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Method for the Comparative Glycomic Analyses of O-Linked, Mucin-Type Oligosaccharides

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A method is presented for the direct relative quantitation of distinct O-linked mucin-type oligosaccharides. Mucin-type oligosaccharides are found in a host of tissues from anuran to humans. Because they are often associated with extracellular matrix, they play important roles in cell−cell recognition. Changes in glycosylation of O-linked oligosaccharides are associated with diseases such as cancer. In fertilization, they play an active role in sperm−egg recognition. We describe a method for observing changes in glycosylation of mucin-type oligosaccharides by incorporating deuterium in the release procedure. Oligosaccharides from two different sources are released separately by sodium tetrahydroborate and sodium tetradeuteroborate. The oligosaccharides are combined and separated into components by HPLC. By observing the ratio of deuterated and undeuterated species, changes in glycosylation are precisely quantified. This method is illustrated with mucin-type oligosaccharides from the egg jelly coat of the anuran *Xenopus laevis*.

The characterization of protein-bound glycans is essential for elucidating the complete structures and understanding the functions of glycoproteins. Glycosylation is the most common form of posttranslational modification. It is estimated that more than 50% of all proteins are glycosylated.[1] Although there is a number of recently developed methods in the fast-evolving field of proteomics for quantitative protein profiling using mass spectrometry,[2] there is effectively no analogous method available for quantitative glycomics.

The term glycomics is analogous to genomics and proteomics and can be used to define the studies of glycans released from an organism or simply a single source. In general, the analytical tools available for glycomics lag that of proteomics because of the inherent difficulties in analyzing oligosaccharides. Unlike the linear structures of proteins and nucleic acids, the structures of glycans are often branched. The complete structural characterization of an oligosaccharide requires not only composition and sequence but also the anomeric character of the glycosidic bond and the position of the linkage. Furthermore, the glycan library released from a tissue or an organism is usually composed of a complicated mixture of oligosaccharides. These factors all contribute to complicate significantly the analysis of glycan structures. A significant effort therefore has focused on these problems.[3]

Quantitative analysis of oligosaccharides has not been widely investigated but is critical for observing changes in glycosylation and expression. A number of studies have been reported for the quantitative determination of oligosaccharides employing different techniques. They rely mainly on HPLC, capillary electrophoresis (CE), ion-exchange chromatography, laser-induced fluorescence (LIF), and MALDI-MS. A method for quantitative analysis of saccharides was introduced by Chen et al. based on the combination of chemical and enzymatic methods coupled with CE separation and LIF detection.[4] This method was demonstrated for the quantitation of *N*-acetylneuraminic acid containing oligosaccharides derived from bovine fetuin. Detection limits of ∼100 pmol were obtained. Routier et al. compared several chromatographic methods including HPLC and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) for the quantitation of oligosaccharides released from immunoglobulin G (IgG).[5] Based on these methods, they further provided characterized standards for use as references for the calibration of other IgG. Wang et al. presented a MALDI-MS method for the quantitative analysis of oligosaccharides found in food.[6] They also compared the quantitation capabilities of MALDI-MS and HPAEC-PAD and concluded that MALDI-MS reflected the true amount of individual oligosaccharides more accurately in the food samples, while the PAD response with HPAE varied a great deal with the samples.

Among those techniques, MALDI-MS shows significant promise due to its high sensitivity specifically with oligosaccharides. Chromatography such as HPLC and other separation methods such as capillary electrophoresis often require derivatization while MALDI-MS does not, but it still provides detection limits in the region of pico- to femtomoles. Quantitative measurement of oligosaccharides using MALDI-MS is based on the linear concentration−response relationship for the analyzed sample. Ideally, the intensity or response of an analyte should be linearly correlated

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to its relative amounts in the MALDI-MS sample. Harvey[7] studied the relationship between the signal strength of a biantennary N-linked glycan and the amount deposited onto the MALDI target and concluded that the signal strength of glycan ionized by MALDI appears to reflect accurately the amount of material on the target, provided that the correct matrix is used. In their experiment, the matrix 2,5-dihydroxybenzoic acid (DHB) displayed a linear correlation (correlation coefficient 0.999) between amount and signal intensity, while 3-amino-4-hydroxybenzoic acid did not and exhibited some saturation effects.[7] To overcome the limitation of matrixes, Bartsch et al. used maltohexaose as an internal standard to quantify the amount of cyclodextrins in the sample. They obtained a similar linear response between sample size and ion intensity when α-cyano-4-hydroxycinnamic acid was used as the matrix.[8] They examined the use of peak heights and peak areas and found no significant differences. Similar relationships were also found for N-linked glycans when 2,6-dihydroxyacetophenone (DHAP) was used as matrix by Pitt and Gorman.[9]

For mixture analyses, it is essential that similar responses be obtained from each constituent that is independent of the molecular weight and structure. For similar types of oligosaccharides, e.g., linear and neutral oligosaccharides, MALDI appears to be sufficient. Stahl et al. reported that no mass-dependent variation in ion abundances had been observed up to 10 000 Da with DHB as matrix.[10,11] Wong et al. used MALDI-MS to quantitate desialylation reaction products.[12] Quantitation of more complex mixtures is complicated by the structural heterogeneity present in samples derived from biological sources. Neutral and anionic oligosaccharides, for example, have widely differing properties that manifest themselves during the release, separation, and ionization. Anionic oligosaccharides do not respond well in the positive mode, while neutral oligosaccharides do not respond well in the negative mode. Even different types of anionic oligosaccharides have differing ionization behavior. Sulfated oligosaccharides tend to suppress sialylated oligosaccharides by as much as 1 order of magnitude.[13]

In this report, a quantitative method is described for monitoring the expression levels of O-linked oligosaccharides. Rather than compare the relative abundances to that of the mixture, each component is compared to the same oligosaccharide species from another mixture. In this way, each oligosaccharide is compared to itself, allowing precise expression levels between mixtures to be obtained. The method employs a stable isotope (deuterium) that is incorporated during the release procedure. It is applied to the mucin-type oligosaccharides found in the egg jelly of the anuran *Xenopus laevis*. In this report, we compare the expression levels of oligosaccharides between individual eggs (cells) of the anuran. However, the method may be applied to mucin-type oligosaccharides from different mammalian tissues, such as in analyses of cancer and normal cells.

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EXPERIMENTAL SECTION

Materials. Carbograph porous graphitized carbon solid-phase extraction (PGC-SPE) cartridges (150 mg, 4 mL) were purchased from Alltech Associates (Deerfield, IL). A Hypercarb porous graphitized carbon HPLC column (100 × 2.1 mm) was obtained from Hypersil Inc. (Bellfonte, PA). Sodium tetrahydroborate and sodium tetradeuterioborate (98%) were purchased from Sigma (St. Louis, MO). DHB and DHAP were obtained from Aldrich (Milwaukee, WI). All other chemical reagents were purchased from Fisher Scientific (Pittsburgh, PA). All reagents were purchased in the highest purity and used without further purification.

Release, Isolation, and Separation of Oligosaccharides. Eggs from *X. laevis* were collected and stored in De Boer buffer (110 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, solid $NaHCO_3$ to yield pH 7.2). An egg was transferred to a 200-μL Eppendorf tube using a plastic pipet and washed with De Boer−Tris buffer solution (pH 8.9) thoroughly (100 μL each time and three times). Then 20 μL of De Boer−Tris buffer solution containing 0.3% (v/v) β-mercaptoethanol was added into the vial to solubilize the jelly. After the egg jelly glycoproteins were completely dissolved (∼5 min), the egg was carefully removed by a micropipet. The glycoprotein solution was dried in a Labconco RapidVac vacuum evaporation system. A 20−μL sample of alkaline tetrahydroborate/tetradeuterioborate solution (mixture of 1.0 M sodium tetrahydroborate or sodium tetradeuterioborate and 0.1 M sodium hydroxide) was added into the tube. The mixture was incubated at 42 °C for 24 h. Afterward, a 1.0 M hydrochloric acid solution was slowly added to stop the reaction and destroy excess sodium tetrahydroborate in an ice bath.

Pairs of egg were selected for comparison. One egg was treated separately with alkaline tetrahydroborate, while the other was treated with alkaline tetradeuterioborate. After the reactions were stopped, the two resulting solutions were combined and treated with solid-phase extraction using a PGC cartridge (PGC-SPE). The procedure is described briefly as follows. The PGC-SPE cartridge was first washed with three column volumes of 80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA)−water solution and three column volumes of water. The sample was applied to the SPE cartridges, and the glycans were allowed to adsorb to the packing. The salts were not retained. The cartridge was then washed thoroughly with three column volumes of water. The glycans were eluted with three column volumes of 40% (v/v) acetonitrile (ACN) in 0.05% (v/v) TFA−water solution. The effluents were collected into fractions and analyzed by MALDI-FTMS. The fractions that contained glycans were identified, pooled, and concentrated. The resulting pooled samples were submitted for further MS or HPLC analysis.

HPLC Separation of Oligosaccharide Mixtures. Glycan mixtures purified from *X. laevis* egg jelly by the above procedures were separated by HPLC on two different narrow-bore columns. For the primary amine-bonded silica column (2.1 × 100 mm), an aqueous ACN gradient (solvent A, H_2O; solvent B, ACN) was used (0−60 min 20−50% A) with a flow rate of 0.25 mL/min. For the porous graphitized carbon column (2.1 × 100 mm), the flow rate was also 0.25 mL/min and the gradient of solvent A, 0.05% TFA−H_2O (v/v), and solvent B, 0.05% TFA−40% ACN−H_2O (v/v), with 0−85 min 12.5−75% B was used. The effluents were monitored at 206 nm by a diode array detector. HPLC effluents were collected as 1-min fractions and analyzed by MALDI-FTMS.

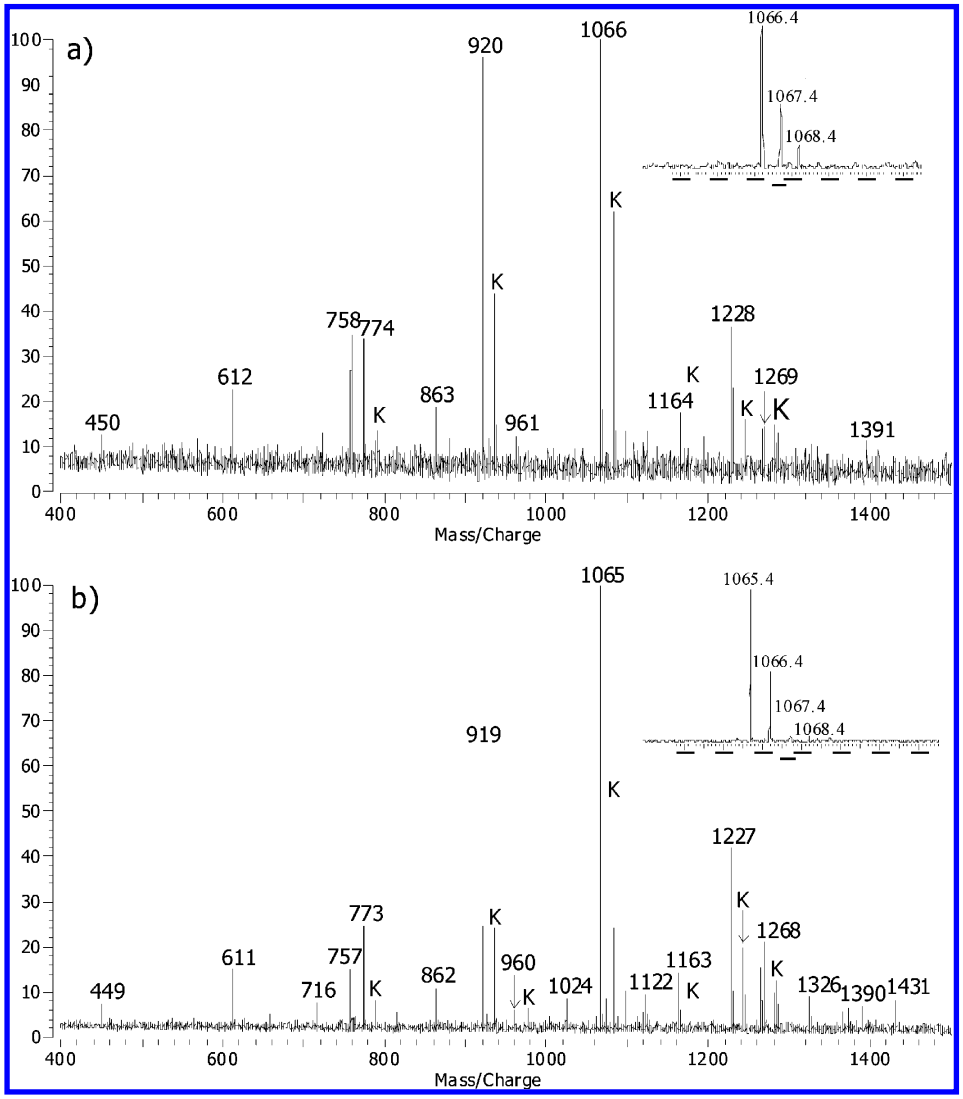
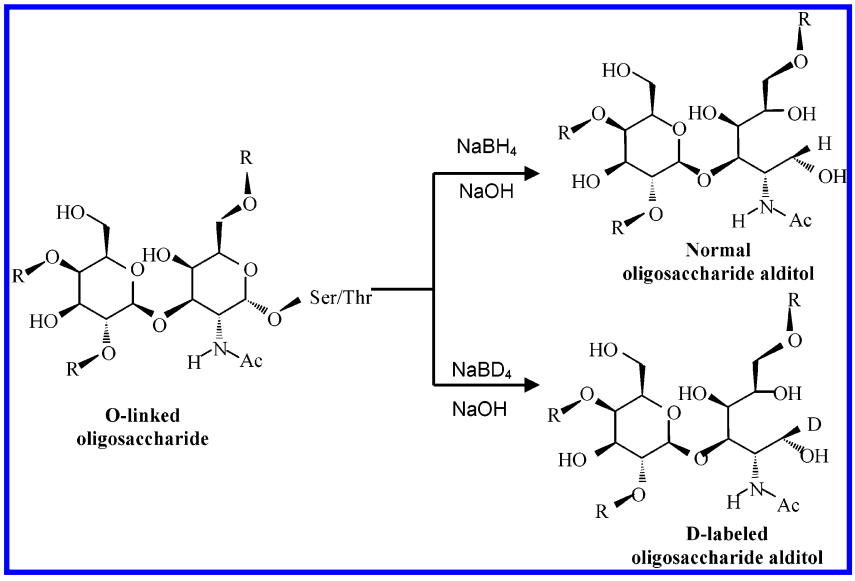


Figure 1. Glycoproteins from two sets of five eggs treated with NaOH/$NaBD_4$ and NaOH/$NaBH_4$ and submitted for PGC-SPE, respectively. (a) Positive mode MALDI-FTMS spectrum of the D-labeled sample. (b) Positive mode MALDI-FTMS spectrum of the unlabeled sample. The peaks for oligosaccharides are labeled with their mass-to-charge ratio. DHAP was the matrix used and hence both Na^+- and K^+-coordinated ions were observed. Peaks corresponding to K^+-complexes were labeled with "K". The expanded region of a peak at *m*/*z* 1065 is shown.

Scheme 1. Release of O-Linked Oligosaccharide from Protein and Reduced into Either an Unlabeled Alditol or a D-Labeled Alditol Using NaOH/$NaBH_4$ or NaOH/$NaBD_4$



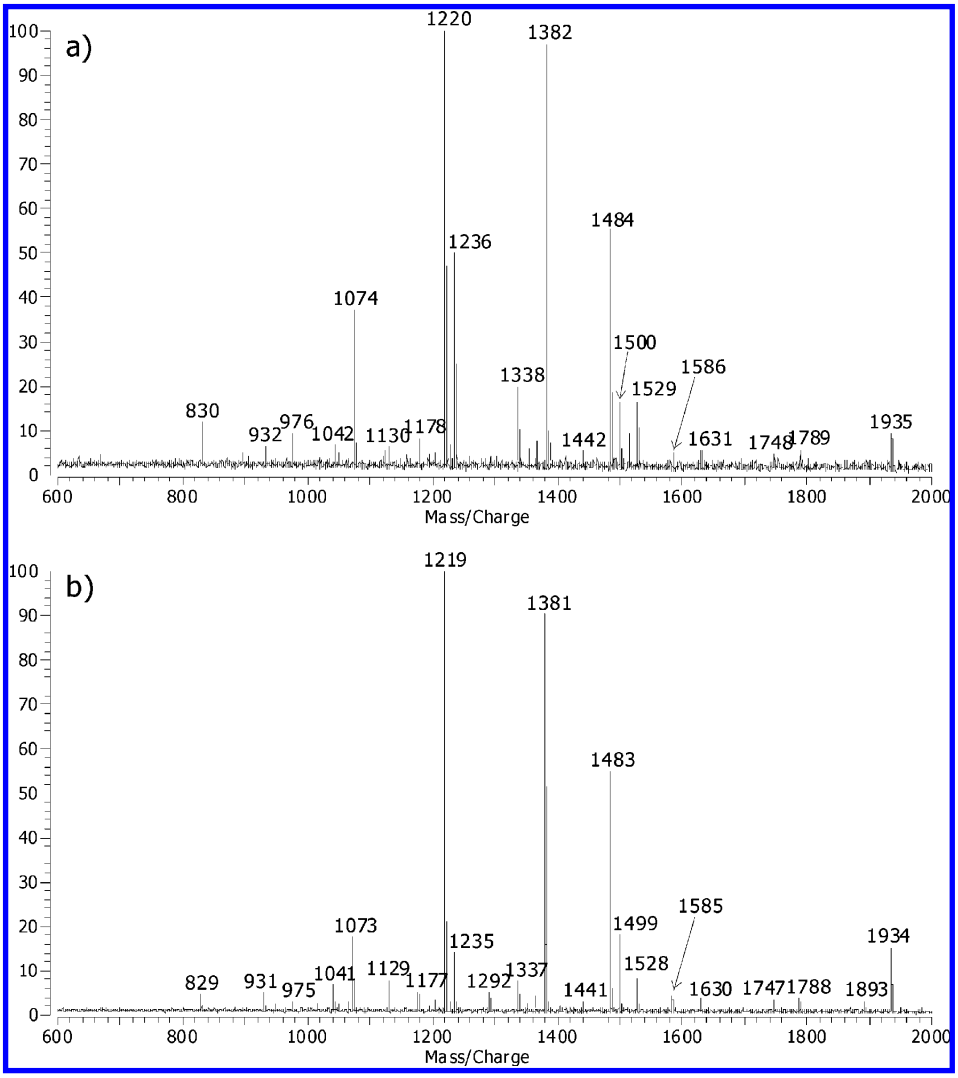


Figure 2. Negative mode MALDI-FTMS spectra taken for the samples in Figure 1. (a) Negative mode MALDI-FTMS spectrum of the D-labeled sample. (b) Negative mode MALDI-FTMS spectrum of the unlabeled sample. The peaks for oligosaccharides are labeled with their mass-to-charge ratio. DHAP was the matrix used.

MALDI-FTMS Analysis. A commercial MALDI-FTMS instrument (IonSpec Corp., Irvine, CA), equipped with an external MALDI source, a 4.7-T superconducting magnet, and a 337-nm nitrogen laser, was used in this study as described in previous publications.[14−16] To analyze the samples, 1−20 μL of HPLC fraction was placed on the MALDI probe. In the positive mode, 1 μL of 0.01 M NaCl in ethanol/water solution (50:50) was added to enrich the Na^+ concentration and produce primarily sodiated species. One microliter of 0.4 M DHB or DHAP in ethanol was added as matrix for the analysis. Warm, forced air was used to quickly evaporate the mixture on the probe. The ions were subjected to chirp excitation and direct-mode broadband detection (128K data points).

Collision-Induced Dissociation (CID) Mass Spectrometry. All CID experiments were performed in the off-resonance mode. The desired ion was isolated in the ion cyclotron resonance cell by using an arbitrary waveform generation and synthesizer excitation. The ions were excited at +1000 Hz offset from their cyclotron frequency for 1000 ms at 2−7 V (base to peak), depending on the desired level of fragmentation and the size of oligosaccharides. Two argon pulses were used during the CID event to maintain a pressure of 10^{-5} Torr.

RESULTS AND DISCUSSION

Incorporation of Deuterium Label into O-Linked Oligosaccharides during the Release Process. Mucin-type oligosaccharides are O-linked and commonly released from glycoproteins by treatment with alkaline tetrahydroborate. During this process, O-linked oligosaccharides are cleaved from the peptide backbone by sodium hydroxide through a β-elimination mechanism. To prevent further degradation of the oligosaccharide through the peeling reaction, they are released as aldehydes and immediately reduced into the corresponding alditols by the sodium tetrahydroborate. A hydrogen atom from the sodium tetrahydroborate is incorporated into the generated alditol (Scheme 1). To compare the relative expression levels of individual oligosaccharides in the two eggs, the oligosaccharide from one egg is released with sodium tetradeuterioborate while the oligosaccharide of another is released with sodium tetrahydroborate.

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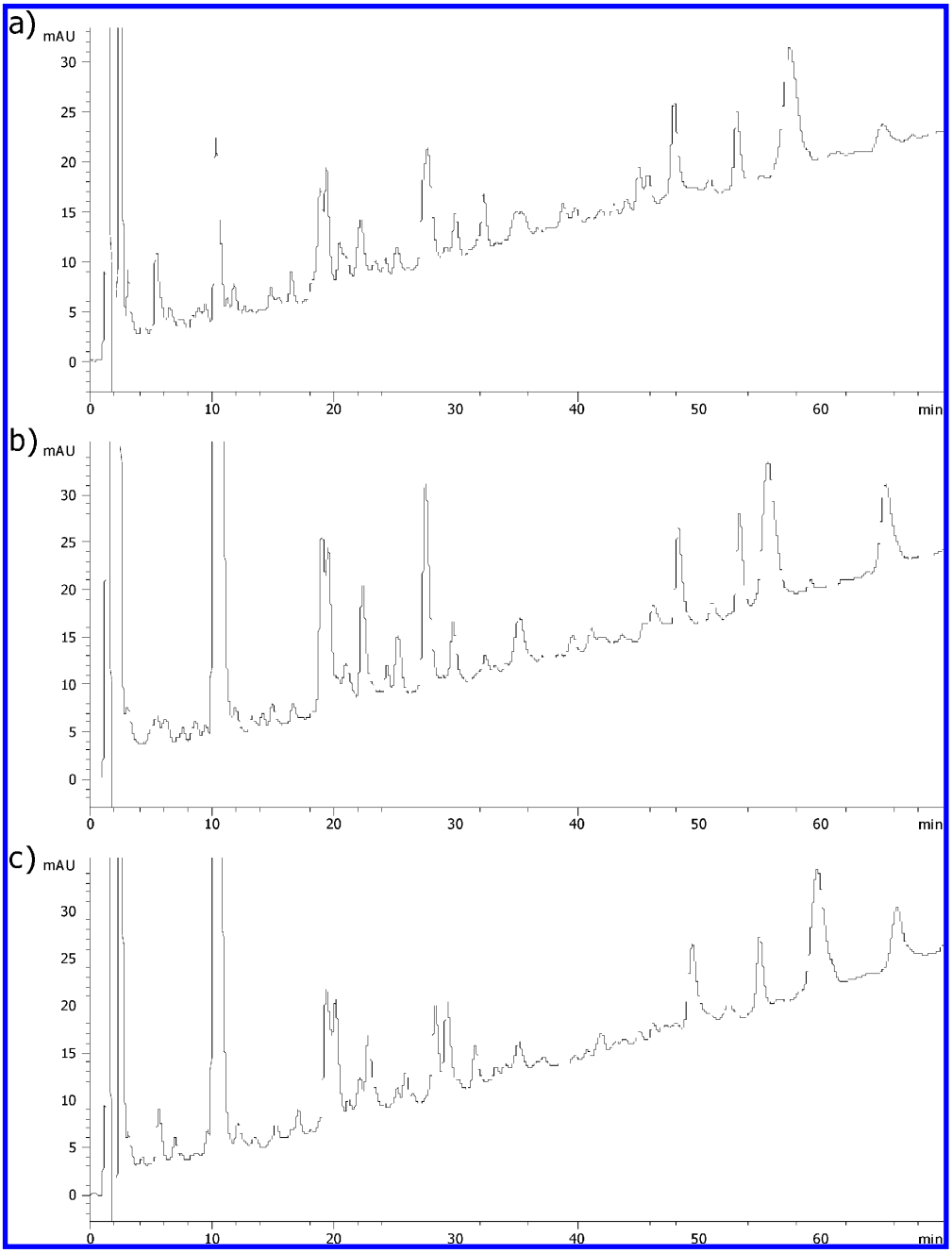

Figure 3. HPLC chromatograms for the above D-labeled and unlabeled sample and a mixture of equal amount of these two samples. The separations were performed on a narrow-bore porous graphitized carbon (PGC) column (2.1 × 100 mm) using a gradient of 0.05% TFA−H_2O and 40% ACN−0.05% TFA−H_2O as described in the Experimental Section.

As a first test of this concept, a group of five eggs from one individual frog was released by sodium tetradeuterioborate as described in the Experimental Section. The oligosaccharide mixture was purified by PGC-SPE, and the resulting mixture was examined directly by MALDI-MS. The mass spectra in both the positive and negative modes are shown in Figure 1a and Figure 2a, respectively. A second set of five eggs was released with sodium tetrahydroborate. The mass spectra are shown in Figure 1b and Figure 2b. The spectral features of the D-labeled oligosaccharides in both the positive and negative modes are nearly identical to the corresponding spectra of the unlabeled oligosaccharides. In both instances, the peaks in the D-labeled oligosaccharides were shifted to one mass unit higher.

For a closer look at one isotopic distribution, the signals of the most abundant ion in the positive mode are expanded in the insets of Figure 1. The deuterated species (m/z 1066.4) is appropriately shifted one mass unit (Figure 1a) relative to the undeuterated species (m/z 1065.4) (Figure 1b).

Several ionic species in the mass spectra were composed of several isomers. To identify each component, the mixtures were separated using HPLC separation on a narrow-bore PGC column (2.1 × 100 mm). Figure 3 shows the HPLC chromatograph of the deuterated sample (Figure 3a), the undeuterated sample (Figure 3b), and a mixture produced by combining equal amounts of deuterated and undeuterated samples (Figure 3c). Baseline separation was achieved for most of the components . The eggs

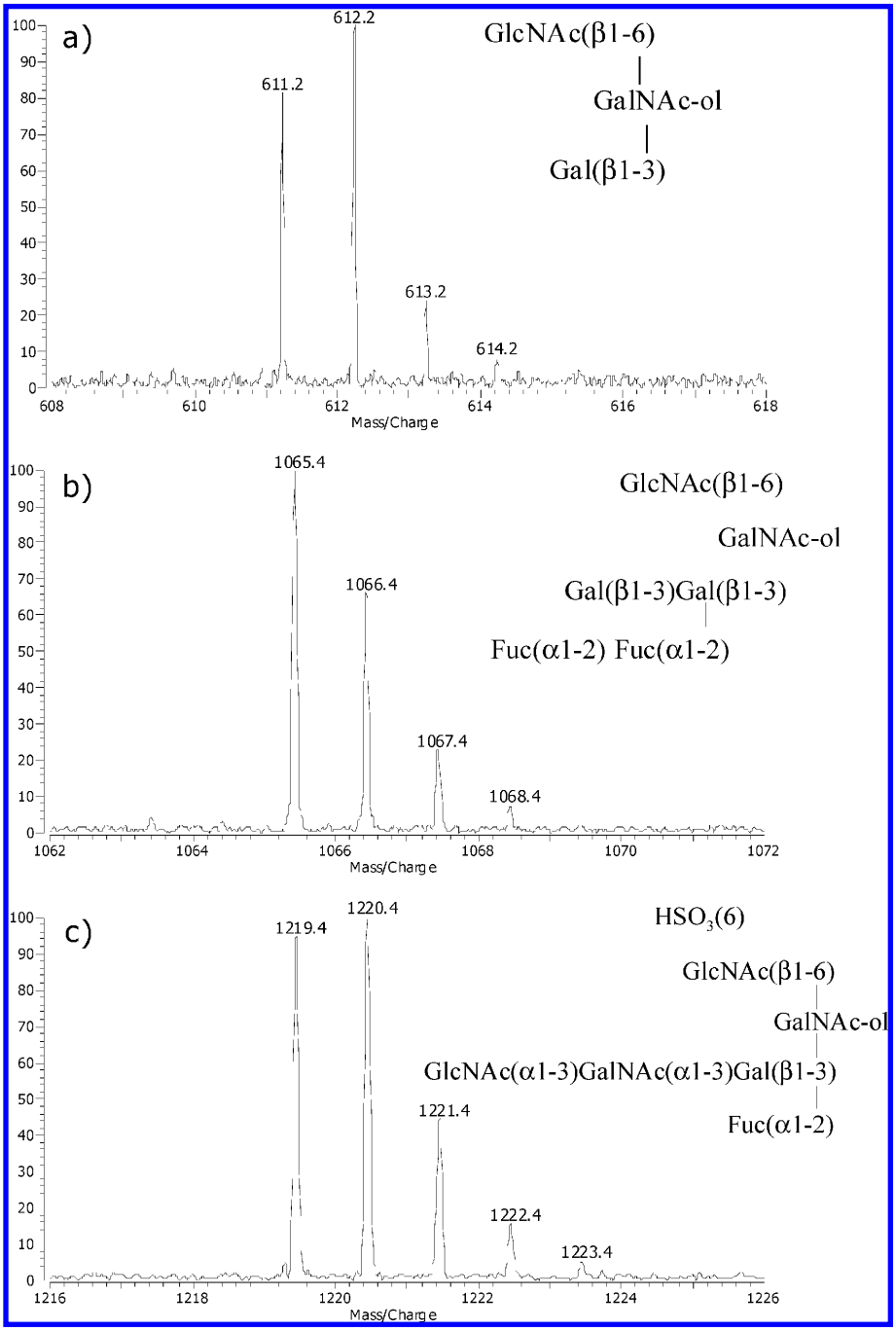

Figure 4. Examples of different abundance levels between the D-labeled and unlabeled oligosaccharide alditols: (a) *m*/*z* 611.2, (b) 1065.4, and (c) *m*/*z* 1219.4. Ratios between the D-labeled and unlabeled compounds were calculated (Table 1) and discussed in the text.

were derived from the same frog and showed nearly all the same peaks with exceptions due to egg-to-egg and chromatographic variability. There are noticeable peak shifts particularly at later retention times, which hide the oligosaccharide variations between samples. The peak shifts at earlier retention times are less pronounced, making the egg-to-egg variability more apparent.

The MS analyses of the HPLC fractions in the mixed sample were individually examined by MALDI-FTMS. Figure 4 shows the spectra of three oligosaccharides with varying degrees of deuterium-labeled components. In Figure 4a, the unlabeled peak is at *m*/*z* 611.2. The deuterium-labeled species is present at *m*/*z* 612.2. The natural isotope contribution of the *m*/*z* 612.2 peak to the *m*/*z* 611.2 peak is 24%. This value was obtained from experimental isotopic distribution of the undeuterated compound in the FTMS. From this spectrum, we calculated the ratio of labeled to unlabeled material to be 0.97 (Table 1). A compound that did not have a significant contribution from the deuterium-labeled species is shown in Figure 4b. The ratio of labeled (*m*/*z* 1066.4) to unlabeled material for this compound was 0.20. The mixture of the oligosaccharides released from *X. laevis* egg jelly glycoproteins is composed of neutral and acidic oligosaccharides. Figure 4c shows that deuterium incorporation is also observed for the anionic species. This compound is shown to have deuterium incorporation with the ratio of 0.54. Note that although the relative intensities of the two compounds in Figure 4a and c are similar, the ^{13}C contribution is significantly greater for the latter.

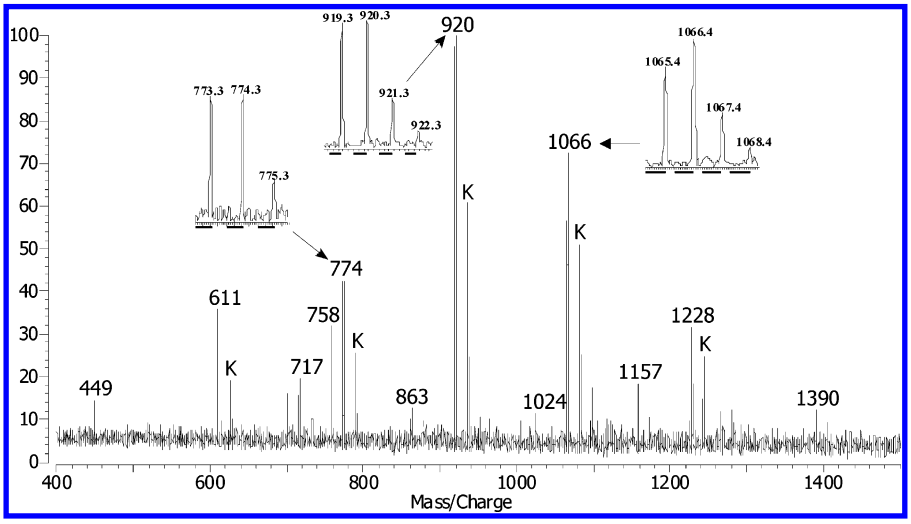

Figure 5. Oligosaccharides released separately from two eggs of two *X. laevis* animals by NaOH/$NaBD_4$ and NaOH/$NaBH_4$ and combined. Shown here is the positive mode MALDI-FTMS spectrum of the pooled PGC-SPE fractions. The expanded isotopic distributions showed mixtures of D-labeled and unlabeled oligosaccharides. The peaks for oligosaccharides are labeled with their mass-to-charge ratio. Peaks corresponding to K^+-complexes were labeled with "K".

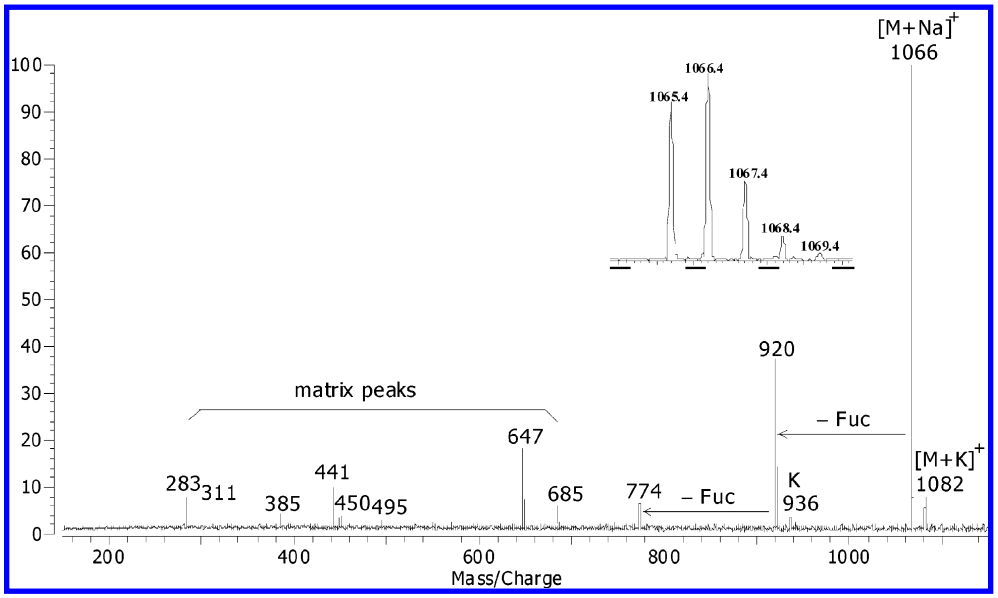

Figure 6. Positive mode MALDI-FTMS spectrum of the HPLC fraction collected between 22 and 23 min. Only one oligosaccharide (*m*/*z* 1066) was found in this fraction with its fragments and a few matrix peaks observed in the spectrum.

Quantitative determination of the relative abundances was performed by calculating the ratio of signal intensity of deuterium-labeled oligosaccharides to that of unlabeled oligosaccharides (D/H). In the calculation, the ^{13}C isotopic component of the unlabeled species was deduced experimentally from unlabeled compounds. We found that the D/H ratio of a specific sample varied depending on the S/N. Variations from the "true" D/H ratio may come from at least two different sources. The first is due to the ion cloud densities. The D/H values with high S/N vary by as much as 15% from those with low S/N. As the pressure is nearly identical during ion excitation, the effect of pressure damping in the variation can be neglected. It has been shown that ions with low densities are prone to shearing and other disruptive forces.[17] Loss of coherence is therefore more likely with weakly intense signals. To obtain accurate values, it was necessary to obtain the highest S/N possible. Variations in S/N occur between different sample spots on the probe. The best possible spots were chosen to provide the highest S/N. When the signal is of sufficient S/N, the theoretical values for ^{13}C abundances were still slightly lower than the experimental value by about 2−3%. Relative peak heights are affected by the relative decay rates in the time domain signal.[17−19] The decay rates are in turn due to a number of factors including the dephasing of the ion cloud by a larger one composed of ions with a different mass. Using the earlier part of the transient, however, readily solves this problem. Indeed, the use of the whole transient yields errors between 5 and 15%. When the early part of the transient is used, this value decreases to between 1 and 5%.

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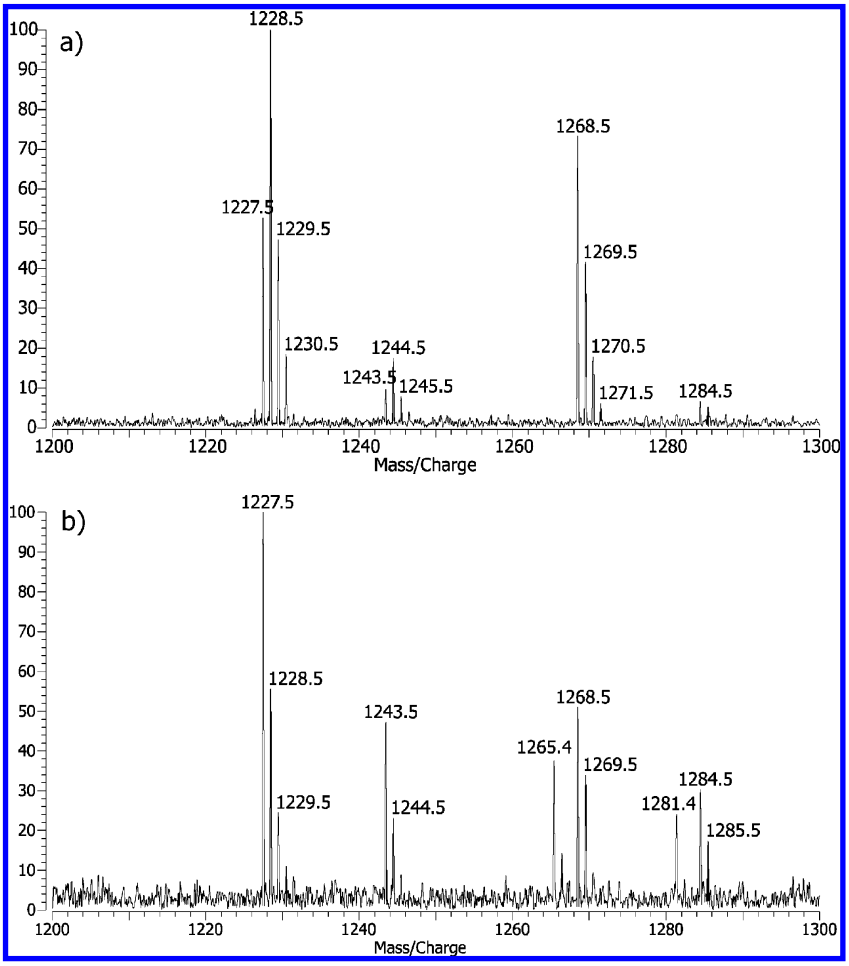


Figure 7. (a) Expanded positive mode MALDI-FTMS spectrum of the HPLC fraction collected at 27−28 min. (b) For comparison, a MS spectrum of a mixture of unlabeled oligosaccharide mixture is illustrated. Two oligosaccharides, *m*/*z* 1227.5 and 1268.5, were found to have a different ratio of D-labeled and unlabeled compounds. The ratio were calculated (Table 2) and discussed in the text.

The relative errors of the measurements when taken in triplicate were less than 1%.

For illustrative purposes, the variations in the D/H ratio for a number of neutral oligosaccharides from a sample composed of two eggs from different frogs are provided in Table 2.

In some cases, the ratios for the same *m*/*z* value showed significant difference (more than 30%) particularly when the samples were obtained from different fractions. The large variations indicated the presence of isomers. Indeed, this method is ideal for finding isomers as the D/H ratio of each isomer generally varied significantly.

Profiling O-Linked Oligosaccharide Expression Levels in the Jelly of Single Eggs. The analyses of individual eggs provide a unique model for comparing variations in glycosylation. The *X. laevis* eggs are ~300 μm in diameter. While the size is larger than most cells, we feel that it nonetheless provides an ideal system for illustrating single-cell analyses. It is also a suitable system for developing this method further.

The jelly solutions of two eggs from two different *X. laevis* animals were treated with $NaOH/NaBH_4$ and $NaOH/NaBD_4$ solutions, respectively, as described in the Experimental Section. The two resulting solutions were mixed together and then purified by PGC-SPE. SPE fractions containing oligosaccharides were pooled, concentrated, and analyzed by MS. MALDI mass spectrum of the pooled PGC fractions in positive mode are shown in Figure 5. Expanded isotopic distributions for some oligosaccharide peaks are inset. The [A + 1] isotopmers shown in Figure 5 all had intensities higher than the [A] isotopmer, which indicated that the labeling was successful.

The mixture was subjected to HPLC separation with a gradient of ACN and water to elute the oligosaccharides as described in the Experimental Section. A typical MS spectrum of one HPLC fraction collected at 22−23 min is shown in Figure 6. It was determined that *m*/*z* 1066 was the quasimolecular ion in this fraction. The other oligosaccharide peaks were either fragments of *m*/*z* 1066 (e.g., *m*/*z* 920 and 774) or the potassium-coordinated parent ion and its fragment (e.g., *m*/*z* 1082 and 936). The expanded isotopic distribution was inset in Figure 6, which showed clearly the presence of D-labeled compound. The ratio between D-labeled and unlabeled compound was calculated to be 0.74 (Table 2).

MALDI-FTMS analyses of the collected HPLC fractions revealed that there were various degrees of deuterium incorporation for individual oligosaccharides in pairs of eggs from two different animals. Shown in Figure 7 is an expanded MS spectrum of one HPLC fraction collected at 27−28 min. Two oligosaccharides were detected by MS in this fraction: *m*/*z* 1227.5 and 1268.5. There was clearly a difference between the isotopic distributions of these two compounds. An expanded MS spectrum of a mixture of unlabeled oligosaccharides released from *X. laevis* is provided as

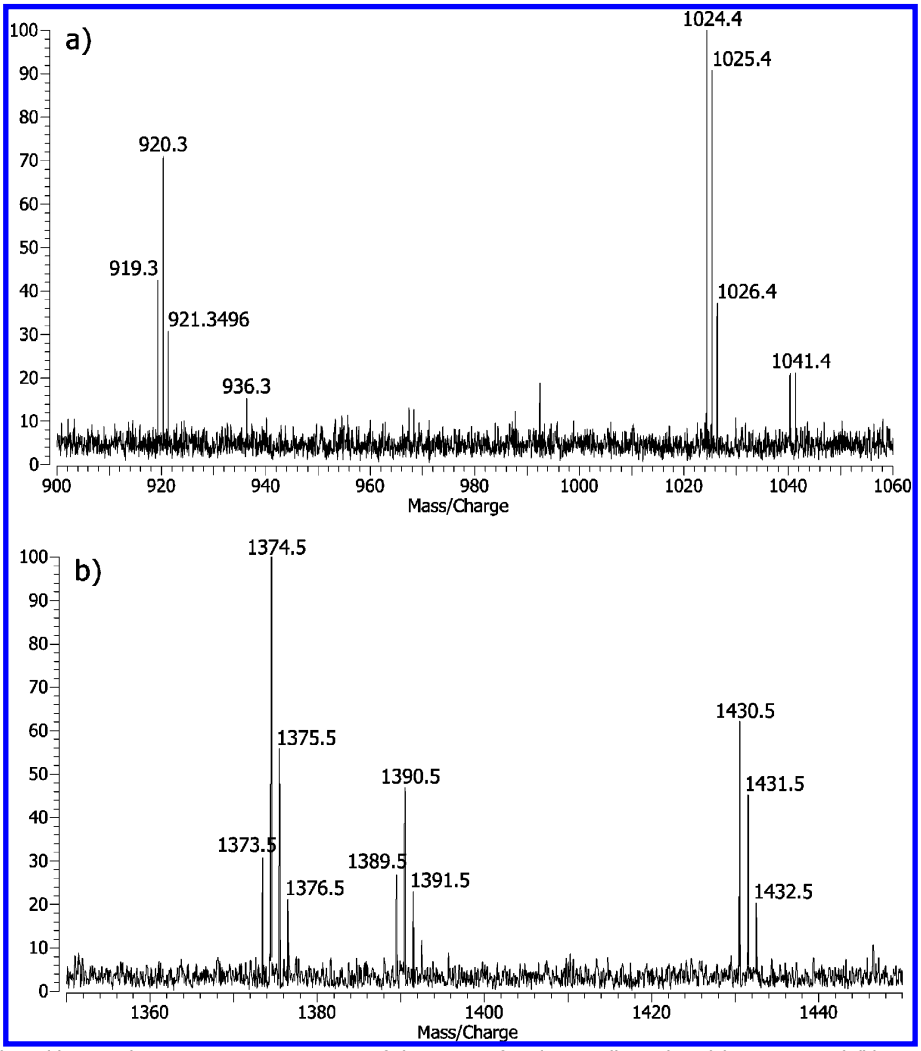


Figure 8. Expanded positive mode MALDI-FTMS spectra of the HPLC fractions collected at (a) 20−21 and (b) 29−30 min. The ratios of D-labeled and unlabeled compounds were calculated (Table 2) and discussed in the text.

Table 1. Ratio of Abundances of Labeled and Unlabled Oligosaccharides in Figure 4

	relative intensities (%)		expected intensities (%)		ratio of two oligo-saccharides
compound	A (m)	A + 1 (n)	A (p)	A + 1 (q)	D/H[a]
a (m/z 611.2)	81.48	100.00	100.00	25.34	0.97
b (m/z 1065.4)	100.00	66.13	100.00	45.81	0.20
c (m/z 1219.4)	91.30	96.55	100.00	51.83	0.54

[a] $D/H = (n - mq/p)/m$.

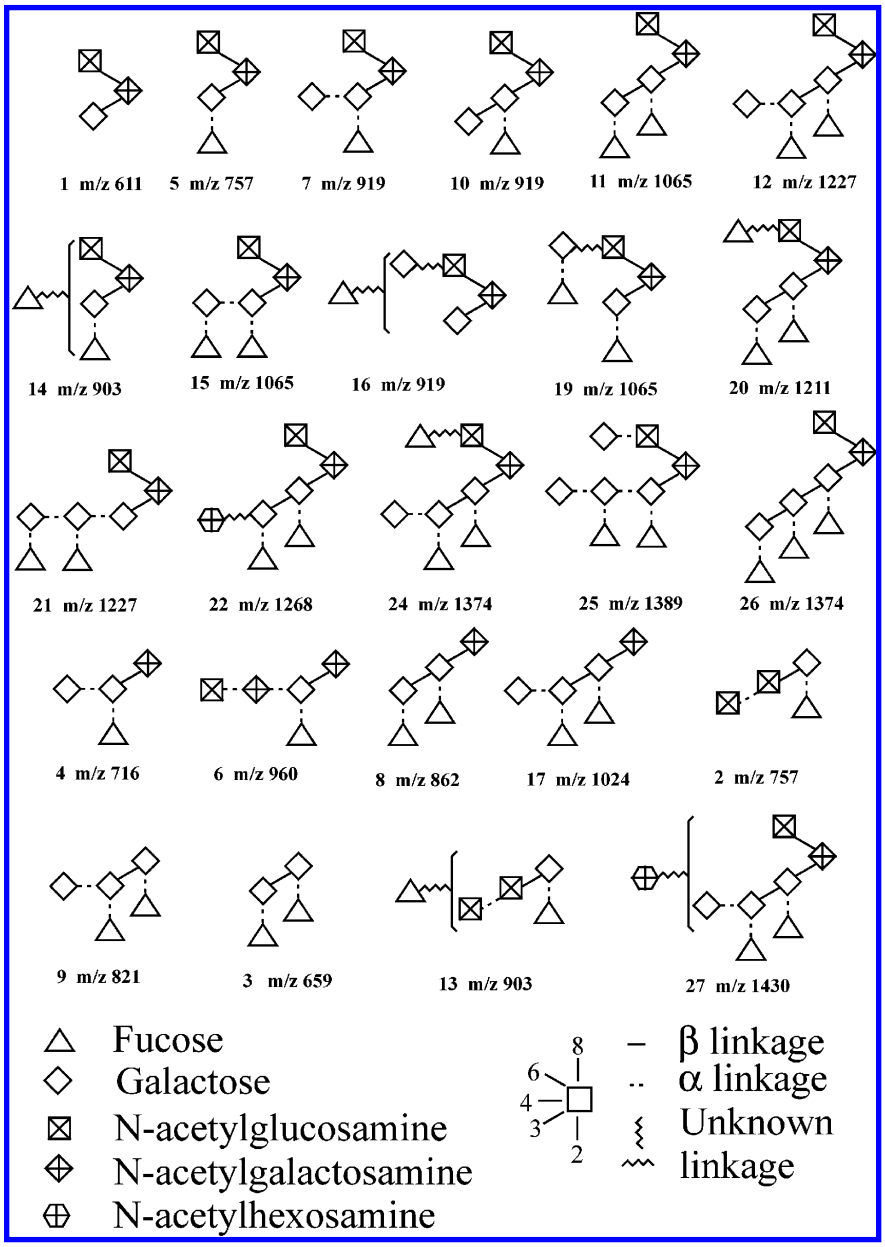
Table 2. Representative Ratios of Labeled and Unlabeled Oligosaccharides

	relative intensities (%)		expected intensities (%)		ratio of two oligo-saccharides
compd (m/z)	A (m)	A + 1 (n)	A (p)	A + 1 (q)	D/H[a]
1065.4	83.71	100.00	100.00	45.81	0.74
1227.5	52.60	100.00	100.00	52.63	1.37
1268.5	72.97	41.37	100.00	55.15	0.02
919.3	42.56	71.00	100.00	39.02	1.28
1024.4	100.00	90.74	100.00	43.27	0.48
1373.5	31.10	100.00	100.00	59.38	2.62
1389.5	26.95	46.99	100.00	59.43	1.14
1430.5	62.15	45.29	100.00	62.00	0.11

Figure 7b. The isotopic distributions of m/z 1227.5 and 1268.5 in Figure 7b reflected the natural abundances of the isotopmers of each compound. Direct comparison of Figure 7b and a revealed that isotopic distribution of m/z 1227.5 was dramatically different from m/z 1268.5. In the former, the presence of D-labeled compound was obvious. For m/z 1268.5, however, the isotopic distribution in Figure 7a was quite similar to that of Figure 7b. Calculation showed that the ratio between the D-labeled and unlabeled compounds was 1.37 for m/z 1227.5 and 0.02 for m/z 1268.5 (Table 2). This indicated that the expression levels of these two compounds were substantially different in these two animals. Other examples of different expression levels are also illustrated in Figure 8. The isotopic distribution of m/z 919.3 and 1373.5 both showed notably higher intensities for the [A + 1] isotopmers, while the intensities of [A] isotopmers for m/z 1024.4 and 1430.5 were higher than the [A + 1] isotopmers. The ratios of D-labeled and unlabeled compounds for a representative group of oligosaccharides were calculated and listed in Table 2.

Variation in Oligosaccharide Expressions in Eggs from *Different* Frogs. The diversity in the oligosaccharide expression between two eggs was performed directly using this method. To facilitate the comparison, oligosaccharides were separated into

Chart 1. Neutral Oligosaccharides from Egg Jelly Coats of *X. Laevis*



"major" and "minor" components. Those compounds with HPLC chromatograph intensities (206 nm with a diode array detector) at least 10% of the most abundant peaks were labeled as major components, while those with less than 10% were minor. For comparison, the MS intensities were also examined, and the relative intensities were found to match closely with the HPLC intensities. A series of bar graphs of the D/H ratios summarizes the results. The structures for all compounds are provided in Chart 1. Figure 9a shows a pair of eggs from two different frogs. Figure 9b shows a second pair from the same frogs, and Figure 9c shows a third pair from two additional frogs. Each bar corresponds to only a single oligosaccharide component. When more than one compound is listed below the bar, the identity of the compound was not determined from the isomers listed. The structures, where available, are provided in the abscissa. Note the similarities in the variation between two mixed pairs from the same two frogs. The variation in Figure 9c from a second pair of frogs is less among the major components but somewhat greater among the minor components. Although the number of frogs (three) is small, the bar graphs in Figure 9 illustrate the different possibilities obtained with different frogs. There can be large variation between the two frogs as evidenced by the large D/H ratio (>2) in both major and minor components. There can also be little variations between the two frogs as illustrated in Figure 9c.

Variation in Oligosaccharide Expression in the Egg from the *Same* Frogs. Eggs from the same frogs are compared in Figure 10. A pair from one frog is shown in Figure 10a, and a pair from another frog is shown in Figure 10b. The structures of each component are included for the discussion. Both major and minor components showed significantly less variations than oligosaccharides from the different frogs. Only one minor component at m/z 960.4 (structure 6) showed a large variation in the first pair with a D/H value equal to 2.3. The average value for all the components is ~1.2.

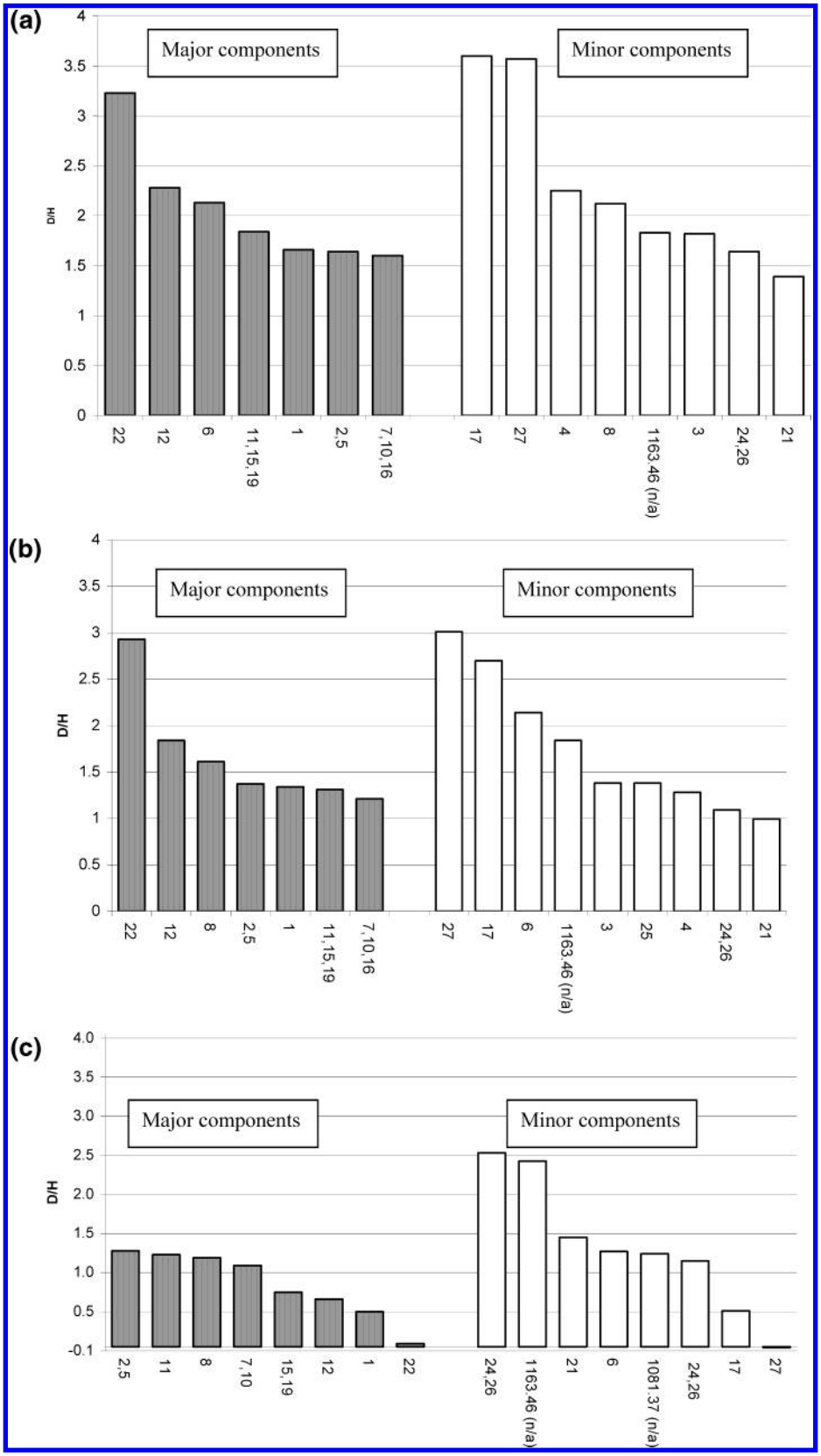


Figure 9. Ratios of oligosaccharides found in two eggs from two different frogs. (a) A pair of eggs from two different frogs. (b) A second pair from the same frogs. (c) A third pair from two additional frog.

A second pair of eggs from another frog shows similarlylittle variations. All the components in Figure 10b showed only minor variation (average D/H = 1.1) with the exception of a single component (structure 20). The oligosaccharide with m/z 1211.5 is a minor component and gave a very small value corresponding to 0.02, indicating that there is no deuterated component to this oligosaccharide.

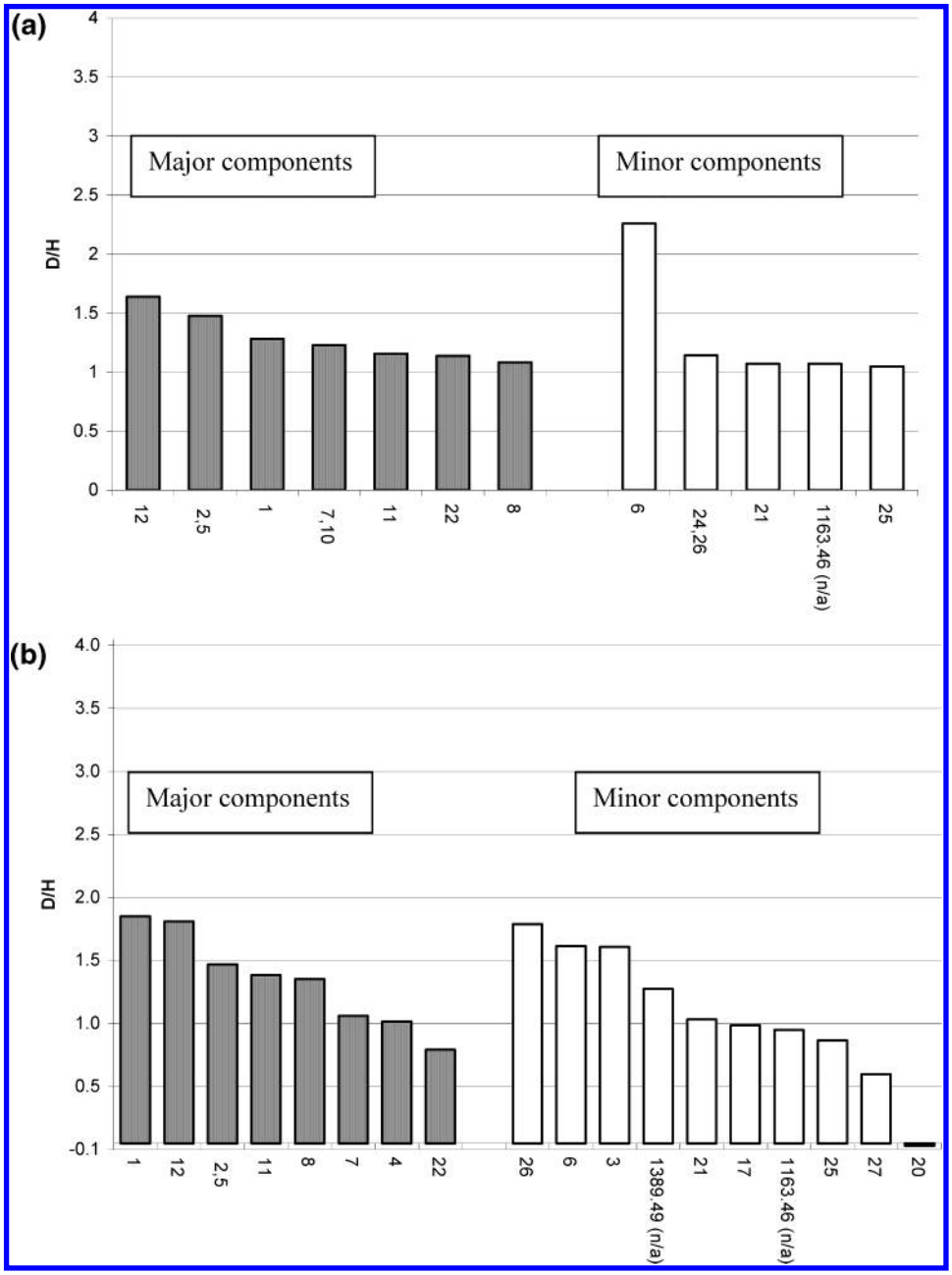


Figure 10. Ratios of oligosaccharides found in two eggs from the same frog. The two graphs (a and b) come from two individual frogs.

CONCLUSION

In this report, a novel comparative glycomic method was demonstrated to profile the expression levels of individual oligosaccharides in *O*-glycan libraries. The method was proved to be fast and sensitive. Introduction of the deuterium label into the O-linked oligosaccharide alditols was simple. There is no problem for modern MS instruments to distinguish the difference between the labeled and unlabeled oligosaccharides. The high resolution of the FTMS instrument used in this study was more than sufficient for this purpose. MS peak intensities reflected the abundances of the components of the *O*-glycan libraries. Using this method, the difference of expression levels between the O-linked oligosaccharides of the extracellular matrix of single eggs from different *X. laevis* animals was examined.

The study in this report was performed on O-linked oligosaccharides. However, this method can be further applied to N-linked oligosaccharides. N-linked oligosaccharides are commonly released from protein backbones as aldehydes by peptide:*N*-glycosidase F. An additional reduction treatment with $NaBD_4$ can convert them into oligosaccharide alditols and introduce the D-label so that comparative studies can be performed. Furthermore, the method can even be extended to oligosaccharides simultaneously labeled with UV and fluorescence markers.

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