Comprehensive Glycosylation Analysis to Meet Regulatory Requirements: Full N-Glycan Characterisation and Quantitation of Sialic Acids

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Introduction

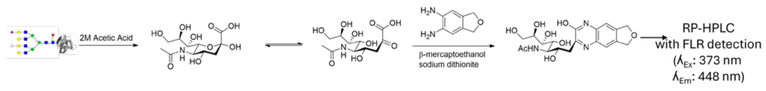
For most therapeutic glycoproteins the glycosylation patterns greatly influence the clinical performance of the drug product, particularly its in vivo safety and efficacy profile.[1] Similarly, in biological tissues glycosylation patterns can correlate with the state of health or disease of the individual.[2] Given this, there is increasing interest in accurately characterizing glycosylation both for biopharmaceutical product development and for biomarker discovery.

Our analytical strategy implements a quality by design (QbD) framework aligned with current FDA, EMA, and ICH expectations for glycosylation assessment. The workflow consists of complementary techniques designed to characterise glycosylation patterns at the required level of detail: quantitative analysis of sialic acid species, quantitative analysis of monosachcaride composition, and full N-glycan and glycopeptide characterisation. Below, we present analytical methodologies required for each technique and summarise the obtained data, using bovine fetuin as a model glycoprotein.

Sialic acid analysis

Biopharmaceuticals often contain two main types of sialic acid; N-acetyl-neuraminic acid (Neu5Ac) and N-glycolyl-neuraminic acid (Neu5Gc). Neu5Ac is found in both human and non-human cells, whereas Neu5Gc not present on human glycoproteins and is immunogenic. The biopharmaceuticals efficacy, serum half-life and immunogenicity are impacted by both the abundance and the type of sialylation (including O-acetylation). Consequently, sialylation is a glycosylation critical quality attribute (GCQA). Sialic acid analysis is in the quality guidelines for registration of biopharmaceuticals (ICHQ6B) and should be performed throughout the product lifecycle.

Workflow: Sialic acids are released from glycoproteins by mild acid hydrolysis using conditions that preserve the N-acetyl, N-glycolyl and O-acetylation. The keto groups of the free sialic acids are derivatised with DMB using the LudgerTag DMB Analysis Kit (LT-KDMB-A1). The DMB-labelled sialic acids are then analysed by RP-HPLC along with DMB-labeled standards to allow for quantitation.



Scheme 1: Workflow for Sialic Acid analysis using Ludger's DMB Kit

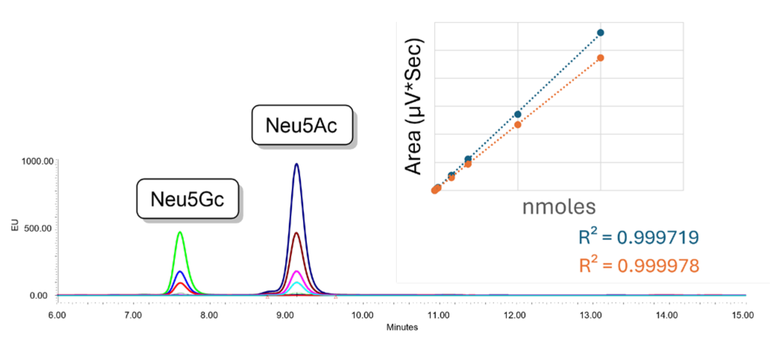


Figure 1X: Overlay of spectra from serial dilutions of Neu5Ac and Neu5Gc standards (Right) used for calibration curves (Left)

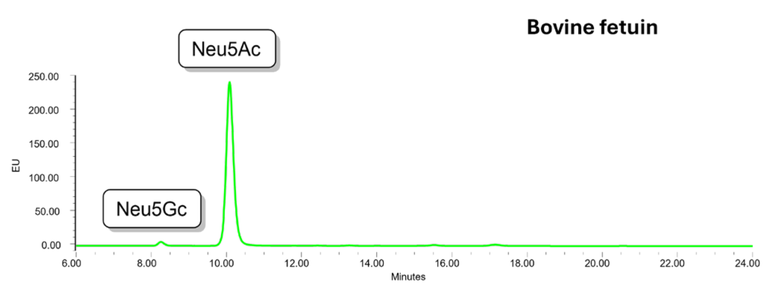


Figure 2: DMB profile of Bovine fetuin

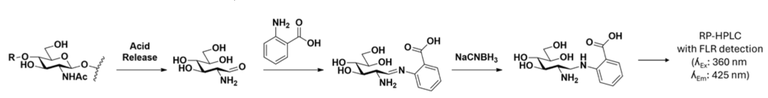
Bovine fetuin (50 µg Glycoprotein)			
Sialic Acid	Amount of Sialic Acids on Protein (nmoles/mg protein)	Amount of Neu5Gc and Neu5Ac (nmoles)	Average Relative Percent (%) of Sialic Acids from Peak Areas
Neu5Gc	8.42	0.051	2.06
Neu5Ac	339.36	2.001	97.94

Table 1: Glycometrics for Sialic Acids in Bovine fetuin

Monosaccharide Analysis

Monosaccharide analysis is a regulatory requirement laid out in the ICH Q6B guidelines for characterisation of biopharmaceuticals and provides absolute or relative quantitation of the neutral (i.e. non-anionic) monosaccharides and information relating to the types of N- and/or O-glycans present on a glycoprotein.

Workflow: Monosaccharides are released from the glycoprotein by acid hydrolysis using 2M trifluoroacetic acid (TFA) (for quantitation of mannose (Man), galactose (Gal), glucose (Glc), fucose(Fuc)) or 6M hydrochloric acid (HCl) (for quantitation of glucosamine (GlcN) and galactosamine (GalN) and labelled with 2-AA using the Ludger Monosaccharide release & labelling kit (LT-MONO-96). The 2-AA-labelled sialic acids are then analysed by RP-HPLC along with 2AA-labelled standards to allow for quantitation.



Scheme 2: Workflow for Monosaccharide analysis using Ludger's Monosaccharide release & labelling kit

HCL & TFA Release Data	nmol/mg protein	
Monosaccharide	Average	%CV
GlcN	346.72	2.1
GalN	63.22	2
Gal	318.38	7.3
Man	190.89	8.9
Glc	12.65	98.4
Fuc	4.18	10.5

Table 2: Glycometrics for Monosacharides in Bovine fetuin

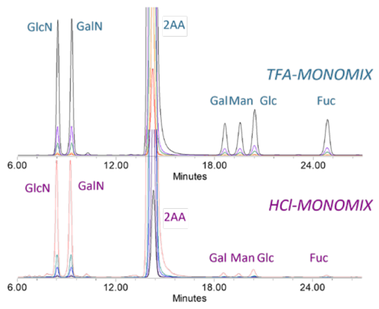


Figure 3: Overlay of chromatograms from serial dilutions of Monosaccharide mix standard contains (GlcN, GalN, Gal, Man, Glc and Fuc) treated with TFA and HCl used for calibration curves.

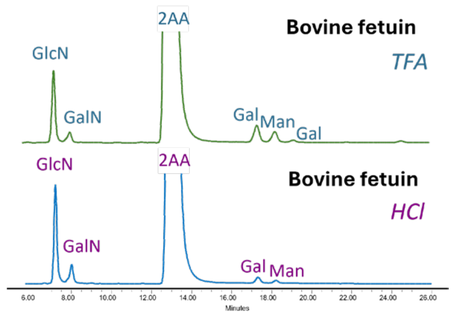
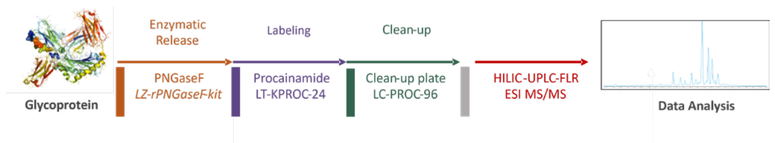


Figure 4: Overlay of chromatograms for 2AA labelled monosaccharides released by TFA (top) and HCl (bottom) from Bovine fetuin.

Detailed N-Glycan Analysis

Provides structural composition and relative amounts of individual N-glycans present on the glycoprotein.

Workflow: N-glycans are released using PNGAse F endoglycosidase then derivatised with fluorescent dye procainamide (PROC). Procainamide labelled glycans are analysed using HILIC (Hydrophilic Interaction Liquid Chromatography) UHPLC and ESI-MS/MS - generally in hyphenated configuration.



Scheme 3. General glycoprofiling workflow for analysis of N-glycans

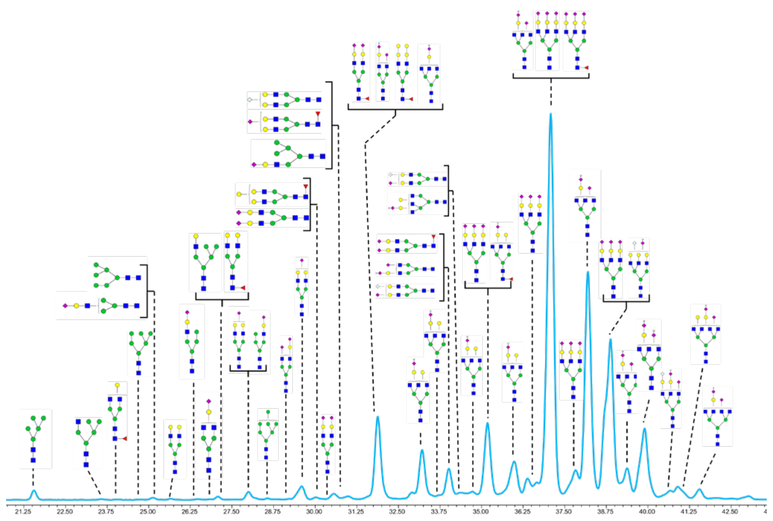


Figure 5. HILIC-UPLC profiles of procainamide labelled N-glycans released from Bovine fetuin. Structures have been assigned by mass spectrometry.

Fetuin																		
HPLC Peak id	Average GU (procainamide)	% Area	Possible structure	LC-ESI-MS														
				Composition									[M/2]$^{2+}$ calculated	[M/2]$^{2+}$ calculated	[M/2]$^{2+}$ registered	[M/2]$^{2+}$ registered	[M/Z] characteristic fragment ions (composition)	
				Hex (H)	HexNAc (N)	Fuc (F)	NeuAc (S)	NeuGc (Sg)									Y ions	B ions
25	9.51	5.41	A3G3S3	6	5	0	3	0	1550.10	1033.74	-	1033.74	441.71 (N1-PROC) 806.28 (H1N2-PROC) 968.55 (H2N2-PROC) 1171.60 (H2N3-PROC) 1333.73 (H3N3-PROC) 1495.66 (H4N3-PROC) 1624.68 (H3NS1-PROC) 1668.67 (H4N4-PROC)	1786.74 (H4NS1-PROC) 1951.77 (H5N4-PROC) 1960.01 (H4NS1-PROC) 2151.81 (H4NS1-PROC) 812.81 (H3NS1-PROC)2+ 1076.48 (H5NS1-PROC)2+ 1140.94 (H5NS1-PROC)2+ 1221.98 (H5NS2-PROC)2+	366.75 (H1N1) 454.57 (H1S1) 528.48 (H2N1) 657.25 (H1N1S1) 819.26 (H2N1S1) 1184.43 (H3N2S1) 1475.57 (H3N2S2) 1711.55 (H3N3S1)	2002.77 (H5NS2) 2077.67 (H6NAS1)
			FA3G3S2	6	5	1	2	0	1477.58	985.39	-	985.37	441.71 (N1-PROC) 587.50 (N1F1-PROC) 644.42 (N2-PROC) 790.76 (N2F1-PROC) 806.42 (H1N2-PROC) 968.80 (H2N2-PROC) 1114.60 (H2N2F1-PROC) 1171.53 (H2N3-PROC) 1333.75 (H3N3-PROC)	1479.65 (H2N2F1-PROC) 1495.55 (H4N2-PROC) 1624.85 (H3NS1-PROC) 1641.77 (H4NS1-PROC) 1786.79 (H4NS1-PROC) 1932.95 (H4NS1F1-PROC)2+ 1149.48 (H6NS1F1-PROC)2+ 1221.01 (H5NS2-PROC)2+	366.73 (H1N1) 819.38 (H2N1S1) 1184.39 (H3N2S1)	
26	9.77	3.78	A3G3S2	6	5	0	2	0	1404.55	936.70	1404.43	936.72	441.70 (N1-PROC) 806.54 (H1N2-PROC) 968.57 (H2N2-PROC) 1130.53 (H3N2-PROC) 1333.68 (H3N3-PROC) 1495.66 (H4N3-PROC) 1624.68 (H3NS1-PROC) 1786.80 (H4NS1-PROC)	1860.78 (H5N4-PROC) 2151.84 (H5NS1-PROC) 812.84 (H3NS1-PROC)2+ 893.99 (H4NS1-PROC)2+ 995.94 (H4NS1-PROC)2+ 1076.52 (H5NS1-PROC)2+ 1222.03 (H5NS2-PROC)2+	366.71 (H1N1) 657.26 (H1N1S1) 819.25 (H2N1S1) 1184.52 (H3N2S1) 1711.57 (H3NS1) 1799.76 (H5NS2) 2002.67 (H5NS2)	

Table 3. Example summary of UPLC, MS, and MS/MS data that allowed for identification of A3G3S3 glycan.

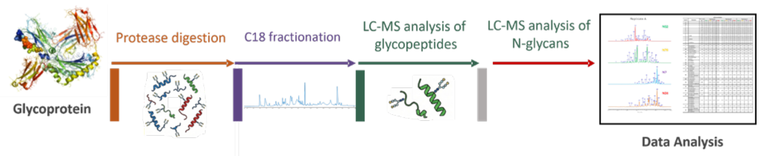
Glycosylation feature	Relative % amount
Fucosylation	15.10
Charged modifications	
Sialylation (total)	97.28
Sialylation (total Neu5Ac)	96.53
Sialylation (total Neu5Gc)	9.18
Sialylation (1 sialic acid)	2.30
Sialylation (2 sialic acids)	16.27
Sialylation (3 sialic acids)	42.60
Sialylation (4 sialic acids)	29.11
Sialylation (4 sialic acids)	7.10
Structural features	
Hybrid/ high mannose	1.34
Alpha-gal	1.43

Table 4. Summary of relative % amounts of N-glycans displaying specific structural features (fucosylation, charged modifications, hybrid/ high mannose and LacdiNAc) detected in Bovine fetuin sample.

Site Specific N-glycosylation Analysis

This analysis provides detailed information on the individual N-glycan sites.

Workflow: Glycoprotein is digested with protease to generate peptides and glycopeptides, which are separated by C18 chromatography and collected as fractions. Portion of each fraction is then treated with PNGase F to release N-glycans, which are subsequently fluorescently labelled and analysed by HILIC-UPLC-FLR to obtain site-specific glycosylation profiles. The corresponding C18 glycopeptide fractions matching each HILIC N-glycan peak are further characterised by LC-MS.



Scheme 4. Glycoprofiling workflow for site specific glycosylation analysis

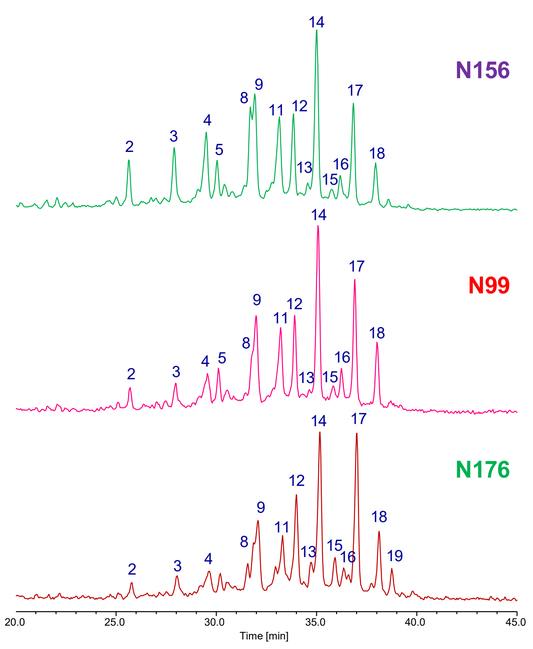


Figure 6. HILIC-UPLC profiles for each N-glycan site for Bovine fetuin

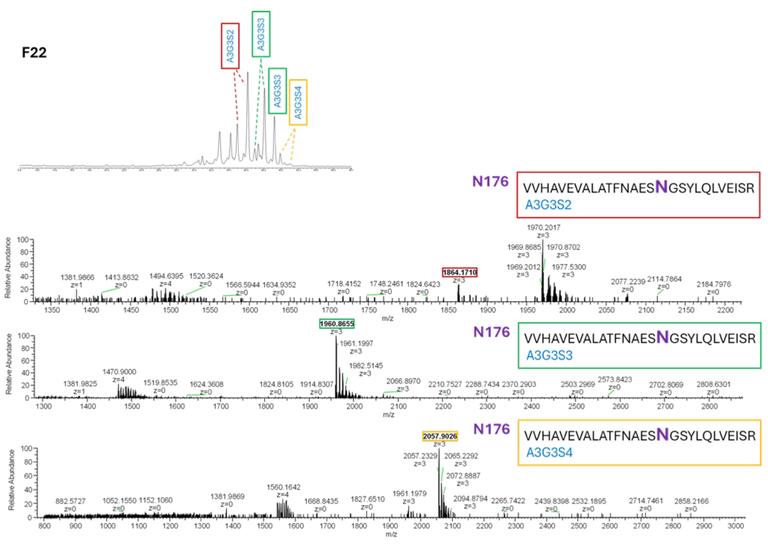


Figure 7. Example LC N-glycan profile and the corresponding glycopeptide MS data for Fraction X from the Bovine fetuin sample.

Peak ID	Average GU	Structure	% Area		
			N156	N99	N176
1	7.53	?	0.51	-	-
2	7.69	A2G2S1 Man4A1G1S1	5.08	2.87	2.71
3	8.14	A2G2S1	9.45	6.72	6.01
4	8.31	Man5A1G1S1 FA2G2S1 A2G2Sg1	3.97	3.96	2.22
5	8.42	A2G2S2	2.04	2.70	-
6	8.51	FA2G2S2	1.40	-	-
7	8.73	A3G3S1	2.29	-	2.93
8	8.81	A2G2S3 A3G2S2 FA2G2S2 FA2G2Ga2	7.51	4.05	3.95
9	8.88	A2G2S2	11.12	10.11	7.72
10	9.19	A2G2S3	-	-	3.99
11	9.28	A2G2S2 A2G2S3 A3G3S2	12.97	13.96	7.72
12	9.51	A2G2S1Sg1 A2G2S3 FA2G2S2	8.69	10.03	10.85
13	9.76	A3G3S2	1.95	-	3.12
14	9.91	A3G3S3 FA3G3S2	16.72	18.94	16.14
15	10.18	A3G3S2	1.53	3.62	4.48
16	10.33	A3G3S3	3.06	4.22	4.53
17	10.57	A3G3S3 FA3G3S3 A3G3S4	8.59	12.87	15.03
18	10.98	A3G3S4	3.14	5.95	6.00
19	11.27	A3G3S4	-	-	2.59

Table 5. Summary of HILIC-UPLC % areas and glycan structures for individual sites for Bovine fetuin

Summary

Each presented workflow provides complementary information required for full characterisation of glycoprotein, and supports the robust detection of glycan specific GCQAs, aligning with the level of understanding increasingly expected by regulatory authorities.

References

1. P. Zhang, S. Woen, T. Wang, B. Liau, S. Zhao, C. Chen, Y. Yang, Z. Song, M.R. Wormald, C. Yu, P.M. Rudd. Drug Discov Today, 2016,21,740-765.
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3. Dotz, A. V. et al. Mass spectrometry for glycosylation analysis of biopharmaceuticals. Trends Anal. Chem. 73, 1–9 (2015).

Contact information

If you'd like a copy or want to know more about our glycomics workflows, please email me (**rad.kozak@ludger.com**) or connect with me on LinkedIn.

Thank you for viewing my poster, Rad.