SHIMADZU
Excellence in Science

Application News

No. LCMS-116

Liquid Chromatography Mass Spectrometry

N-Linked Glycans Analysis of Monoclonal Antibodies, Biosimilars, and Glycoproteins with High Resolution Mass Spectrometry and Fluorescence Detection using Restek's Raptor Polar X Column and Protein Metrics' Software Suite

■ Introduction

Therapeutic antibodies have quickly become one of the predominant classes of new drugs developed over the years, with revenue predicted to be over $300 billion by the year 2025[1]. Biosimilar therapy drugs are designed to have similar properties compared to their native monoclonal antibody (mAb) but are far less expensive to produce.

Quality by Design (QbD) standards are implemented throughout the pharmaceutical manufacturing process of biosimilars to ensure consistency. Critical Quality Attributes (CQA) are benchmark criteria used in QbD standards that can be continuously monitored and measured to ensure final products are within acceptable quality limits[2].

Ensuring consistent glycosylation during process validation is an important CQA because N-linked glycans can affect a glycoprotein's stability, solubility and recognition by glycan-binding proteins. N-glycans are attached to proteins at asparagine (Asn) residues by a N-glyosidic covalent bond. In monoclonal antibodies and biosimilars, this occurs at the crystallizable fragment (Fc), as shown in **Figure 1**.

Characterization of released glycans from monoclonal antibodies, biosimilars, and glycoproteins were completed using the Shimadzu LCMS-9030 high resolution quadrupole time of flight (QToF) mass spectrometer with fluorescence detection using the Restek Raptor Polar X column.

The Raptor Polar X column was chosen in this study due to its unique stationary phase which balances ion exchange and hydrophilic interaction liquid chromatography (HILIC) to retain and separate polar analytes. This balanced retention for polar compounds should provide a powerful separation of glycans. The released glycan data was processed using Detached Glycan (N-Linked) Workflow from Protein Metrics.

This workflow can be combined with previously published workflows for complete mAb characterization[3].

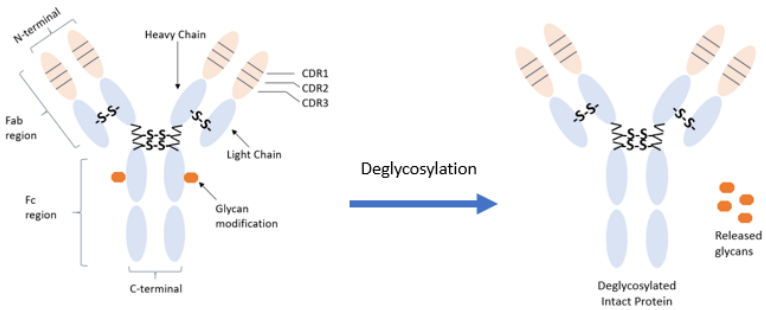


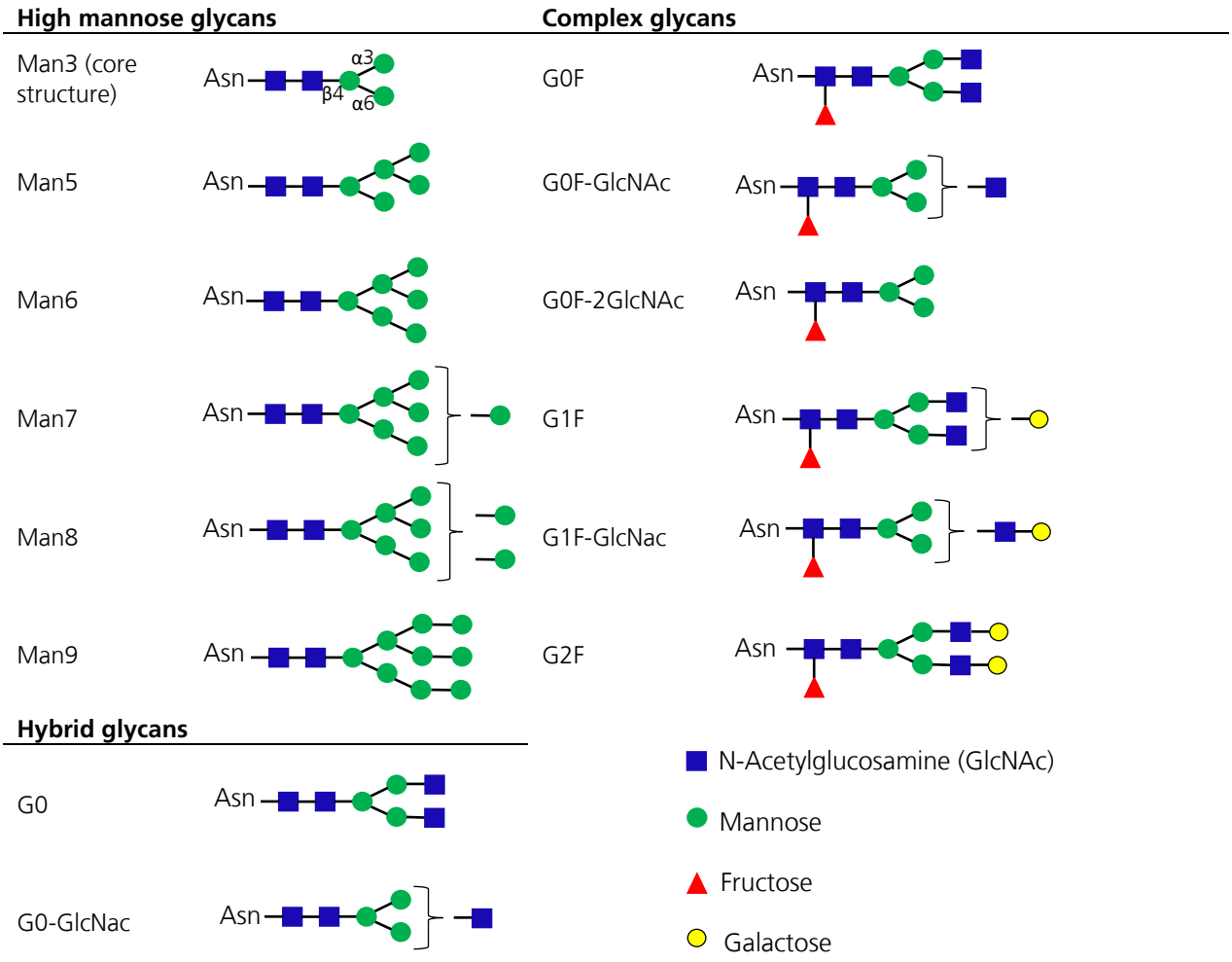
Figure 1: N-linked glycans are attached to proteins at Asn residues on the Fc region

■ Glycan Structure and Nomenclature

N-linked glycans share a common pentasaccharide core structure containing three mannose residues attached to two N-Acetylglucosamine (GlcNAc) residues (**Table 1**). One GlcNAc is attached to an Asn side chain of the protein. The Asn residue is recognized when asparagine is followed by any amino acid, except for proline, and ends with serine or threonine (Asn-X-Ser/Thr). N-linked Glycans can be further classified into three types as shown in **Table 1**.

High mannose glycans contain only unsubstituted terminal mannose sugars, while hybrid and complex glycans both contain GlcNac substituted "antennae" linked to the mannose core. In hybrid glycans, only mannose residues are attached to the Manα6 arm of the core. In complex glycans, the added GlcNac is on both Manα6 and Manα3 sites.

Table 1: N-Linked glycan structure



■ Experimental

Materials and Methods

NIST mAb Humanized IgG1κ monoclonal antibody reference material 8671 was purchased from the National Institute of Standards & Technology (NIST). Bevacizumab biosimilar (brand name Avastin) was donated by Intertek Pharmaceuticals (San Diego, CA). High mannose glycoprotein RNase B was purchased from Sigma-Aldrich (St. Louis, MO). Proteins were treated with PNGase F purchased from New England Biolabs (Ipswich, MA) and analyzed using Restek's Raptor Polar X column (part # 9311A12) with the conditions listed in **Table 2**.

Table 2: Liquid Chromatography (LC), fluorescence and MS conditions

LC Conditions	
Column	Restek Raptor Polar X (2.1 × 100 mm × 2.7 µm) Part #: 9311A12
Mobile Phase A	0.5% formic acid in water
Mobile Phase B	90:10 Acetonitrile:water, 20mM ammonium formate, pH 3
Flow Rate	0.4mL/min
Column Temp	40 °C
Gradient	0min-0.5min, 88%B, 0.5min-30.5min, 75%B, 30.5-30.6min, 10%B, 30.6min-32.6min, 10%B, 32.6-32.7min, 88%B, 37.7min, end
Fluorescence Conditions	
Detector	RF-20Axs
Excitation	310
Emission	370
Cell Temperature	40 °C
Q-ToF Conditions	
Mode	DDA
TOF Start m/z	500.0000
TOF End m/z	1100.0000
DDA Start m/z	100.0000
DDA End m/z	2200.0000
CE	50
CE Spread	25
Event Time (s)	0.100
Pulser Inj, Time	193
Nebulizing Gas Flow	3.0L/min
Heating Gas Flow	10L/min
Interface Temp	300 °C
Drying Gas Flow	10L/min

Deglycosylation

Intact proteins were solubilized in 50mM ammonium bicarbonate at a 1mg/mL concentration, followed by treatment with PNGase F to remove N-linked glycans. 100µg of protein was combined with 10µL (100U) PNGase F and incubated for 4 hours at 37 °C.

The released glycans were extracted using ZipTip C4 pipette tips by eluting with 0.1% formic acid in water and were dried down by a centrifugal evaporator.

Procainamide labeling and purification

Released glycans were labeled with LudgerTag™ Procainamide Glycan Labeling Kit[4] using 10uL of procainamide dye, DMSO, glacial acetic acid, and 2-picoline borane. Procainamide labeled glycans were purified using Discovery® Glycan SPE Tubes[5] and dried down by a centrifugal evaporator. Samples were reconstituted in 100µL 90:10 ACN:Water.

■ Instrumentation

All data from this application note were obtained on a Shimadzu UHPLC in conjunction with a fluorescence detector and a Q-ToF Mass Spectrometer, LCMS-9030, connected in series. The specific configuration includes LC-30AD x2 solvent delivery pumps, DGU-20A5R online degassing units, SIL-30AC autosampler, CTO-20AC column oven, CBM-20A system controller, RF-20AXS fluorescence detector, LCMS-9030 QTOF, LabSolutions Ver. 5.97SP1 chromatography workstation, and Detached Glycan (N-linked) Workflow from Protein Metrics.

■ Results

RNAse B

Figure 2 shows the fluorescence chromatogram (left) and extracted ion chromatogram (right) for the high mannose (Man5-Man9) glycans which are characteristic of RNaseB glycoprotein The Restek Polar X column achieved excellent baseline separation for all glycans.

Table 3 shows the mass accuracy of the Shimadzu LCMS-9030 for RNAse B procainamide labeled glycans. The data was processed using Protein Metrics Detached Glycan (N-Linked) workflow and tabulated automatically. All glycans show better than 5ppm mass accuracy.

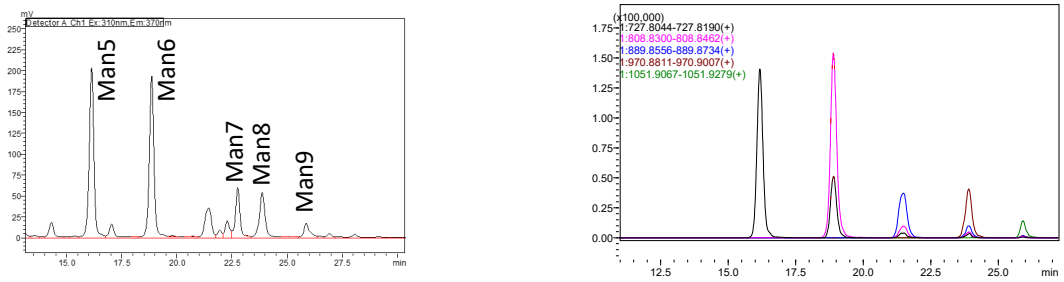


Figure 2: Fluorescence chromatogram (left) and extracted ion chromatogram (right) for procainamide labeled RNaseB Glycans

Peak #	Apex time	[Glycan Name]	Glycans	Obs.M	Calc.M	[ppm]	GlycanClassification
1	16.1955	Man5	Procainamide HexNAc(2)Hex(5)	1453.605	1453.607	-1.60	High manose
2	18.9061	Man6	Procainamide HexNAc(2)Hex(6)	1615.658	1615.660	-0.96	High manose
3	21.4623	Man7	Procainamide HexNAc(2)Hex(7)	1777.711	1777.713	-1.11	High manose
4	23.9079	Man8	Procainamide HexNAc(2)Hex(8)	1939.761	1939.765	-2.06	High manose
5	25.904	Man9	Procainamide HexNAc(2)Hex(9)	2101.814	2101.818	-1.82	High manose

Table 3: Mass accuracy of LCMS-9030 for RNaseB glycans

Avastin

Figure 3 shows the fluorescence chromatogram (left) and extracted ion chromatogram (right) for glycans found on Avastin biosimilar.

Table 4 shows the mass accuracy of the Shimadzu LCMS-9030 for Avastin procainamide labeled glycans. The data was processed using Protein Metrics Detached Glycan (N-Linked) workflow All glycans show better than 5ppm mass accuracy.

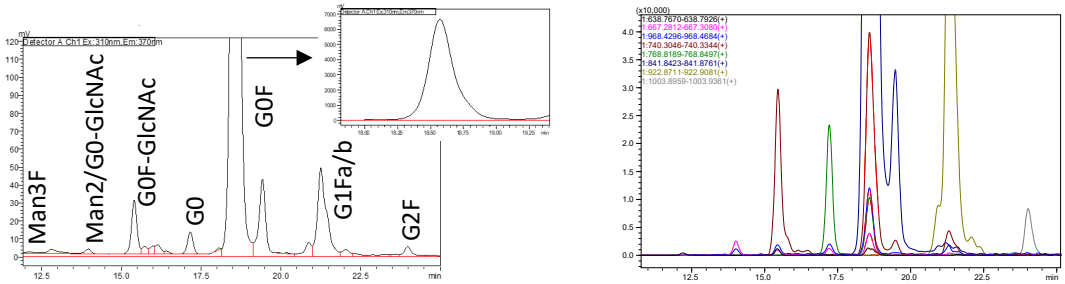


Figure 3: Fluorescence chromatogram (left) and extracted ion chromatogram (right) for procainamide labeled Avastin glycans.

Peak #	Apex time	[Glycan Name]	Glycans	Obs.M	Calc.M	[ppm]	Glycan Classification
1	12.3509	Man3F	Procainamide HexNAc(2)Fuc(1)Hex(3)	1275.56	1275.56	-2.50	Neutral fucosylated
2	14.0537	G0 - GlcNAc	Procainamide HexNAc(3)Hex(3)	1332.58	1332.58	-3.04	Neutral a-fucosylated
		Man2	Procainamide HexNAc(2)Hex(2)	967.449	967.449	0.00	High manose
3	15.4923	G0F - GlcNAc	Procainamide HexNAc(3)Fuc(1)Hex(3)	1478.64	1478.64	-1.33	Neutral fucosylated
4	17.223	G0	Procainamide HexNAc(4)Hex(3)	1535.66	1535.66	-1.97	Neutral a-fucosylated
5	18.6017	G0F	Procainamide HexNAc(4)Fuc(1)Hex(3)	1681.72	1681.72	-1.75	Neutral fucosylated
6	21.3463	G1F	Procainamide HexNAc(4)Fuc(1)Hex(4)	1843.77	1843.77	-0.85	Neutral fucosylated
7	24.0609	G2F	Procainamide HexNAc(4)Fuc(1)Hex(5)	2005.82	2005.82	-0.69	Neutral fucosylated

Table 4: Mass accuracy of LCMS-9030 for Avastin glycans

NIST mAb

Figure 4 shows the fluorescence chromatogram (left) and extracted ion chromatogram (right) for glycans found on NIST mAb.

Table 5 shows the mass accuracy of the Shimadzu LCMS-9030 for NIST mAb procainamide labeled glycans. The data was processed using Protein Metrics Detached Glycan (N-Linked) workflow. All glycans show better than 6ppm mass accuracy.

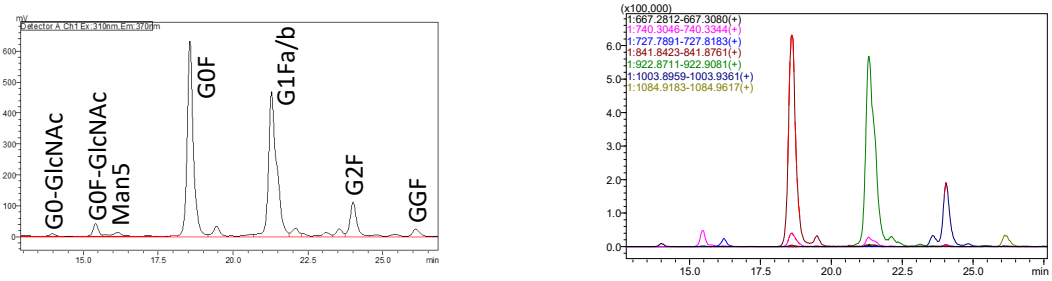


Figure 4. Fluorescence chromatogram (left) and TIC (right) for procainamide labeled NIST mAb glycans.

Peak #	Apex time ↑	[Glycan Name]	Glycans	Obs.M ↑	Calc.M ↑	[ppm] ↑	Glycan Classificatin ↑
1	13.9697	G0 - GlcNAc	Procainamide HexNAc(3)Hex(3)	1332.57	1332.58	-4.69	Neutral a-fucosylated
		Man3 + GlcNAc	Procainamide HexNAc(3)Hex(3)	1332.57	1332.58	-4.69	Neutral a-fucosylated
2	15.4397	G0F - GlcNAc	Procainamide HexNAc(3)Fuc(1)Hex(3)	1478.63	1478.64	-2.95	Neutral fucosylated
3	16.2145	Man5	Procainamide HexNAc(2)Hex(5)	1453.6	1453.61	-5.87	High manose
4	18.575	G0F	Procainamide HexNAc(4)Fuc(1)Hex(3)	1681.72	1681.72	-1.75	Neutral fucosylated
5	21.3306	G1F	Procainamide HexNAc(4)Fuc(1)Hex(4)	1843.77	1843.77	-1.50	Neutral fucosylated
6	24.0371	G2F	Procainamide HexNAc(4)Fuc(1)Hex(5)	2005.82	2005.82	-0.49	Neutral fucosylated
7	26.1079	GG F	Procainamide HexNAc(4)Fuc(1)Hex(6)	2167.87	2167.88	-0.83	Neutral fucosylated

Table 5: Mass accuracy of LCMS-9030 for NIST mAb glycans

Normalized Peak Areas for each glycan in each sample

The fluorescence chromatogram was integrated, and the normalized area for each released glycan was automatically plotted as a pie chart using Protein metrics report generator. **Figure 5** shows the pie chart for RNase B (left), NIST mAb (middle) and Avastin (right).

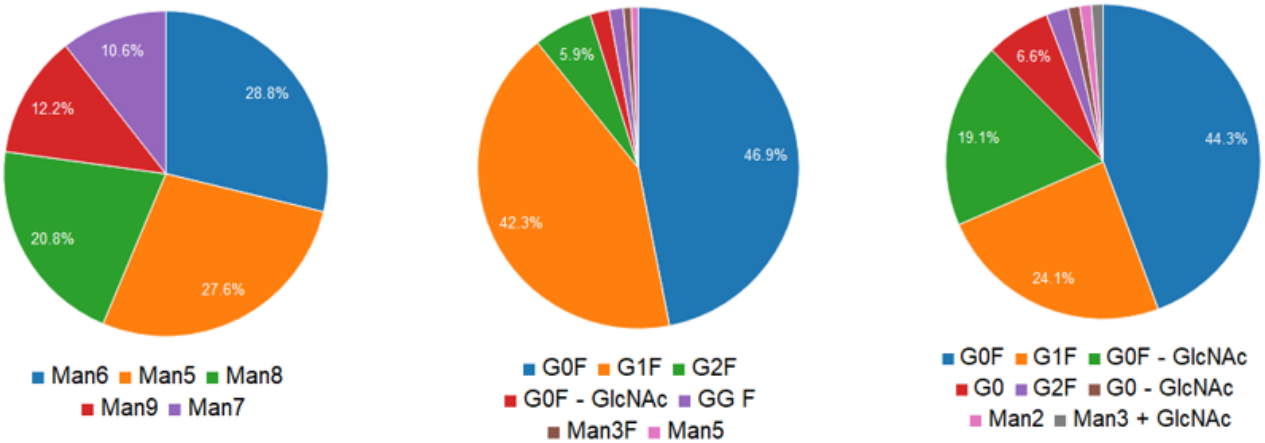
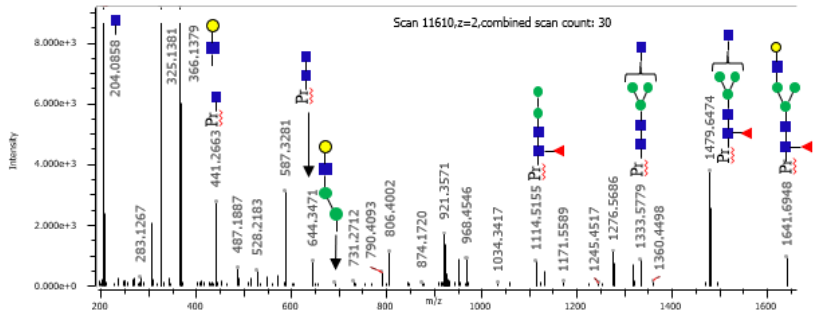


Figure 5: Normalized area for glycans released from RNase B (left), NIST mAb (middle) and Avastin (right)

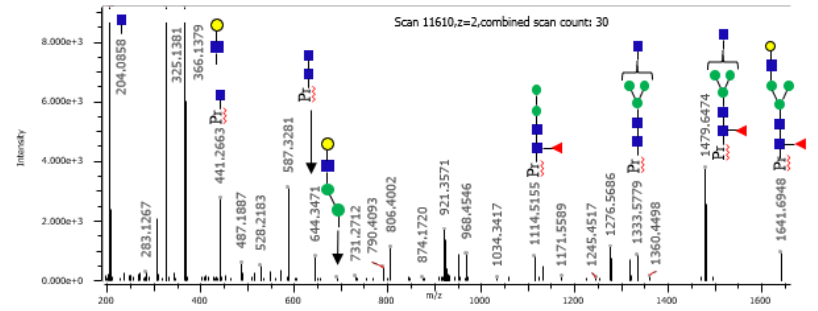
MS/MS Spectra

To determine the spectra of high abundant glycans, MS/MS fragmentation was performed, and the spectra were analyzed for glycan fragments. **Figure 6** shows the MS/MS spectra of abundant glycans.

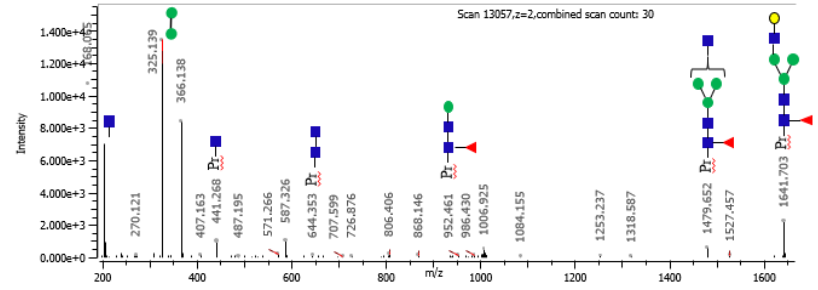
G0F



G1F



G2F



Man5

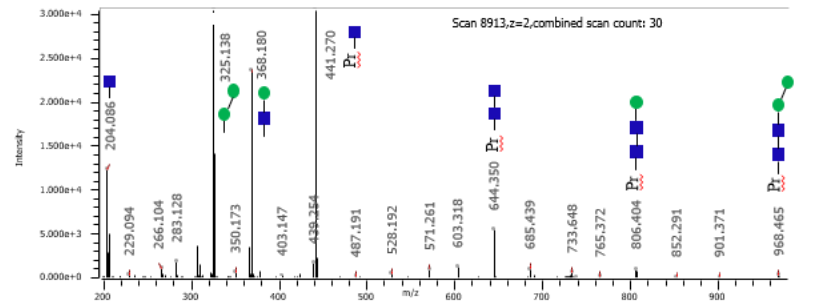


Figure 6: MS/MS spectra for selected procainamide labeled glycans

■ Conclusion

The Shimadzu LCMS-9030 has shown excellent mass accuracy for N-linked glycans on several proteins. The Restek Raptor Polar X, an innovative hybrid ion-exchange/HILIC column, is an ideal column for glycans as reflected by the baseline separation of the structurally similar N-glycans. Protein Metrics Glycan Workflow offers additional workflows to other published methods[3].

■ References

1. https://jbiomedsci.biomedcentral.com/articles/10.1186/s12929-019-0592-z
2. https://www.gxp-cc.com/news/fda-european-regulations-for-life-sciences/2014/07/15/defining-critical-quality-attributes-in-the-pharmaceutical-manufacturing-process
3. https://www.ssi.shimadzu.com/sites/ssi.shimadzu.com/files/Products/literature/lcms/Shimadzu_LCMS-103_NIST_mAB_WorkflowQTOF_0624.pdf
4. https://www.ludger.com/docs/products/lt/lt-kproc/ludger-lt-kproc-vp24-guide.pdf
5. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/General_Information/1/T415070.pdf

Special thanks to Restek for method development assistance with this new column phase

ULTRA FAST MASS SPECTROMETRY


LCMS-8040


LCMS-8045


LCMS-8050


LCMS-8060NX


LCMS-2020


Q-TOF LCMS-9030

Founded in 1875, Shimadzu Corporation, a leader in the development of advanced technologies, has a distinguished history of innovation built on the foundation of contributing to society through science and technology. Established in 1975, Shimadzu Scientific Instruments (SSI), the American subsidiary of Shimadzu Corporation, provides a comprehensive range of analytical solutions to laboratories throughout North, Central, and parts of South America. SSI maintains a network of ten regional offices strategically located across the United States, with experienced technical specialists, service and sales engineers situated throughout the country, as well as applications laboratories on both coasts.

For information about Shimadzu Scientific Instruments and to contact your local office, please visit our Web site at www.ssi.shimadzu.com

SHIMADZU

Shimadzu Corporation
www.shimadzu.com/an/

SHIMADZU SCIENTIFIC INSTRUMENTS, INC.
Applications Laboratory
7102 Riverwood Drive, Columbia, MD 21045
Phone: 800-477-1227 Fax: 410-381-1222
URL https://www.ssi.shimadzu.com

First Edition: September 2020