Application Note: 471

Analysis of Sialylated Glycans with the MALDI LTQ Orbitrap Mass Spectrometer using DHB/N,N-Dimethylaniline Matrix

Julian Saba[1], Sergei Snovida[2], Deb Charych[3] and Rosa Viner[1]
[1]Thermo Fisher Scientific, [2]University of Manitoba, [3]Five Prime Pharmaceuticals

Key Words

- DHB, DHB/DMA
- Glycoproteins
- LTQ Orbitrap XL
- MALDI
- Sialylated glycans

Introduction

Studies have shown that the alterations in glycan structures are associated with various developmental and pathological states of glycoproteins and have great biological significance.[1-3] The direct involvement of glycans in biochemical processes has been widely studied and the general consensus is that they have two significant roles. First, glycans confer certain physicochemical properties on to proteins, and second, they act as signals in cell-surface recognition phenomena.[4] However, unlike nucleic acid biopolymers and linear proteins, the intricate nature of oligosaccharides found in glycoproteins poses a challenge for analysis. In addition to the characterization of the sugar sequence, the analysis must elucidate the branching, the linkages between monosaccharide units, the anomeric configuration, and the location of possible sulfate or phosphate groups.[5] The apparent functional significance of the carbohydrate moieties creates a need for reliable and sensitive methods for their detection and analysis.

The development of matrix-assisted laser desorption/ionization (MALDI) for mass spectrometry (MS) has proven to be extremely useful for the analysis of this type of biomolecules.[6,7] MALDI provides several advantages over other ionization techniques, mainly in terms of sample amount requirements, relative simplicity of spectral interpretation, and the possibility of preserving the samples for later use.

Particular attention is being paid to sialylated glycans due to their involvement in many important biological phenomena, including cell-cell adhesion and cell-pathogen interaction.[8-10] Sialylation expression is often dependent on the cell type, developmental stage of an organism, or a disease state. Ionic signal suppression is often a major issue in the analysis of sialylated glycans by MS, particularly due to the presence of a carboxyl group at the anomeric carbon, which is usually ionized at physiological pH, thus resulting in a negative charge above pH value of ~2.6. As the number of sialic moieties increase on a particular glycan, it becomes inherently difficult to detect these glycans, which can lead to incomplete characterization of a given sample. The labile nature of sialic acid also poses a challenge for MS characterization. In-source and post-source losses of sialic acid often lead to inaccurate representation of the degree of sialylation. To avoid these problems, sialylated glycans are typically characterized in the negative mode while asialo glycans are characterized in the positive mode. An alternative approach is to chemically modify sialylated glycans, such that it stabilizes sialic acids for MS analysis. Additionally, the choice of chemical modification can also improve the ionization efficiency of the glycans.

In this study, we examined the utility of 2,5-dihydroxybenzoic acid/N,N-dimethylaniline (DHB/DMA) matrix[11] to improve analysis of *N*-linked sialylated glycans from human α_1-acid glycoprotein by MALDI.

Goal

The goal of this work is to develop a simple and reliable sample preparation method for the study of sialylated and asialylated glycans by employing DHB/DMA matrix and taking advantage of the high resolving power and mass accuracy of the Thermo Scientific MALDI LTQ Orbitrap XL hybrid mass spectrometer.

Experimental

Sample Preparation

Glycans from human α_1-acid glycoprotein (AGP) released upon PNGase F treatment were labeled with 2-aminobenzoic acid at reducing conditions according to the method reported previously.[12]

The preparation of DHB/DMA matrix solution was as described by Snovida/Perreault[11]. An initial solution of the DHB matrix (LaserBiolabs, France) was prepared by dissolving 20 mg of DHB in 200 µL of 1:1, acetonitrile (ACN)/water. This was followed by adding 4 µL of DMA (Sigma, St. Louis, MO) to the DHB solution, such that the molar ratio of DHB to DMA was about 3:1. Samples were deposited onto a stainless steel MALDI sample plate by mixing the analyte and matrix solutions (0.5 µL each) on plate and allowing the mixture to dry at room temperature. DHB matrix in ethanol was prepared as 30 mg/mL solution in 70% ethanol/30% 0.1% trifluoroacetic acid (TFA) in water and for standard DHB matrix solution was prepared as 30 mg/mL solution in 50% ACN/0.1% TFA in water.

Mass Spectrometry

All MALDI experiments were performed on a Thermo Scientific MALDI LTQ Orbitrap XL hybrid mass spectrometer. For details on its functionalities and in particular the MALDI source design of the instru-

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mentation see Strupat et.al.[13] The mass spectrometer was operated in either negative or positive modes with Oribitrap™ full-scan setting using resolving power of 60000 or 100000 at *m/z* 400 (FWHM). MS/MS analysis was conducted in the linear ion trap with Orbitrap mass analyzer or ion trap detection. For automated data acquisition, automatic gain control (AGC), automatic spectral filtering (ASF) and the crystal positioning system (CPS) were used to automatically locate optimal sample regions. Software detection of the neutral loss of sialic acid (*m/z* 291) was used to automatically perform MS^3 analysis of sialylated glycans. For MS/MS scans the following settings were used: isolation width of 3 amu, normalized collision energy of 35%, 5 microscans, and 5-50 laser shots/microscan.

Results and Discussion

Raju and co-workers have highlighted the necessity of taking into account the effect of the relative p*K*a values of analytes in a mixture on ionization efficiency in MALDI, and have described the use of negative-ion mode MALDI conditions for the analysis of acidic *N*-linked glycans.[14] Although, the negative mode logically offers better sensitivity than the positive mode for sialylated compounds, asialo oligosaccharides remain more sensitive under positive mode conditions owing to the presence of *N*-acetylated residues. A methodology that uses a single ionization polarity rather than having to switch between polarities would be an attractive analytical protocol.

Recently, Snovida and co-workers have demonstrated that incorporation of DMA into a conventional DHB matrix solution has resulted in significant improvement in sensitivity for native oligosaccharides when analyzed by MALDI-TOF MS.[11] They have shown the use of DHB/DMA matrix solution resulted in sample spots composed of very fine crystals compared to those of DHB alone and led to a more uniform sample distribution. They, also, found that DMA, a tertiary amine, could not form the Schiff base derivatives with reducing end of an oligosaccharide, but can interact with DHB.[15]

Human AGP was chosen as the model glycoprotein for our studies because its glycan content has been the object of extensive studies over the years and there is wide availability of data in the literature pertaining to its structural characterization.[16-18] Human AGP contains five *N*-glycosylation sites with complex-type glycan structures, with a significant portion being highly sialylated multi-antennary.[19-21] Thus, this glycoprotein provides an ideal model system for our method development.

To evaluate the DHB/DMA matrix, the *N*-linked glycans released from human AGP were divided into three equimolar proportions (calculated volumetrically), and spotted onto the MALDI target using three different matrices, DHB, DHB in 70% ethanol, and DHB/DMA. Mass spectra were acquired in the positive mode to evaluate ionization efficiency of all three matrices. Figure 1 shows a list of the structures for oligosaccharides found in a human AGP sample, and Figure 2 shows the comparative spectra obtained using the three different matrices as well as the carbohydrate compositions identified with each matrix.

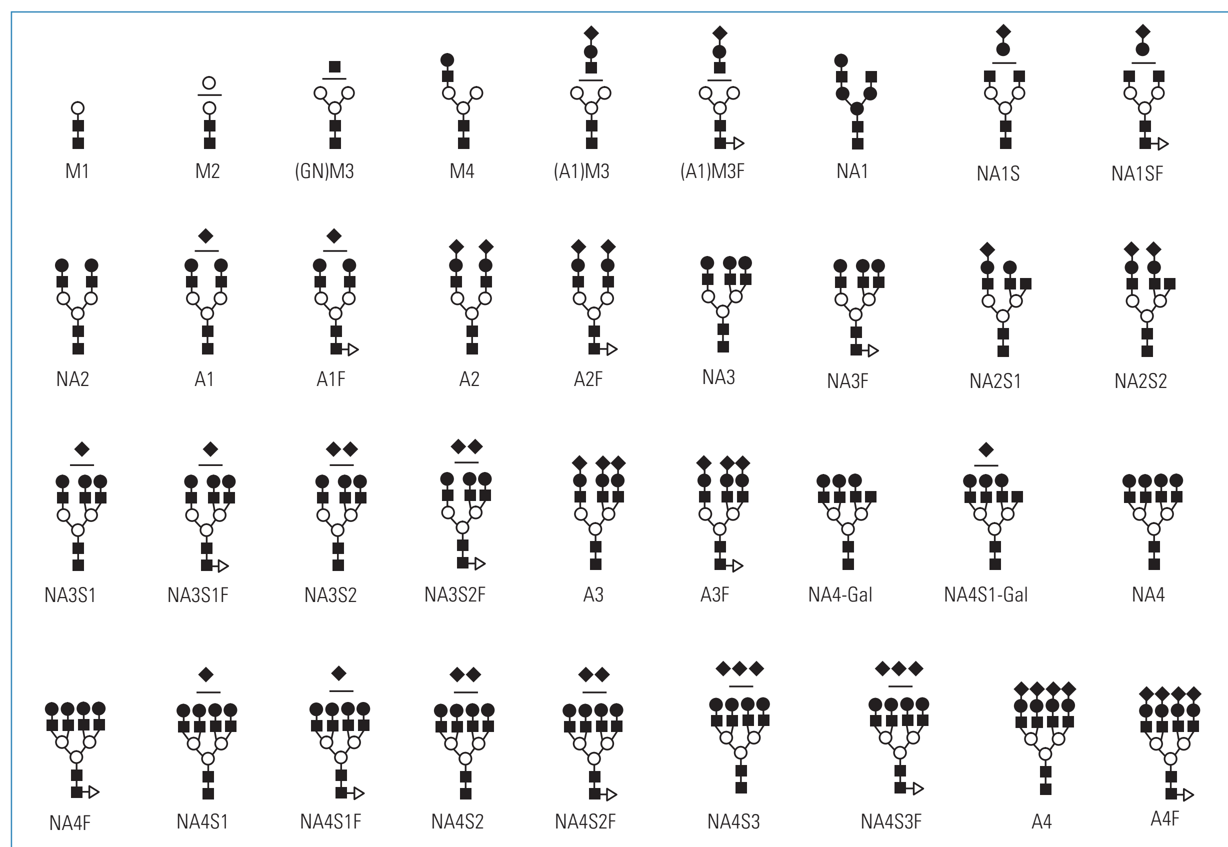

Figure 1: Structures of complex *N*-linked glycans described in this study.

Glycans analyzed using DHB and DHB in 70% ethanol mainly produced $[M+H]^+$ ions for asialyl glycans and $[M+Na]^+$ for sialylated glycans. Samples spotted using DHB as the matrix resulted in detection of mainly asialyl glycans with few monosialylated glycans (Figure 2a). Molecular masses of the glycans were measured within 2 ppm of their calculated monoisotopic values and the ions were of sufficient abundance to allow MS/MS for structural assignment.

A previous publication[11] suggests that using 70% ethanol as DHB matrix solvent improves samples spot quality, but in this case, due to efficiency with which CPS identified crystals, no improvement in analyte detection was observed. Similar to the conventional DHB matrix, DHB dissolved in 70% ethanol resulted in the identification mainly of asialyl glycans with few monosialylated glycans (Figure 2b), with the exception of the ion at *m/z* 2344.843 that was characterized by MS/MS as a disialylated glycan.

In contrast to these two matrices, DHB/DMA provided much more complete glycan characterization in terms of the number of sialyl and asialyl glycans detected (Figure 2c). The ionization efficiency of MALDI strongly relies on similar chemical properties among analytes in a mixture, e.g., carbohydrates different in composition by one GlcNAc residue will have different ionization efficiencies in the positive ion mode, whereas a difference of one Gal instead would not be as influential. The presence of sialic acid residues greatly enhances negative ion production, but will decrease the number of ions observed in the positive ion mode. The use of DHB matrix clearly shows this phenomenon. Even though, we can detect asialyl glycans using this matrix, as the number of sialyl acid residues increased, the number of possible sialylated glycans detected decreased. It was only possible to detect mainly monosialylated glycans using DHB as the matrix in the positive ion mode. In contrast to this, DHB/DMA enabled detection of di- and triantennary sialylated glycans that were not possible to detect using DHB or DHB in 70% ethanol (Figure 2c). Based on this observation, the difference in performance of DHB versus DHB/DMA matrices is due to real improvement of ionization and stabilization of the negative charge of sialylated glycans and not due to the labile nature of sialic acid.

Additional spectra were acquired for DHB/DMA at resolving power 100,000 at *m/z* 400. The inset in Figure 2c shows glycans at *m/z* 2731 and *m/z* 3023 at resolving power 100,000 at *m/z* 400, highlighting the resolution and high mass accuracy achieved. Acquisition at this resolution made it possible to see ions at this high mass area with a resolution (FWHM) of ~ 48,000. In order to detect sialylated glycans that were observed using DHB/DMA in the positive ion mode, negative ion mode MALDI was required using DHB (Figure 3). Glycans analyzed using DHB /DMA produced mainly $[M+Na]^+$ ions in the positive mode. Molecular masses of the glycans were measured within 2 ppm of their calculated monoisotopic mass values allowing for structural assignment without acquiring MS/MS spectra (Table 1). However, we did perform MS/MS analysis for structural confirmation. Figures 4a and 4b show MS/MS spectra for the asialyl digalactosyl biantennary glycan at *m/z* 1784.37 and the disialyl biantennary glycan at *m/z* 2344.843, representative of fragmentation observed in MS/MS experiments in this study. The fragment ions peaks are labeled according to the nomenclature proposed by Domon and Costello.[21] MALDI-MS spectra were also acquired in the negative mode to evaluate the performance of the DHB/DMA. Even though the MALDI LTQ Orbitrap XL™ mass spectrometer was able to acquire trisialylated glycans with sufficient signal in the positive

Glycan Structure	Observed $[M+H]^+$	Theoretical $[M+H]^+$	Mass Accuracy (ppm)
M1	708.282	708.282	-0.24
M2	870.335	870.335	-0.23
(GN)M3+Na	1257.449	1257.449	0.12
M4+Na	1419.504	1419.501	1.61
(GN)M3+SA+Na	1548.546	1548.544	1.10
(A1)M3	1688.616	1688.615	0.21
NA2/G2+Na	1784.637	1784.634	1.71
G2S1/A1+Na	2075.734	2075.729	2.2
G2S2/A2+Na	2366.824	2366.826	-0.64
NA3+Na	2149.771	2149.766	2.27
NA3F+Na	2295.825	2295.824	0.80
NA3S1+Na	2440.870	2440.861	3.63
NA3S2+Na	2731.959	2731.957	0.91
NA3S2F+Na	2878.016	2878.014	0.57
A3/G3S3+Na	3023.057	3023.052	1.71
NA4+Na	2514.900	2514.898	0.78
NA4F+Na	2660.949	2660.956	-2.46
NA4S1+Na	2805.996	2805.993	1.08
NA4S2+Na	3097.088	3097.089	-0.17
NA4S3+Na	3388.184	3388.184	-0.14

Table 1. AGP glycans (AA derivative) detected by MALDI Orbitrap XL in positive ion mode with DHB/DMA matrix

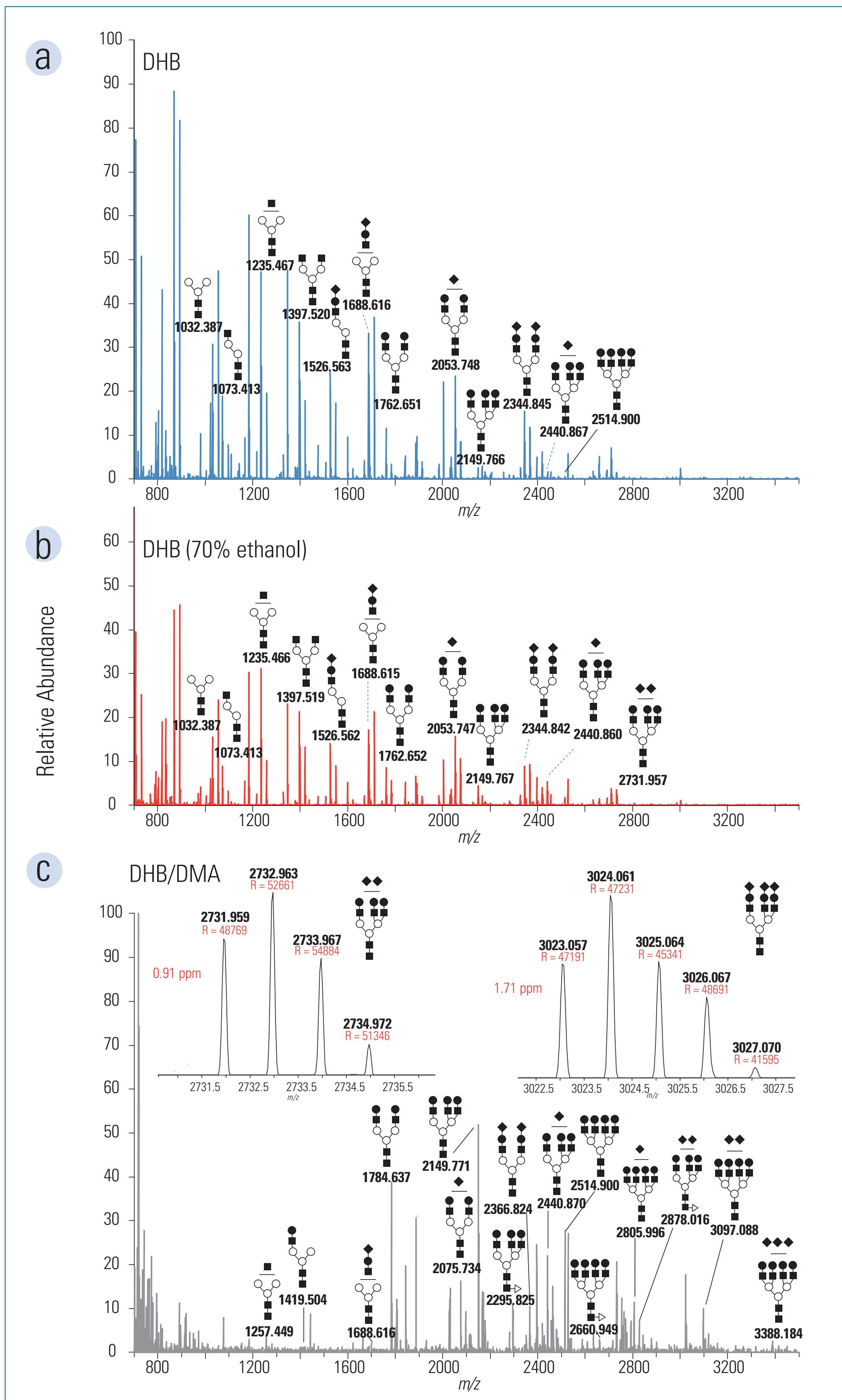

Figure 2: Orbitrap detector full mass spectrum at resolving power 60,000 at *m/z* 400 of an equimolar mixture of human α1-acid glycoprotein glycans derivatized with 2-aminobenzoic acid in (a) DHB (average of 3 individual scans, total of 9 laser shots), (b) DHB in 70% ethanol (average of 3 individual scans, total of 9 laser shots) and (c) DHB/DMA matrix (average of 9 individual scans, total of 20 laser shots) respectively, as well as the carbohydrate compositions identified for each matrix by MS/MS. The inset in (c) shows the mass range *m/z* 2770-2900 at resolving power 100,000 at *m/z* 400, highlighting the resolution and high mass accuracy provided in the high mass area.

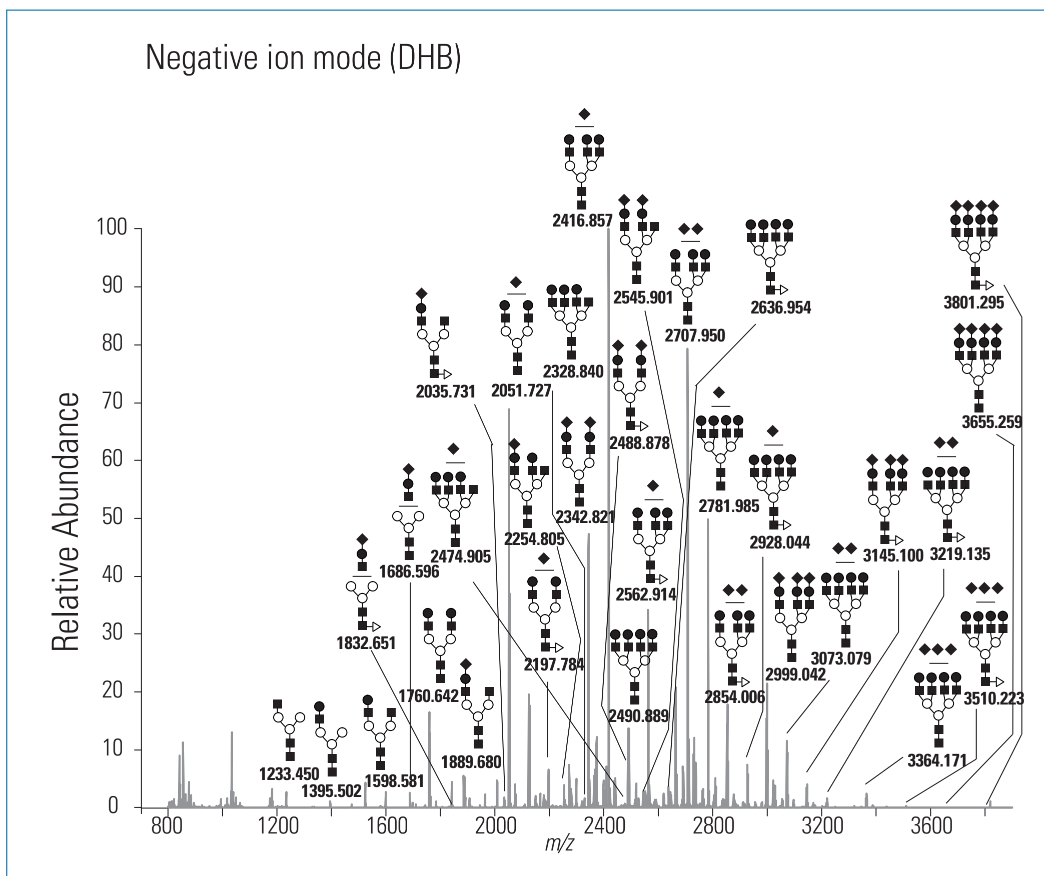

Figure 3: Orbitrap detector full mass spectrum at resolving power 60, 000 at *m/z* 400 of an *N*-linked glycans derivatized with 2-aminobenzoic acid from human α1-acid glycoprotein analyzed in DHB matrix (average of 2 individual scans, total of 47 laser shots) by negative ion mode.

Glycan Structure	Observed [M-H]⁻	Theoretical [M-H]⁻	Mass Accuracy (ppm)
NA1S	1889.676	1889.676	-0.05
NA2/G2	1760.642	1760.637	2.58
G2S1/A1	2051.736	2051.733	1.91
G2S2/A2	2342.833	2342.828	1.98
NA3S1	2416.866	2416.865	0.69
NA3S1F	2562.926	2562.923	1.39
NA3S2	2707.962	2707.960	0.70
NA3S2F	2854.019	2854.018	0.37
A3/G3S3	2999.057	2999.056	0.41
NA4	2490.906	2490.902	1.87
NA4S1	2781.998	2781.997	0.43
NA4S1F	2928.056	2928.055	0.24

Table 2. AGP glycans (AA derivative) detected by MALDI orbitrap XL in negative ion mode with DHB/DMA matrix.

ion mode, we also wanted to ensure that we were able to characterize all possible sialylated glycans present in human AGP. Figure 5 shows the comparison between positive and negative ion mode MALDI-MS spectrum for human AGP as well as the carbohydrate compositions identified for each mode. Table 2 highlights the mass accuracy obtained in the negative ion mode for the molecular masses of the glycan structures observed.

Figure 6a shows the sialylation profile observed by MALDI LTQ Orbitrap, for mono-, di- and trisialylated glycan structures detected in human AGP reported as relative percentages to the total amount of sialylated glycan components found, while Figure 6b highlights the major components identified. It should be noted here that

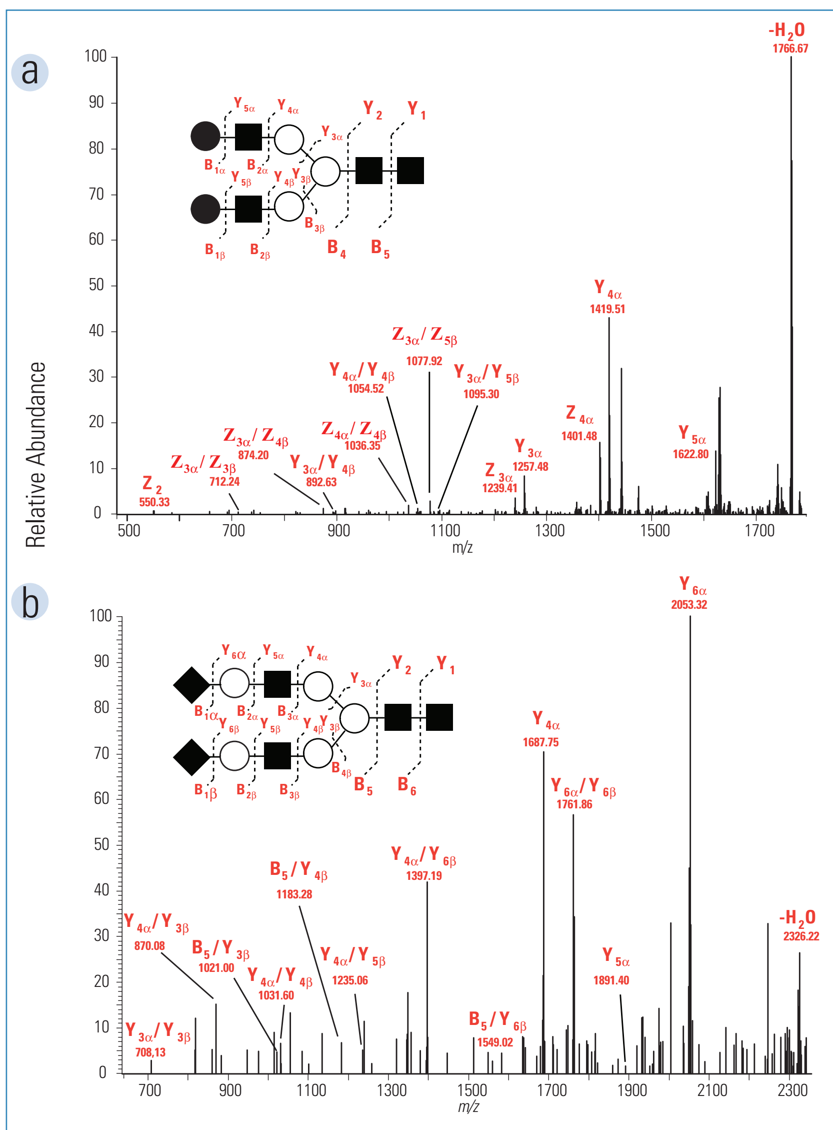

Figure 4: Ion trap MS/MS of (a) asialyl digalactosyl biantennary glycan-AA at *m/z* 1784.37 (single scan, total of 5 laser shot) and (b) disialyl biantennary glycan-AA at *m/z* 2344.843 (single scan, total of 9 laser shots) from human α1-acid glycoprotein.

even the labile nature of sialic acid poses a challenge for MS characterization.[15] Nevertheless, as shown in Figures 2 and 3, no significant in-source losses of sialic acid were observed during data acquisition by MALDI LTQ Orbitrap which enables us to perform relative quantification of detected glycans.

Others have explored quantification of sialylated glycans in the past, but majority of these approaches have relied on exploring sialylation in the absence of asialylated glycans due to the inability to see both while using the same MS ionization mode. Typically these quantification results have been reported on the basis of comparing sialylations relative to each other. Thanks to our ability to profile both sialylated and asialylated in the same mode or in the same spectrum, we were able to quantify sialylated glycans relative to the total amount of glycans present in a sample. Overall, the majority of the glycans detected in the study were sialylated, which is in agreement with what is observed in literature.

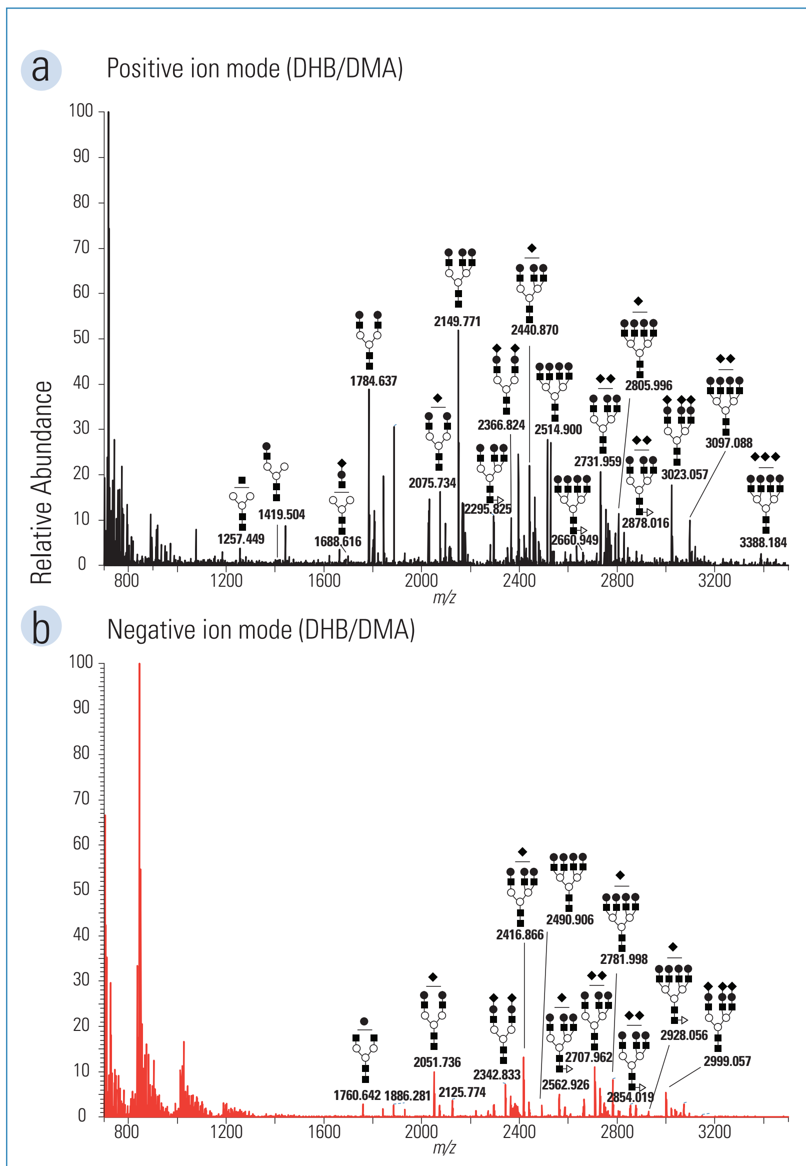

Figure 5: Orbitrap detector mass spectrum at resolving power 60,000 at *m/z* 400 of an *N*-linked glycans derivatized with 2-aminobenzoic acid from human α1-acid glycoprotein analyzed in (a) DHB/DMA matrix (average of 9 individual scans, total of 20 laser shots) by positive ion mode, (b) DHB/DMA matrix (average of 2 individual scans, total of 40 laser shots) by negative ion mode.

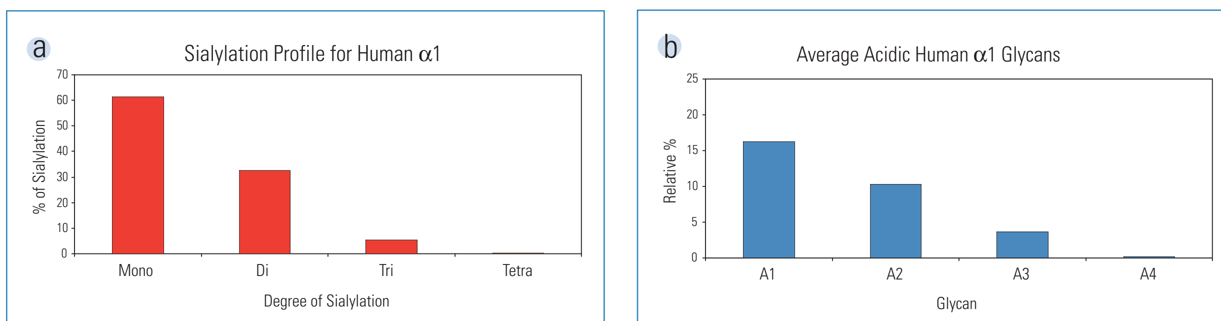

Figure 6: (a) Bar chart showing the distribution of sialylated structures found in human α1-acid glycoprotein. Values for the different glycan structures were reported as relative percentages to the total amount of glycan components found. (b) Showing the quantities of major components found.

Conclusion

We have developed a simple, fast, and reliable workflow for profiling *N*-linked glycans. It combines the benefits of MALDI LTQ Orbitrap technology with the homogeneity of sample distribution throughout the crystal layer provided by the DHB/DMA matrix. The high mass accuracy and mass resolution provided by the Orbitrap detector enables confident structural discrimination without the aid of tandem MS. In addition, ions are produced with sufficient abundance for further characterization, if needed. The ability to profile both sialylated and asialylated glycans with a single ionization mode in MALDI experiments introduces a previously lacking practicality into the workflow. This ability is useful in determining the degree of silaylation relative to overall glycan composition. Finally, more glycans were identified and characterized using the DHB/DMA matrix, than the much studied DHB matrix.

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