture of fucopentaoses Gal$_2$-Fuc-GlcNAc-Glc, (m/z 1100.3:1116.3), and a mixture of difucohexaoses Gal$_2$-Fuc$_2$-GlcNAc-Glc, (m/z 1274.4:1292.4).

pairs are the peaks at m/z 940.3, 1116.3, and 1292.4. Since the ^{12}C and ^{13}C methylated glycans were mixed in equal proportions, the height of the peaks of each isotopic pair was similar. The mass differences between the isotopic pairs described earlier were 14, 16, and 18 Da, respectively. These values matched the theoretical values calculated for the oligosaccharides present in the mixture.

Development and validation of the quantitation method

The aim of these experiments was to develop a rapid procedure to measure differences and compare the relative amounts of specific oligosaccharides between two populations of oligosaccharides extracted from two different samples utilizing stable isotopic labeling. In practice, these two samples could be obtained, for example, from cells at two different developmental stages or tissues from wild-type and mice genetically null for a specific enzyme. The principle of the isotopic labeling procedure investigated in this study is presented in Figure 2. In this scheme, oligosaccharides are isolated from two populations (I and II) in which the amount of oligosaccharide B in population II differs. Each population of oligosaccharides is separately methylated with either ^{12}C or ^{13}C enriched methyl iodide, followed by combination of these two populations and MS analysis. Differences in the ion abundances between the isotopic pairs should reflect differences in the concentration between the two populations. In this
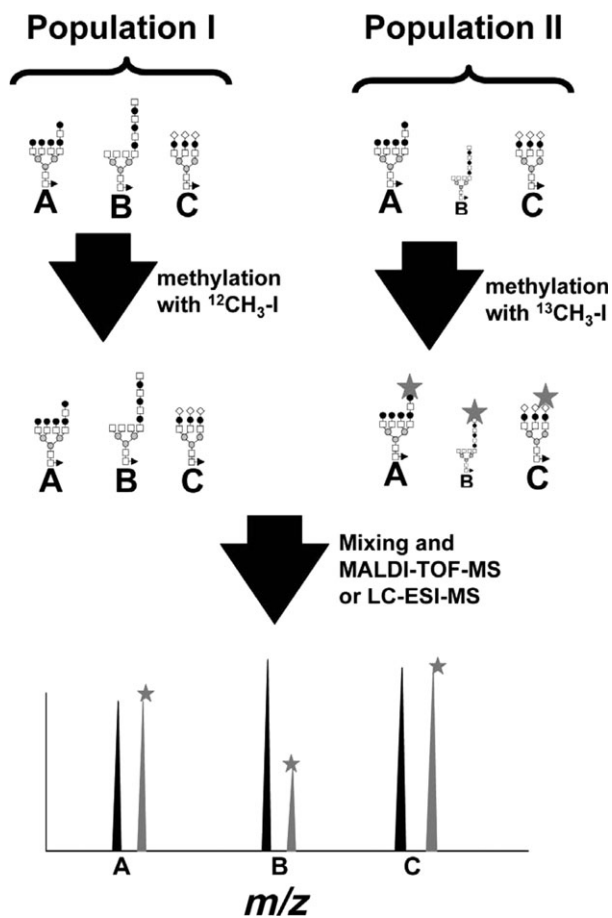
example, the amount of oligosaccharides A and C in both populations are the same, and therefore, their ^{12}C- and ^{13}C-labeled derivatives should have nearly identical peak areas. The lower abundance of the isotopically labeled oligosaccharide B in population II, however, is reflected by a decrease in its peak height.

Figure 3 shows that the difference in signal intensity among the components of the isotopic pair of the tetrasaccharides was consistent with the different proportions of ^{12}C and ^{13}C permethylated derivatives present in the mixtures. A similar pattern was observed in the measurements obtained from the pentasaccharide and hexasaccharide components of the spectrum (Table I). In order to test the reproducibility of the isotopic labeling procedure, MALDI-TOF-MS spectra were recorded after the isotopically labeled methylated oligosaccharides
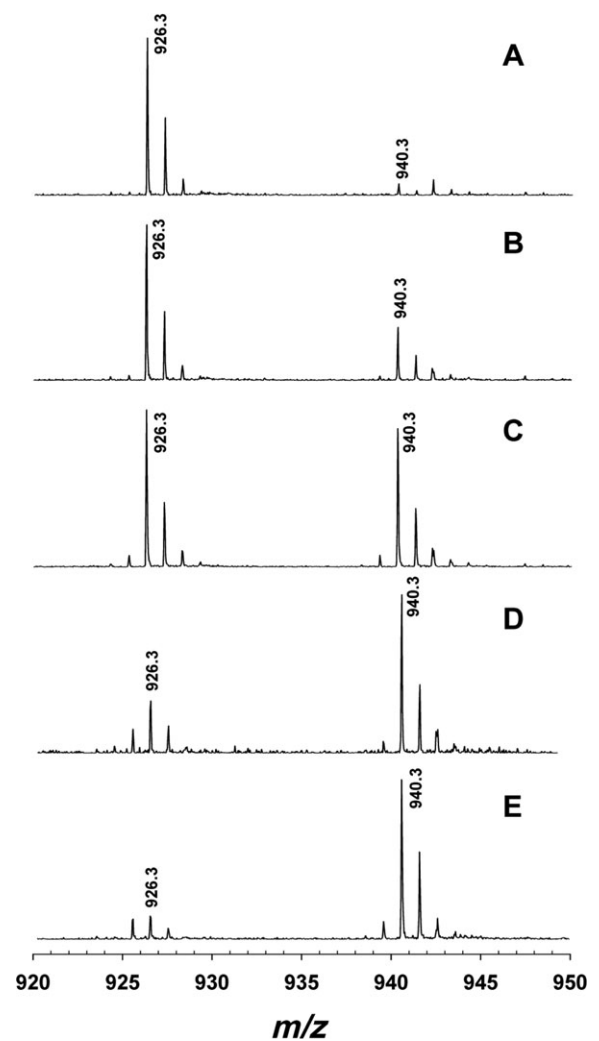

Fig. 3. MALDI-TOF MS spectra showing the isotopic pairs for the tetrasaccharide mixture (Gal$_2$-GlcNAc-Glc) labeled with different proportions of ^{12}C and ^{13}C methyl iodide. For this experiment, two aliquots with similar amounts of oligosaccharides from human milk were separately labeled with either ^{12}C or ^{13}C methyl iodide. Before MS, these aliquots were mixed in the following ^{12}C:^{13}C proportions: 9:1 (**A**), 7.5:2.5 (**B**), 1:1 (**C**), 2.5:7.5 (**D**), and 1:9 (**E**). The monoisotopic m/z for the ^{12}C- and ^{13}C-labeled glycans are 926.3 and 940.3, respectively. Quantitative data from the size of the monoisotopic peaks are shown in Table II for the isotopic pairs of all three oligosaccharides contained in the mixture.

Fig. 2. Scheme for the isotopic labeling procedure for the relative quantitation of oligosaccharides released from two glycoprotein populations.

Table I. Consistency and variability of the isotopic labeling procedure. Identical aliquots of neutral oligosaccharides from human milk were permethylated in triplicate, with ^{12}C or ^{13}C methyl iodide. Five mixtures were prepared with different proportions of ^{12}C/^{13}C methylated oligosaccharides and analyzed by MALDI-TOF-MS. The ^{12}C:^{13}C ratios were measured and compared to the theoretical values

Theoretical ^{12}C:^{13}C ratio	Structure	Measured ^{12}C:^{13}C ratio[a]	Standard deviation	CV (%)
9.00	Tetrasaccharide	9.41	0.829	8.8
	Pentasaccharide	9.16	1.077	11.8
	Hexasaccharide	10.78	1.542	14.3
3.00	Tetrasaccharide	3.26	0.419	12.9
	Pentasaccharide	3.50	0.876	25.0
	Hexasaccharide	3.53	0.616	17.4
1.00	Tetrasaccharide	1.04	0.083	8.0
	Pentasaccharide	1.09	0.023	2.1
	Hexasaccharide	1.06	0.026	2.4
	Tetrasaccharide	0.39	0.027	6.9
0.33	Pentasaccharide	0.30	0.066	21.8
	Hexasaccharide	0.33	0.046	13.6
	Tetrasaccharide	0.12	0.006	5.2
0.11	Pentasaccharide	0.11	0.021	18.4
	Hexasaccharide	0.10	0.014	13.3
Average				12.7

[a]Averaged from three measurements.

were mixed at different ^{12}C:^{13}C proportions in triplicate. Data from these measurements (Table I) showed that the observed ^{12}C:^{13}C proportions were consistent with the theoretical compositions of the different components of the mixture. The variability of the measurements was smallest [coefficient of variation (CV) less than or equal to 8.0] for the glycans mixed in a 1:1 ratio of ^{12}C:^{13}C and was the greatest (CV less than or equal to 25.0%) for the glycans mixed in a ratio of 3:1, 12C:13C. The average CV was 12.7%, and for most of the measurements shown in Table II, this value was smaller than 16.0%.

In order to illustrate the linearity of the procedure, triplicate measurements corresponding to data for the tetrasaccharide at different ^{12}C:^{13}C proportions were plotted against their respective theoretical values. The resulting graph (Figure 4) showed linearity with an R^2 of 0.9998 and a slope of 1.04 for this oligosaccharide over a dynamic range that was close to two orders of magnitude. Similar linearity was observed for the isotopic pairs of the pentassacharide and hexasaccharide components of the mixture (data not shown).

Quantitation of N-linked oligosaccharides from human α1-acid glycoprotein using ^{13}C labeling and MALDI-TOF MS

In order to test the isotopic labeling procedure developed in the section *Development and validation of the quantitation method*, a set of N-linked oligosaccharides was prepared from human α-1 acid glycoprotein (*h*-AGP), a 43 kDa protein abundant in human blood serum which contains five glycosylation sites (Shiyan and Bovin 1997). Oligosaccharides from this heavily glycosylated protein were

obtained by peptide *N*-glycosidase (PNGase F) digestion. One aliquot of these glycans was methylated with ^{12}C methyl iodide and the other aliquot was isotopically labeled with ^{13}C as described in the section *Development and validation of the quantitation method*. Then, the ^{12}C and ^{13}C methylated glycans were mixed and analyzed by MALDI-TOF MS. Figure 5A shows the MALDI-TOF-MS spectrum with the monoisotopic masses of a mixture of ^{12}C- and ^{13}C-labeled oligosaccharides mixed in a 1:1 proportion. In this spectrum, the isotopic pairs of seven oligosaccharides were observed. The isotopic pair at m/z 2792.7 and 2831.8 corresponds to a disialylated diantennary oligosaccharide (NeuAc$_2$ Hex$_5$ HexNAc$_4$). The pair at m/z 3603.2 and 3553.4 is a trisialylated triantennary structure (NeuAc$_3$ Hex$_6$ HexNAc$_5$), whereas the pair at m/z 3777.3 and 3829.5 is a monofucosylated trisialylated triantennary structure (NeuAc$_3$ Hex$_6$ HexNAc$_5$ Fuc$_1$). The next isotopic pair at m/z 4052.5 and 4108.7 is a trisialylated tetraantennary structure (NeuAc$_3$ Hex$_7$ HexNAc$_6$) and the pair at m/z 4226.6 and 4284.8 a monofucosylated trisialyl-tetraantennary structure (NeuAc$_3$ Hex$_7$ HexNAc$_6$ Fuc$_1$). The peaks at m/z 4413.7 and 4474.9 correspond to the isotopic pair of a tetrasialyl-tetraantennary oligosaccharide (NeuAc$_4$ Hex$_7$ HexNAc$_6$) and the peaks at m/z 4587.9 and 4651.1 are those of a monofucosylated tetrasialyl-tetraantennary structure (NeuAc$_4$ Hex$_7$ HexNAc$_6$ Fuc$_1$). Previous studies by other groups have found a similar oligosaccharide composition in this glycoprotein (Treuheit et al. 1992; Shiyan and Bovin 1997).

Figure 5B shows an enlargement of the region of the spectrum which corresponds to the isotopic pair of the trisialylated triantennary oligosaccharide. In this spectrum, the isotopic cluster of the ^{13}C methylated oligosaccharide shows the presence of two less abundant peaks that are 1 and 2 mass units smaller than the monoisotopic peak for the ^{13}C methylated glycan (m/z 3653.4). When the heights of all the peaks in the isotopic distribution are summed, these smaller peaks constitute about 11.0% of the cluster.

To test if these smaller peaks were due to the presence of a small amount of ^{12}C in the ^{13}C methyl iodide reagent (approximately 1% as reported by the vendor), the isotopic clusters of the trisialyl-triantennary oligosaccharide were modeled in silico from the theoretical isotopic proportions determined from the elemental composition of the oligosaccharide and the ^{12}C and ^{13}C methyl iodide reagents. When the modeled spectrum was overlaid with the spectrum that resulted from MALDI-TOF-MS analysis shown in Figure 5B, the simulated and experimental spectra were very similar for the ^{12}C isotopic cluster (Figure 6A), demonstrating that the experimental data was consistent with the simulated isotopic distribution. The simulated and experimental spectra of the ^{13}C isotopic cluster showed the presence of two peaks that were 1 and 2 m/z units smaller than the monoisotopic peak which constituted between 11 and 12% of the cluster (Figure 6B), confirming that these peaks resulted from the presence of a small amount of ^{12}C in the ^{13}C-labeling reagent. However, in contrast to the data for the ^{12}C cluster, the simulated spectrum showed significant differences with the experimental data in the height of the peaks of the isotopic distribution. This observation suggests that, in addition to the smaller peaks, the presence of ^{12}C in the ^{13}C methyl iodide reagent also affected the distribution of the isotopic

Table II. Relative quantification of the isotopic pairs present in the 1:1 mixture of oligosaccharides methylated with ^{12}C and ^{13}C methyl iodide and analyzed by MALDI-TOF-MS (spectrum shown in Figure 5). □-GlcNAc, ○- Gal, ○- Man, ◇-NeuNAc, and ◄ Fuc

Glycan structure	[12]C m/z[a] measured (estimated)	[13]C m/z[a] measured (estimated)	Δ Mass[b]	Calculated [12]C:[13]C ratios from mono-isotopic peak height	Calculated [12]C:[13]C ratios from sum of peaks
	2792.7 (2792.4)	2831.8 (2831.4)	39.0	0.99	1.15
	3603.2 (3602.8)	3653.4 (3652.8)	50.0	0.99	1.24
	3777.3 (3776.8)	3829.5 (3828.8)	52.0	0.88	1.14
	4052.5 (4052.0)	4108.7 (4108.0)	56.0	1.16	1.29
	4226.6 (4226.1)	4284.8 (4284.1)	58.0	1.27	1.27
	4413.7 (4413.2)	4474.9 (4474.2)	61.0	0.64	1.20
	4587.9 (4587.2)	4651.1 (4650.2)	63.00	0.64	1.10
Average ^{12}C/^{13}C ratios				0.94	1.20
Standard deviation				0.24	0.07
CV				25.7%	6.0%

[a]Values correspond to monoisotopic masses.
[b]Difference between the monoisotopic masses of the ^{13}C and ^{12}C methylated derivatives.
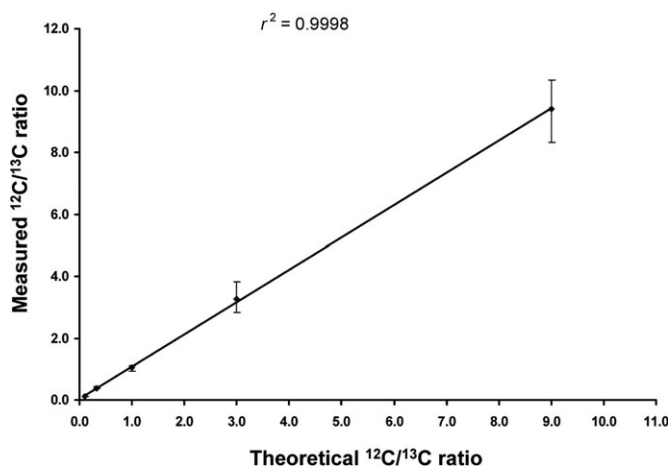
Fig. 4. Comparison of the measured ^{12}C/^{13}C ratios with their respective theoretical values. A straight line ($r^2 = 0.9998$) was observed when the ^{12}C/^{13}C ratios of the tetrasaccharide (Gal$_2$-GlcNAc-Glc) from permethylated human milk oligosaccharide mixtures that were analyzed by MALDI-TOF-MS in triplicate were graphed against their theoretical ^{12}C/^{13}C mix ratios. Error bars represent the difference between the highest and the lowest values measured for each ratio.

cluster of the ^{13}C-labeled oligosaccharide. This tendency was also observed for the rest of the oligosaccharides present in the mixture (data not shown).

Table II shows the relative glycan quantification obtained from the intensities of the peaks in the isotopic pairs detected in Figure 5, expressed as ^{12}C/^{13}C ratios. When this ratio was calculated from the peak heights of the monoisotopic masses of each component of the isotopic pair, the estimated ^{12}C/^{13}C ratios were close to the theoretical 1:1 proportion for the two smaller oligosaccharides. As the size of the glycans increased, the difference between the theoretical and measured ratios also increased, and for the two largest oligosaccharides, the difference between theoretical and experimental ratios was more than 35%. The overall dispersion in the relative quantitation values calculated from the height of the monoisotopic peaks had a CV of 25.7%.

Since the simulated data presented in Figure 6 suggested that the height of the monoisotopic peak in the ^{13}C-labeled glycan is affected by the presence of ^{12}C in the isotopic labeling reagent, and that this effect is dependent on the mass of the glycan, a second approach was taken for the relative quantitation of the labeled glycan populations in which the ^{12}C/^{13}C ratios were calculated from the sum of the heights of all the peaks of the isotopic distribution of each component of the isotopic pair. The resulting ^{12}C/^{13}C ratios, presented in Table II, showed less variation
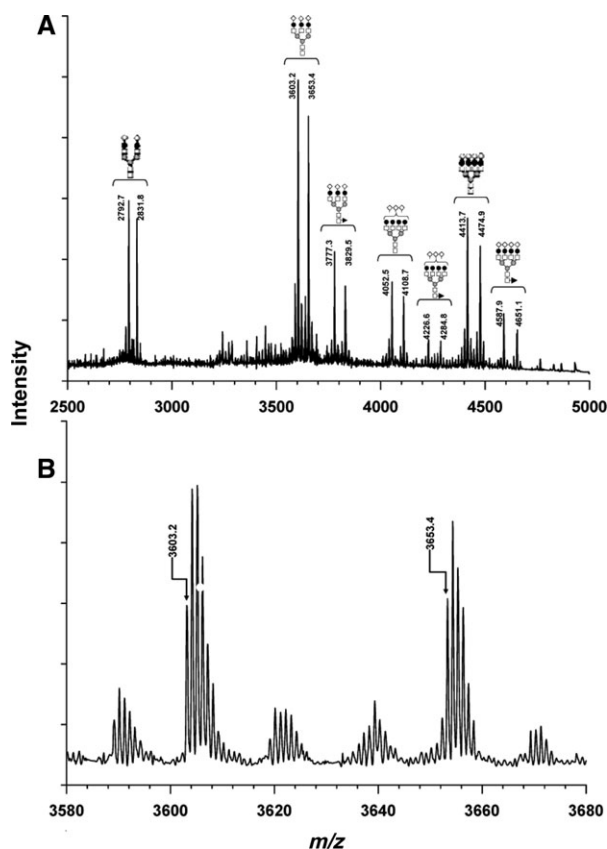
Fig. 5. Isotopic labeling of oligosaccharides from human α-1 acid glycoprotein. (**A**) A MALDI-TOF MS spectrum showing the isotopic pairs of a 1:1 ^{12}C:^{13}C mixture of oligosaccharides released from human α-1 acid glycoprotein by PNgase F treatment. Quantitative data for the isotopic pairs are shown in Table III. (**B**) A region of the spectrum was enlarged that corresponds to the trisialylated- triantennary oligosaccharide (NeuAc$_3$ Hex$_6$ HexNAc$_5$). This spectrum shows the isotopic clusters of the ^{12}C and ^{13}C methylated derivatives of this oligosaccharide (m/z 3603.2 and 3643.4 respectively). □-GlcNAc, ●- Gal, ●- Man, ◇-NeuNAc, and ◀-Fuc.

(CV = 6.0%). These results indicate that the sum of the peak heights of the isotopic distributions is a more useful method for estimating reliable ^{12}C/^{13}C ratios of isotopically labeled glycan mixtures.

Quantitation of N-linked oligosaccharides from glycoprotein mixtures using ^{13}C labeling and ESI–FT-MS analysis

In order to test the this method using glycoprotein mixtures, two preparations of ^{12}C- and ^{13}C-labeled N-linked oligosaccharides released from different glycoproteins were prepared by permethylation and analyzed by ion cyclotron resonance Fourier transformation-MS with ESI–FT-MS. In addition to *h*-AGP, two additional glycoproteins were mixed in these experiments: human transferrin (h-tf) a approximately 90 kDa protein that possesses two N-glycosylation sites occupied by sialylated diantennary oligosaccharides (Yamashita et al. 1993), and bovine fetuin (b-fet), a 43 kDa protein with three N-glycosylation sites that are occupied predominantly with sialylated triantennary oligosaccharides (Yet et al. 1988). For these experiments, ^{12}C- and ^{13}C-labeled fractions were prepared with the oligosaccharides released from two identical aliquots of mixed glycoproteins that contained 1 mg
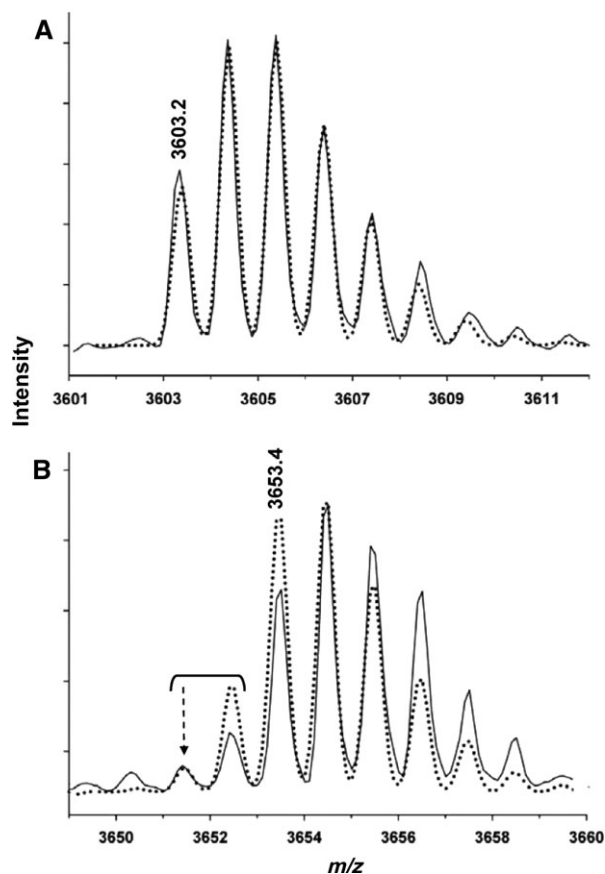

Fig. 6. Comparison of ^{12}C and ^{13}C isotopic clusters of the MALDI-TOF-MS spectrum from the trisialylated triantennary glycan obtained from experimental and simulated data. In order to evaluate the effect of a small amount of ^{12}C (approximately 1.0%) in the ^{13}C methyl iodide label in the isotopic distribution of the methylated oligosaccharides, the isotopic cluster of the ^{13}C-labeled trisialylated triantennary oligosaccharide (solid spectrum) was overlaid on an isotopic cluster modeled in silico from the theoretical composition of isotopes of the glycan and the methyl iodide label (dotted spectrum). The monoisotopic peak (m/z 3653.4) is indicated and the peaks under the bracket that are 1 and 2 m/z units smaller that the monoisotopic peak, reflect the presence of ^{12}C in the isotopic label.

(approximately 23 nmol) of *h*-AGP, 1 mg (approximately 26 nmol) of b-fet, and 1 mg (approximately 11 nmol) of h-tf. Then, two isotopic mixtures were prepared at ^{12}C:^{13}C ratios of 1:3 (0.33) and 1:1 (1.00). Each mixture was analyzed by ESI–FT-MS.

The resulting spectra are presented in Figure 7. These spectra revealed more than one ionization state for some of the oligosaccharides in the mixture. For example, the disialylated diantennary oligosaccharide showed two isotopic distributions that were doubly and triply charged (monoisotopic m/z values of the ^{12}C-labeled ions were 1407.7 and 946.1 respectively). The trisialylated triantennary structure showed two ion species with three and four positive charges (with ^{12}C m/z values of 1216.3 and 918.0 respectively), while the tetrasialylated- triantennary structure showed two ion species ions that had three (^{12}C m/z of 1336.9) and four charges (^{12}C m/z of 1008.5). The trisialyl- tetraantennary oligosaccharide gave two ions with ^{12}C m/z values of 1365.9 and 1030.3, having three and four charges, respectively.
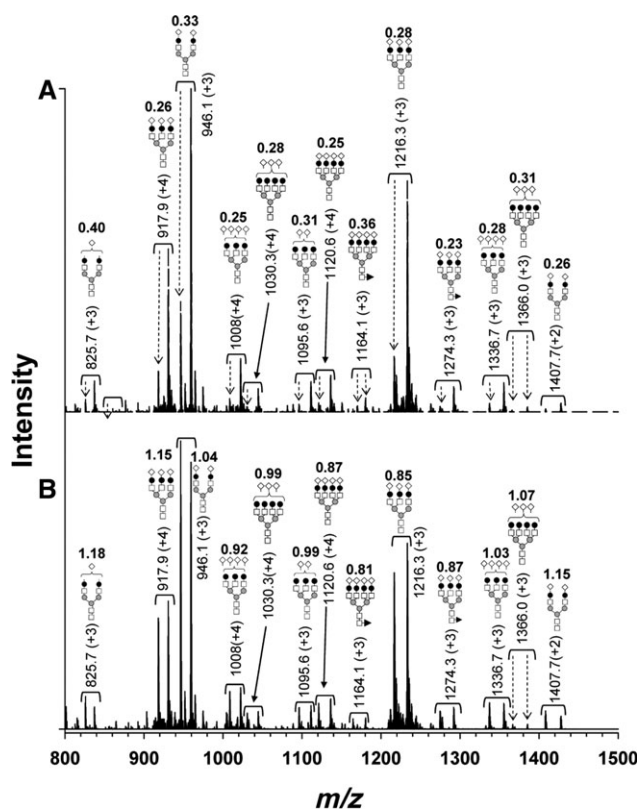
Fig. 7. ESI–MS mass spectrum of isotopically labeled oligosaccharides from a mixture of α-1 acid glycoprotein, b-fet and h-tf. (**A**) A MALDI-TOF MS spectrum showing the isotopic pairs of a 1:3, ^{12}C:^{13}C mixture of oligosaccharides is shown. (**B**) A 1:1, ^{12}C:^{13}C mixture. The monoisotopic m/z values of the ^{12}C species, the structures and the experimental ^{12}C:^{13}C ratios are indicated for each detected isotopic pair. □-GlcNAc, ●-Gal, ●-Man, ◇-NeuNAc, and ◄-Fuc.

Table III. Relative quantification of oligosaccharides released from mixtures of glycoproteins and then labeled with ^{12}C or ^{13}C methyl iodide. (□-GlcNAc, ●- Gal, ●- Man, ◇-NeuNAc, and ◄- Fuc)

Structure	z	m/z[a]	Mass[a]	^{12}C/^{13}C mixture I	^{12}C/^{13}C mixture II
	3	825.8	2431.3	1.07	1.01
	2	1407.7	2792.5	1.00	1.16
	3	946.5	2793.4	1.00	1.01
	3	1095.8	3241.5	1.31	1.21
	3	1216.3	3605.5	1.35	1.37
	4	918.0	3602.8	1.40	1.42
	3	1274.7	3778.0	1.00	2.85
	3	1336.9	3964.6	5.73	1.04
	4	1008.5	3964.9	6.63	1.03
	3	1365.9	4051.7	1.03	3.54
	4	1030.3	4052.4	1.08	3.32
	4	1120.8	4414.1	1.02	2.47
	4	1163.8	4586.3	1.18	3.0

[a]Values correspond to monoisotopic masses of the ^{12}C-labeled oligosaccharide measured by FT-MS.

The spectrum of the mixture combined at a 1:3 (0.33) proportion of ^{12}C:^{13}C (Figure 7A) showed a smaller size of the ^{12}C isotopic cluster with an average ^{12}C:^{13}C ratio of 0.29 and a CV of 16.2%. The spectrum of the 1:1 proportion of ^{12}C:^{13}C showed similar sizes of the ^{12}C and ^{13}C isotopic clusters with an average ^{12}C:^{13}C ratio of 0.97 and a CV of 12.4%. These spectra showed that the ^{12}C:^{13}C ratios obtained from these measurements are consistent with their respective theoretical values.

In order to test if the isotopic labeling method could detect differences of relative glycan concentrations between two samples, two ^{12}C- and ^{13}C-labeled methylated glycan mixtures were prepared for analysis by ESI–FT-MS. In Mixture I, the ^{12}C-labeled glycan portion was prepared from the PNGase F released oligosaccharide fraction of 1 mg (approximately 23 nmol) of h-AGP, 1 mg (approximately 26 nmol) of b-fet and, 1 mg (approximately 11 nmol) of h-tf. The ^{13}C portion of this mixture was obtained from the deglycosylation of a combination of 1 mg (approximately 23 nmol) of h-AGP, 0.2 mg (approximately 5 nmol) of b-fet, and 1 mg (approximately 11 nmol) of h-tf. Similar to Mixture I, Mixture II also contained a ^{12}C methylated portion with the glycans obtained from 1 mg (approximately 23 nmol) of h-AGP, 1 mg (approximately 26 nmol) b-fet and 1 mg (approximately 11 nmol) of h-tf. However, this mixture contained a ^{13}C

methylated fraction with 0.3 mg of h-AGP (approximately 8 nmol), 1 mg (approximately 26 nmol) of b-fet and 1 mg (approximately 11 nmol) of h-tf. On the basis of the proportions described earlier, in Mixture I the oligosaccharides from b-fet should have a theoretical ^{12}C:^{13}C ratio of 5.00, while h-AGP and h-tf have a ratio of 1.00. On the other hand, in Mixture II the oligosaccharides from h-AGP should have a theoretical ^{12}C:^{13}C ratio of 3.00 and the ratios for b-fet and h-tf are 1.00.

Table III shows the relative glycan quantitation of the mixtures described earlier expressed as ^{12}C:^{13}C ratios. In Mixture I, the diantennary oligosaccharides that are released mainly from h-tf, as well as the monofucosylated- trisialylated triantennary oligosaccharides and the tetraantennary structures, which were released exclusively from h-AGP showed a ^{12}C:^{13}C ratio that was very close to 1.00. In contrast, the di- and tri-sialylated triantennary oligosaccharides, which were released from both h-AGP and b-fet, showed a ^{12}C:^{13}C ratio that ranged from 1.30 and 1.40. Moreover, the tetrasialyl- tetraantennary structure, expressed exclusively in b-fet, showed an average ^{12}C:^{13}C ratio of 6.18 for the two charge states detected in the spectrum. Figure 8 presents the ESI–FT-MS spectrum obtained from Mixture I, in which two regions that show the isotopic distributions of the tetrasialyl triantennary from b-fet have been enlarged.
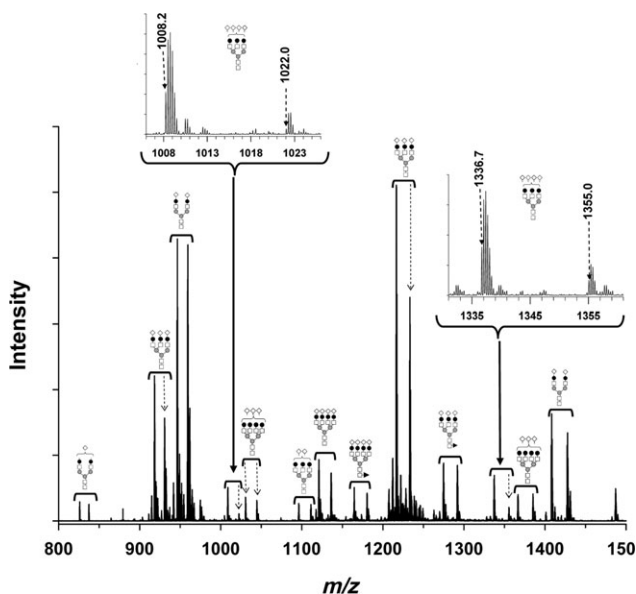
Fig. 8. Relative quantitation of a mixture of [12]C- and ^{13}C-labeled oligosaccharides released from three different glycoproteins. The ^{12}C portion of the fraction contained the oligosaccharides released from a mixture of 1 mg (approximately 23 nmol) of α-1 acid glycoprotein (*h*-AGP), 1 mg (approximately 26 nmol) of bovine fetuin (b-fet) and 1 mg (approximately 11 nmol) of human transferring (h-tf). The ^{13}C portion of this mixture was obtained from the deglycosylation of a combination of 1 mg (approximately 23 nmol) of *h*-AGP, 0.2 mg (approximately 5 nmol) of b-fet and 1 mg (approximately 11 nmol) of h-tf. The two enlarged areas show the isotopic distributions for the ions with +3 ([12]C *m/z* 1336.7) and +4 ([12]C *m/z* 1008.2) charges of the tetrasialylated triantennary oligosaccharide from b-fet, which shows a [12]C:[13]C ratio much larger than the rest of the oligosaccharides in the mixture. □- GlcNAc, ●- Gal, ⬤-Man, ◇-NeuNAc, and ◄- Fuc.

Similar to the results for Mixture I, the FT-MS spectrum of Mixture II showed a [12]C:[13]C ratio of approximately 1.00 for the diantennary structures (presumably from h-tf), and between 1.21 and 1.42 for the disialylated and trisialylated triantennary structures (from *h*-AGP and b-fet). However, the tetrasialyl-triantennary oligosaccharide (exclusively from b-fet) showed a [12]C:[13]C ratio of 1.00. The monofucosyl-trisialyl-triantennary and the tetraantennary structures showed [12]C:[13]C ratios that ranged between 2.47 and 3.54.

The results presented above indicated that the measured [12]C:[13]C ratios reflected the differences in the glycoprotein concentration among the ^{12}C and ^{13}C portions of the mixtures and that these values were consistent among the different charge states presented by some of these oligosaccharides. For example, the oligosaccharides that were present predominantly or exclusively in one glycoprotein (i.e., the diantennary structures from h-tf, the tetrasialyl-triantennary structure from b-fet, and the monofucosyl-trisialyl triantennary as well as the tetraantennary structures from *h*-AGP) showed either a [12]C:[13]C ratio of approximately 1.00, which reflected no difference in the glycoprotein concentration between the ^{12}C and ^{13}C portions, or a large [12]C:[13]C ratio which reflected substantial differences in the concentration of that particular glycoprotein in the ^{13}C methylated glycan pool. The oligosaccharides that were present in two of the glycoproteins of the mixture (i.e., the di- and trisialyl-triantennary structures present in both *h*-AGP and b-fet) showed [12]C:[13]C ratios that reflected moderate differences in the concentration of these glycans

among the isotopic pools. These results indicated that the isotopic labeling procedure developed in this report is useful for detecting changes in the relative oligosaccharide concentration between two samples.

Discussion

Given the important functions that glycoprotein glycans play in many physiological contexts, the knowledge of hundreds of genes that encode for enzymes involved in the glycosylation biosynthetic machinery has allowed the quantitation by reverse transcription polymerase chain reaction (RT–PCR) or DNA arrays of changes in the expression of transcripts from these genes (Comelli et al. 2006). Understanding how relative expression levels of individual glycosylation genes affect the levels of actual oligosaccharide structures found in a cell or tissue is still a major challenge in the field of glycoscience. A technique that allows the rapid, quantitative analysis of the changes in the oligosaccharide repertoire of a cell or tissue is critical, therefore, to begin to integrate glycogene expression with glycan structural analysis. Moreover, such technique must possess high sensitivity and resolving power in order to detect subtle changes in glycan expression.

An example of such an analytical technique is the use of fluorescent tags for labeling of glycans (Rice 2000) which are then analyzed by high-performance liquid chromatography (HPLC) (Nakagawa et al. 1995) or capillary electrophoresis and fluorescence detection (Oefner and Chiesa 1994; Zieske et al. 1996). The fluorescent tags most frequently used in this approach are 2-aminopyridine (Hase 1994), 2-aminobenzamide (Townsend et al. 1996), and anthranilic acid (Anumula and Dhume 1998), although there are other fluorescent groups suitable for glycan labeling (Rice 2000). The main advantages of the fluorescent tagging-HPLC method for glycan analysis are its sensitivity and the ability to obtain quantitative data with good precision and reproducibility. The significant drawbacks of this procedure are that the derivatization procedure is time consuming and requires the availability of standards for every glycan to be identified. Additionally, the resolution of this procedure is dependent on the HPLC or capillary electrophoresis column, which in many instances is not capable of resolving all the structures in the sample and therefore is limited.

Another approach for glycan analysis is the use of MS, particularly of ESI-MS and matrix assisted laser desorption/ionization-time of flight (MALDI-TOF)-MS. During the last decade, these mass spectrometric techniques have found a wide array of applications in complex carbohydrate analysis and characterization (Dell et al. 1994; Novotny and Mechref, 2005; Wuhrer et al. 2005; Budnik et al. 2006; Morelle et al. 2006). Advantages of these mass spectrometric techniques are their speed, sensitivity, and high resolution. The inability to utilize mass spectrometric techniques for glycan quantitation purposes, however, is an important limitation in the use of these techniques for glycoproteomic studies.

Mass spectrometric analysis of isotopic labeled peptides has been very useful in quantitative proteomics (Tao and Aebersold 2003), and the advantages of this technique can be applied to glycan analysis. Isotopic labeling provides quantitative data by comparing the abundance of particular components in two molecular populations, one labeled with a

"heavy" isotope, and the other with a "light" isotope. The resulting mass difference determined by MS provides a method to distinguish isotopic clusters from each population of molecules which are otherwise identical, thus allowing relative quantitation of specific components with very good resolution. It should be noted, however, that isotopic labeling does not provide information on the absolute amount of a compound and is useful when two samples are being compared.

In a recent study, hexadeuterium 2-aminopyridine was used to generate tetradeuterium-labeled pyridylamino oligosaccharides which were then analyzed by HPLC-ESI-MS (Yuan et al. 2005). This approach presented the advantage that the oligosaccharides could be separated in a chromatography column coupled to the mass spectrometer, thus increasing substantially the resolution. This technique had the disadvantage, however, that only the reducing end of the oligosaccharides were modified and, therefore, sialylated structures would still ionize less efficiently than neutral glycans, which could result in a heterogeneous signal from the components of a sialylated oligosaccharide mixture.

In the present study, a procedure was developed for the relative quantitation of glycans from two different samples by MS, in which a population of glycans was labeled with ^{13}C methyl iodide using standard permethylation techniques. The ^{13}C-labeled methylated glycans were then mixed with another population of ^{12}C methylated glycans, and the mixture was analyzed by MALDI-TOF-MS or ESI–FT-MS. The results presented here demonstrate that this procedure was capable of providing accurate, relative quantitative data on the differences in the abundance of oligosaccharides between two samples. The variability of the data obtained by using this technique was small. Using a mixture of oligosaccharides from human milk, we were able to obtain a CV smaller than 25%; and in most measurements, this value was smaller than 15% (Table I). This reproducibility is similar to that reported for isotopic labeling techniques for the relative quantitation of proteins (Washburn et al. 2002; Hsu et al. 2003; Hicks et al. 2005).

Regarding its sensitivity, this method provided adequate linearity over a dynamic range of two orders of magnitude and the data showed strong reproducibility among the different batches of human milk oligosaccharide mixtures that were analyzed. When the procedure was tested with oligosaccharides released from glycoproteins, the results revealed that the presence of a small amount of ^{12}C in the ^{13}C-labeled methyl iodide reagent affected the isotopic distribution of the ^{13}C-labeled glycans and thus, the height of the monoisotopic peak. Therefore, in order to estimate an accurate ^{12}C:^{13}C ratio, the sum of the heights of the peaks in the isotopic clusters was utilized to compute the ratios.

The isotopic labeling procedure was tested in oligosaccharide mixtures analyzed by two different modes of MS, MALDI-TOF-MS and ESI–FT-MS. Similar to other isotopic labeling techniques reported in proteomics, moderate variations in the ^{12}C:^{13}C ratios were observed among the components of individual samples (Washburn et al. 2002; Hsu et al. 2003; Hicks et al. 2005). These differences can be attributed to factors such as the manipulation of submicroliter volumes, the presence of contaminants in the sample or fluctuations in the ionization of the oligosaccharide fragments during the MS analysis. However, the resulting spectra showed

uniformity between the theoretical and experimental ^{12}C:^{13}C ratios and the method was able to detect variations in the relative abundance of specific oligosaccharides consistently.

The results presented in this study demonstrate that the isotopic labeling technique possessed all the advantages of the methylation reaction for oligosaccharide analysis such as its high resolution and a more homogenous ionization of all the acidic and neutral glycans in the sample, adding the ability of this reaction to be used for quantitative purposes. Moreover, this procedure has the added advantage of generating larger mass differences between the components of the isotopic pairs, thereby facilitating their assignment. For these reasons, the isotopic labeling procedure described here is an important step in the integration of quantitative glycan expression data with those obtained from measuring the levels of glyco-gene transcript expression. Once these types of glycomic data are generated from a large number of samples from different tissues, cell lines, and organisms at different developmental stages, it may be possible ultimately to predict with reasonable accuracy the glycan expression patterns of a particular cell population based on the patterns of glyco-transcript expression. This information will make it possible to better understand the function of the glycosylation machinery and its role in physiological processes such as development and disease progression.

Recent studies have shown that permethylated oligosaccharides can be fractionated efficiently by HPLC using a C-18 reverse phase column (Delaney and Vouros 2001). The possibility of coupling the isotopic labeling procedure described in this study to LC-ESI-MS analysis should increase substantially the resolution and sensitivity of this procedure for quantitating relative amounts of glycans in a sample.

Materials and methods

Isolation of oligosaccharides

Neutral oligosaccharides (tetrasaccharide to hexasaccharide) were isolated from human milk as described in (Kobata et al. 1978). Briefly, milk was delipidated by centrifugation and protein was precipitated with 68% ethanol overnight at 0°C. Supernatant was filtered and chromatographed on Sephadex G-25. The voided volume, which contained oligosaccharides larger than fucose, was pooled and applied to a DEAE-cellulose column to separate neutral from sialylated oligosaccharides. This oligosaccharide fraction contained a mixture of lacto-N-tetrasaccharides (Hex$_3$ HexNAc) lacto N-fucopentaoses (Hex$_3$ deoxyHex HexNAc) and lacto-N-difucohexaoses (Hex$_3$ deoxyHex$_2$ HexNAc). The neutral oligosaccharides were lyophilized and dissolved in water at a concentration of 1 mg/mL.

Oligosaccharides were released from mixtures of human α-1 acid glycoprotein (h-AGP, Sigma, St. Louis, MO), b-fet (b-fet, Sigma), and (h-tf, Research Diagnostics International, Flanders, NJ) by dissolving 1 mg of protein mixture in 47.5 μL of 0.2 M Tris, pH 7.5, with 0.1% sodium dodecyl sulfate (SDS) and 0.1 M β-mercaptoethanol. The dissolved protein was then denatured by incubating at 100°C for 7 min. The mixture was cooled and NP-40 was added to a concentration of 0.5%. To achieve deglycosylation, 1500 units of PNGase F (New England BioLabs, Ipswich, MA) were added

and the reaction was incubated overnight at 37°C. The reaction was stopped by adding 250 µL of 5% acetic acid and applied to a 60 mg OASIS-HLB solid phase extraction catridge (Waters, Milford, MA), previously activated with 1 mL of methanol and equilibrated with 2 mL of water. The glycans were eluted with 1 mL of water and evaporated to dryness under reduced pressure.

Exhaustive methylation of glycans and MS analysis

Dried glycans (30 µg aliquots) were permethylated with modifications of the procedure by Ciucanu and Kerek (Ciucanu and Kerek 1984). Glycans were suspended in dimethyl sulfoxide (DMSO) (0.1 mL) and NaOH (20 mg in 0.1 mL of dry DMSO) was added. After strong mixing, 0.1 mL of ^{12}C- or ^{13}C-labeled methyl iodide (Aldrich, St Louis, MO) were added. According to the manufacturer, ^{13}C-labeled methyl iodide contained 99% of the ^{13}C isotope. After 10 min incubation in a bath sonicator 1 mL of water was added, and the excess of methyl iodide was removed by bubbling with a stream of N_2. One milliliter of methylene chloride was added with vigorous mixing, and after phase separation the upper aqueous layer was removed and discarded. The organic phase was then extracted three times with water. Methylene chloride was evaporated under a stream of N_2, and the methylated glycans were dissolved in 25–50 µL of 50% methanol. Before MS analysis, ^{13}C- and ^{12}C-labeled methylated glycans were mixed in different ^{12}C:^{13}C proportions, and each of these mixtures was evaporated to dryness.

Before MALDI-TOF-MS analysis, mixtures of methylated oligosaccharides from human milk were redissolved in 100 µL of 80% acetonitrile and the mixtures of methylated glycans from h-AGP were dissolved in 25 µL of 50% methanol, 1 mM NaOH. The MALDI-TOF-MS matrix was prepared by dissolving 13 mg of dihydrohybenzoic acid (DHB, Sigma) in 1 mL 50% acetonitrile in water. Then, 0.5 µL of methylated glycan sample were mixed with 0.5 µL of matrix solution and 0.5 µL of the mixture were applied to the MS probe and crystallized by evaporating solvents at room temperature. The samples were then analyzed in an Applied Biosystems 4700 Proteomics Analyzer working in TOF-reflector mode.

For the analysis of oligosaccharides by ESI, the dried methylated glycan ^{12}C:^{13}C mixtures were dissolved in 25 µL of 50% methanol and then diluted 1:25 in a solution that contained 50% methanol and 1 mM NaOH. The glycans were then analyzed on a hybrid linear ion trap Fourier transform mass spectrometer (LTQ-FT, Thermo Fisher Scientific, Waltham, MA). Each glycan mixture was infused into the LTQ-FT at a flow rate of 0.3 µl/min and electrosprayed through a 15 µm pulled silica capillary (New Objective, Woburn, MA) at 1.9 kV. MS^n experiments in the LTQ were carried out in positive ion and profile mode using a normalized collision energy of 29%, activation Q of 0.25, and activation time of 30 ms. FTMS experiments were carried out using a mass range of 500–2000 Da and a resolution of 50 000.

Computer simulation of isotopic clusters from mass spectra

The isotopic distributions in the mass spectra of methylated oligosaccharides were simulated by an algorithm (WS York, unpublished) that calculates the probability of each isotopomer and uses the results to calculate the theoretical abundance versus m/z function. Input parameters include the glycosyl composition of the oligosaccharide, the natural isotope abundances of each element in the oligosaccharide, and the enriched isotope abundances in the chemically added methyl groups. The abundance of each group of isotopomers that have the same isotopic composition is calculated using probability theory, taking into account the number W of distinct isotopomers that have this isotopic composition. (W is the well-known parameter from statistical mechanics that describes the number of ways of arranging N objects with n1 in one group, $n2$ is the second group, etc.) The isotopomer theoretical populations are then used to calculate a theoretical mass spectrum based on Gaussian distributions of ions centered at m/z of each theoretical group of isobaric ions. The peak width and digitization for this simulation are chosen to match the experimental data that is being evaluated.

Conflict of interest statement

None declared.

Abbreviations

b-fet, bovine fetuin; CV, coefficient of variation; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; FT-MS, Fourier transformation mass spectrometry; h-AGP, human α1-acid glycoprotein; HPLC, high-performance liquid chromatography; h-tf, human transferrin; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MS, mass spectrometry; PNGase F, peptide-N-glycoamidase F; RT–PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate.

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