

Analysis of Sulfated Glycans by MALDI Orbitrap and DHB/N,N-Dimethylaniline Matrix

Julian Saba¹, Penelope Drake², Akraporn Prakobphol², Sarah Robinson², Susan J. Fisher², and Rosa Viner¹

¹Thermo Fisher Scientific, San Jose, CA ²University of California San Francisco, San Francisco, CA

Overview

Purpose: To develop a simple and reliable sample preparation method for the study of sulfated O-linked glycans by employing 2,5-dihydroxybenzoic acid/N,N-dimethylaniline (DHB/DMA) matrix and MALDI LTQ Orbitrap™ XL hybrid mass spectrometer.

Methods: Sulfated O-linked glycans from mucin were analyzed using DHB/DMA and DHB matrix on a MALDI LTQ Orbitrap XL mass spectrometer. Pulsed-Q dissociation (PQD) and collision-induced dissociation (CID) MSⁿ was conducted in the LTQ ion trap for ions of sufficient abundance with detection in the ion trap or Orbitrap detector.

Results: The DHB/DMA matrix enhanced the detection of sulfated O-linked glycans in the negative mode as compared to traditional DHB matrix. The high mass accuracy and mass resolution provided by the Orbitrap detector enabled confident structural discrimination between phosphorylated and sulfated species without the aid of tandem MS. FTMS PQD analysis confirmed the structural composition and enabled site localization of the sulfated group.

Introduction

Studies have shown sulfation is a key structural feature regulating numerous biological functions. For example, leukocyte tethering and rolling is mediated by interactions between L-selectin and its oligosaccharide ligand 6-sulfo sialyl Lewis x (the MECA-79 antigen). The apparent functional significance of the carbohydrate moieties creates a need for rapid, reliable and sensitive methods for their detection and characterization. Analysis of sulfated glycans by mass spectrometry (MS) has been a challenge for many years due in part to the limited capacity of these compounds to ionize as well as the labile nature of these substituents. A further challenge in MS strategies is the ability to distinguish between sulfation and phosphorylation, since these groups are isobaric. A factor complicated by the identical data generated by MS/MS analysis of these species.

Here we demonstrate the use of DHB/DMA matrix¹ for the analysis of sulfated O-linked glycans by MALDI-Orbitrap mass spectrometry. This matrix offers significant improvements over the conventional DHB matrix. Furthermore, the use of the MALDI-Orbitrap instrument provides accurate and reliable MS and MS/MS information which, combined with high mass resolution, brings definiteness to the analysis.

Methods

Sample Preparation

High-molecular-weight mucins were isolated by size-exclusion chromatography. The O-linked glycans were released by β -elimination. Borates were removed with glacial acetic acid and the sample was desalted on an ion-exchange column. Oligosaccharides were separated on a porous graphitized carbon solid-phase extraction cartridges (Alltech Associates, Deerfield, IL) into 10 and 20% ACN fractions for MS analysis.

The preparation of DHB/DMA solution was as follows: an initial solution of the DHB matrix (LaserBioLabs, France) was prepared by dissolving 20 mg of DHB in 200 μ L of 1:1, ACN/water. This was followed by adding 4 μ L of DMA (Sigma, St. Louis, MO) to the DHB solution, such that the molar ratio of DHB to DMA was about 3:1. DHB matrix in ethanol was prepared as 30 mg/mL solution in 70% ethanol/30% 0.1% TFA in water. Samples were deposited onto a MALDI target by mixing the analyte and matrix solutions (DHB in 70% ethanol and DHB/DMA respectively) (0.5 μ L each) on-target and allowing the mixture to dry at room temperature.

MALDI-MS

All MALDI experiments were performed on a Thermo Scientific MALDI LTQ Orbitrap XL hybrid mass spectrometer. Details on its functionalities and in particular the MALDI source design are described elsewhere.² The mass spectrometer was operated in negative ion mode with Orbitrap full scan setting using resolving power of 60000 or 100000 at m/z 400 (FWHM). For MS/MS analysis, both PQD and CID were conducted in the linear ion trap with Orbitrap or ion trap detection. For CID scans, the following settings were used: isolation width of 3 amu, normalized collision energy of 35%, 5 microscans and 5-50 laser shots/microscan. PQD collision energies were optimized on a 6-sulfo sialyl Lewis x standard that was spiked in the sample. An optimized normalized collision energy of 38% was used for all PQD spectra. Additionally, isolation width of 3 amu, 5 microscans and 5-50 laser shots/microscan were employed for PQD scans settings. For automated data acquisition, automatic gain control (AGC), automatic spectral filtering (ASF) and the crystal positioning system (CPS) were used to locate optimal sample regions.

FIGURE 1. Orbitrap full mass spectrum at resolving power 60,000 at m/z 400 of an equimolar mixture of O-linked glycans from mucin in DHB/DMA (top panel) and DHB (bottom panel). The asterisks denote the most intense glycans detected in each matrix. The red and blue boxes highlight mass range m/z 765-835 shown in Figure 2.

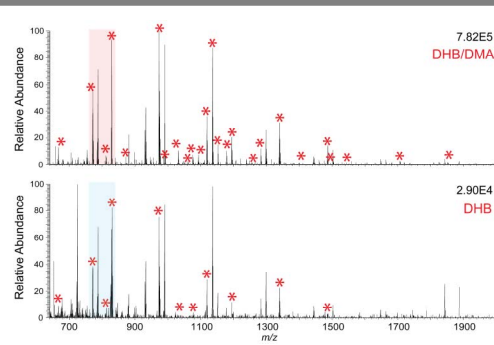
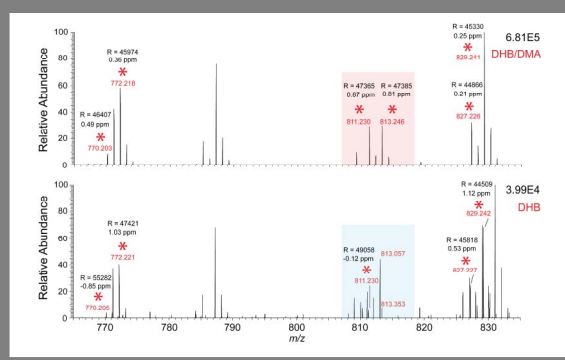


FIGURE 2. Orbitrap mass spectrum at mass range m/z 765-835, highlighting the O-linked glycan detected at m/z 813.246 in DHB/DMA matrix but not in DHB matrix as well as the cleaner spectrum obtained due to higher ionization efficiency of the DHB/DMA matrix vs. DHB matrix.



Results

- O-linked glycans from mucin were separated into two equimolar proportions (calculated volumetrically), and spotted onto the MALDI target using two different matrices, DHB (in 70% ethanol) and DHB/DMA. Mass spectra were acquired in the negative mode to evaluate ionization efficiency of the two matrices (Figure 1).
- 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. Studies have shown that using 70% ethanol as DHB matrix solvent improves samples spot quality, but in our case, due to efficiency with which CPS identified crystals, no improvement in analyte detection was observed.¹
- In contrast to DHB matrix, substantial improvements in sensitivity were observed using DHB/DMA. On average, a 25-fold increase in signal intensity was observed for detected glycans (equimolar sample calculated volumetrically).
- Due to the low ionization efficiency of sulfated O-linked glycans in conventional DHB, the spectrum contained extensive number of ions relating to the matrix as well as other ionizable species in the sample. DHB/DMA spectrum consisted mainly of glycan ions making structural assignment straightforward. (Figure 2).
- Alkaline borohydride treatment during the release of glycans from mucins can convert the HexNAc attached to Ser/Thr of the peptide core to HexNAcol, thus increasing the mass by 2.016 Da. We see evidence of glycans with HexNAc and HexNAcol in the identified structures (Figure 2 and Table 1).
- DHB/DMA also provided efficiency in detection of higher MW glycans ($m/z > 1400$) that DHB failed to do so. (Figure 3).
- Additionally, the number of sulfated O-linked glycans detected far exceeded those detected using the conventional DHB matrix (41 to 19).
- Molecular weights of the glycans were measured within 0.8 ppm of calculated monoisotopic values allowing for differentiation between sulfation and phosphorylation without MSⁿ analysis (Table 1).
- Ions were of sufficient abundance to allow MS/MS (PQD and CID) for further structural assignment
- Both PQD and CID mainly produced y and z ions, and provided overlapping fragmentation information. However, PQD data confirmed the presence and localization of sulfate groups (Figure 4).
- The presence of a sulfate group was indicated by the m/z 96.960 [HSO₄⁻] and the localization of sulfates group on a Hex or HexNAc was indicated by the presence of either m/z 241.002 [Hex-SO₃⁻] or m/z 282 [HexNAc-SO₃⁻].

FIGURE 3. Orbitrap mass spectrum of purified sulfated O-glycans over mass range m/z 1400-2000.

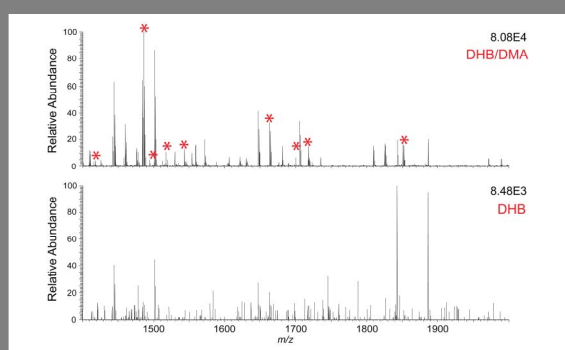
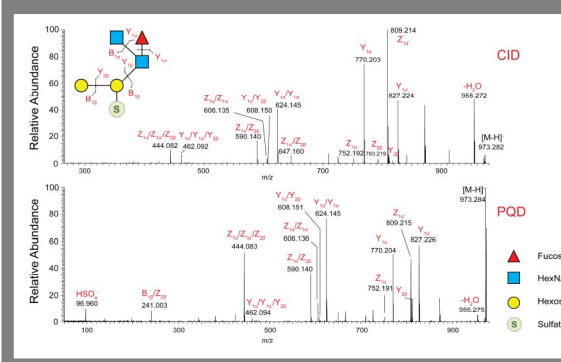


TABLE 1. Identified mucin sulfated O-linked glycans.

Glycan Structure	Observed [M-H] ⁻	Theoretical [M-H] ⁻	MassAccuracy (ppm)
HexNAc(HexNAc)	888.117	888.117	0.0
HexNAc(HexNAc)	889.117	889.117	0.0
HexNAc(HexNAc)	890.117	890.117	0.0
HexNAc(HexNAc)	891.117	891.117	0.0
HexNAc(HexNAc)	892.117	892.117	0.0
HexNAc(HexNAc)	893.117	893.117	0.0
HexNAc(HexNAc)	894.117	894.117	0.0
HexNAc(HexNAc)	895.117	895.117	0.0
HexNAc(HexNAc)	896.117	896.117	0.0
HexNAc(HexNAc)	897.117	897.117	0.0
HexNAc(HexNAc)	898.117	898.117	0.0
HexNAc(HexNAc)	899.117	899.117	0.0
HexNAc(HexNAc)	900.117	900.117	0.0
HexNAc(HexNAc)	901.117	901.117	0.0
HexNAc(HexNAc)	902.117	902.117	0.0
HexNAc(HexNAc)	903.117	903.117	0.0
HexNAc(HexNAc)	904.117	904.117	0.0
HexNAc(HexNAc)	905.117	905.117	0.0
HexNAc(HexNAc)	906.117	906.117	0.0
HexNAc(HexNAc)	907.117	907.117	0.0
HexNAc(HexNAc)	908.117	908.117	0.0
HexNAc(HexNAc)	909.117	909.117	0.0
HexNAc(HexNAc)	910.117	910.117	0.0
HexNAc(HexNAc)	911.117	911.117	0.0
HexNAc(HexNAc)	912.117	912.117	0.0
HexNAc(HexNAc)	913.117	913.117	0.0
HexNAc(HexNAc)	914.117	914.117	0.0
HexNAc(HexNAc)	915.117	915.117	0.0
HexNAc(HexNAc)	916.117	916.117	0.0
HexNAc(HexNAc)	917.117	917.117	0.0
HexNAc(HexNAc)	918.117	918.117	0.0
HexNAc(HexNAc)	919.117	919.117	0.0
HexNAc(HexNAc)	920.117	920.117	0.0
HexNAc(HexNAc)	921.117	921.117	0.0
HexNAc(HexNAc)	922.117	922.117	0.0
HexNAc(HexNAc)	923.117	923.117	0.0
HexNAc(HexNAc)	924.117	924.117	0.0
HexNAc(HexNAc)	925.117	925.117	0.0
HexNAc(HexNAc)	926.117	926.117	0.0
HexNAc(HexNAc)	927.117	927.117	0.0
HexNAc(HexNAc)	928.117	928.117	0.0
HexNAc(HexNAc)	929.117	929.117	0.0
HexNAc(HexNAc)	930.117	930.117	0.0
HexNAc(HexNAc)	931.117	931.117	0.0
HexNAc(HexNAc)	932.117	932.117	0.0
HexNAc(HexNAc)	933.117	933.117	0.0
HexNAc(HexNAc)	934.117	934.117	0.0
HexNAc(HexNAc)	935.117	935.117	0.0
HexNAc(HexNAc)	936.117	936.117	0.0
HexNAc(HexNAc)	937.117	937.117	0.0
HexNAc(HexNAc)	938.117	938.117	0.0
HexNAc(HexNAc)	939.117	939.117	0.0
HexNAc(HexNAc)	940.117	940.117	0.0
HexNAc(HexNAc)	941.117	941.117	0.0
HexNAc(HexNAc)	942.117	942.117	0.0
HexNAc(HexNAc)	943.117	943.117	0.0
HexNAc(HexNAc)	944.117	944.117	0.0
HexNAc(HexNAc)	945.117	945.117	0.0
HexNAc(HexNAc)	946.117	946.117	0.0
HexNAc(HexNAc)	947.117	947.117	0.0
HexNAc(HexNAc)	948.117	948.117	0.0
HexNAc(HexNAc)	949.117	949.117	0.0
HexNAc(HexNAc)	950.117	950.117	0.0
HexNAc(HexNAc)	951.117	951.117	0.0
HexNAc(HexNAc)	952.117	952.117	0.0
HexNAc(HexNAc)	953.117	953.117	0.0
HexNAc(HexNAc)	954.117	954.117	0.0
HexNAc(HexNAc)	955.117	955.117	0.0
HexNAc(HexNAc)	956.117	956.117	0.0
HexNAc(HexNAc)	957.117	957.117	0.0
HexNAc(HexNAc)	958.117	958.117	0.0
HexNAc(HexNAc)	959.117	959.117	0.0
HexNAc(HexNAc)	960.117	960.117	0.0
HexNAc(HexNAc)	961.117	961.117	0.0
HexNAc(HexNAc)	962.117	962.117	0.0
HexNAc(HexNAc)	963.117	963.117	0.0
HexNAc(HexNAc)	964.117	964.117	0.0
HexNAc(HexNAc)	965.117	965.117	0.0
HexNAc(HexNAc)	966.117	966.117	0.0
HexNAc(HexNAc)	967.117	967.117	0.0
HexNAc(HexNAc)	968.117	968.117	0.0
HexNAc(HexNAc)	969.117	969.117	0.0
HexNAc(HexNAc)	970.117	970.117	0.0
HexNAc(HexNAc)	971.117	971.117	0.0
HexNAc(HexNAc)	972.117	972.117	0.0
HexNAc(HexNAc)	973.117	973.117	0.0
HexNAc(HexNAc)	974.117	974.117	0.0
HexNAc(HexNAc)	975.117	975.117	0.0
HexNAc(HexNAc)	976.117	976.117	0.0
HexNAc(HexNAc)	977.117	977.117	0.0
HexNAc(HexNAc)	978.117	978.117	0.0
HexNAc(HexNAc)	979.117	979.117	0.0
HexNAc(HexNAc)	980.117	980.117	0.0
HexNAc(HexNAc)	981.117	981.117	0.0
HexNAc(HexNAc)	982.117	982.117	0.0
HexNAc(HexNAc)	983.117	983.117	0.0
HexNAc(HexNAc)	984.117	984.117	0.0
HexNAc(HexNAc)	985.117	985.117	0.0
HexNAc(HexNAc)	986.117	986.117	0.0
HexNAc(HexNAc)	987.117	987.117	0.0
HexNAc(HexNAc)	988.117	988.117	0.0
HexNAc(HexNAc)	989.117	989.117	0.0
HexNAc(HexNAc)	990.117	990.117	0.0
HexNAc(HexNAc)	991.117	991.117	0.0
HexNAc(HexNAc)	992.117	992.117	0.0
HexNAc(HexNAc)	993.117	993.117	0.0
HexNAc(HexNAc)	994.117	994.117	0.0
HexNAc(HexNAc)	995.117	995.117	0.0
HexNAc(HexNAc)	996.117	996.117	0.0
HexNAc(HexNAc)	997.117	997.117	0.0
HexNAc(HexNAc)	998.117	998.117	0.0
HexNAc(HexNAc)	999.117	999.117	0.0
HexNAc(HexNAc)	1000.117	1000.117	0.0

FIGURE 4. Orbitrap CID (top panel) and PQD (bottom panel) MS/MS spectra of O-linked sulfated glycan FucHex₂HexNAc₂ at m/z 973.282. The fragment ions peaks are labeled according to the nomenclature proposed by Domon and Costello.³



Conclusion

- We developed a simple, fast, and reliable workflow for profiling sulfated O-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.
- Improvements in detection and ionization efficiency were demonstrated.
- The high mass accuracy and mass resolution provided by the Orbitrap detector enabled confident structural discrimination without the aid of tandem MS.
- PQD provides the "low mass" mass region for fragment ions in the LTQ ion trap that is required for localization of sulfate group.

References

- Snovida, S. I.; Perreault, H. A 2,5-dihydroxybenzoic acid/N,N-dimethylaniline matrix for the analysis of oligosaccharides by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom.* **2007**, *21* (22), 3711-5.
- Strupat, K.; Kovtoun, V.; Bui, H.; Viner, R.; Stafford, G.; Horning, S., MALDI produced ions inspected with a linear ion trap-Orbitrap hybrid mass analyzer. *J Am Soc Mass Spectrom* **2009**, *20*, (8), 1451-63.
- Domon, B.; Costello, C. E., A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate J* **1988**, *5*, 397-409.

Acknowledgements

The authors would like to thank Sergei Snovida at University Of Manitoba for helpful assistance in DHB/DMA matrix preparation.

All trademarks are the property of Thermo Fisher Scientific and its subsidiaries.