A Sweet Solution for Mass Spectrometric Glycan Analysis

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Introduction

Glycans can significantly influence the stability, activity, and immunogenicity of biologics, making their analysis crucial for assessing the quality of antibody therapeutics, especially biosimilars. Presented here is a tool to streamline mass spectrometry-based glycan analysis using automated glycan grouping with Protein Metrics™. From released glycans to peptide-level multi-attribute method (MAM) workflows, Protein Metrics generates intuitive visualizations with automated glycan grouping that can transform complex datasets into actionable insights. Work presented here shows the utility of glycan grouping in bridging data from multiple analytical methods and in comparing day of culture samples from different bioreactors using mass spectrometry (MS)

Methods

For MAM, samples were reduced, alkylated, and digested with proteases and the resulting peptides produced were separated by reversed phase chromatography on an Agilent Zorbax® C18 column. Eluted peptides were detected using a Thermo Orbitrap Exploris® 240 Mass Spectrometer. Peptide sequences with and without glycans were identified and quantitated using a Protein Metrics™. Glycosylation was quantified by determining the Extracted Ion Chromatogram (XIC) peak area of the MS signal and calculating the glycosylation percentage. A glycan grouping tool was developed in collaboration with Protein Metrics® to help visualize glycosylation trends. For released glycan analysis, samples were deglycosylated and the resultant glycan mixture was separated on a Hydrophilic Interaction Chromatography (HILIC) column coupled to a fluorescence (FLR) detector.

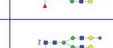
Results and Discussion

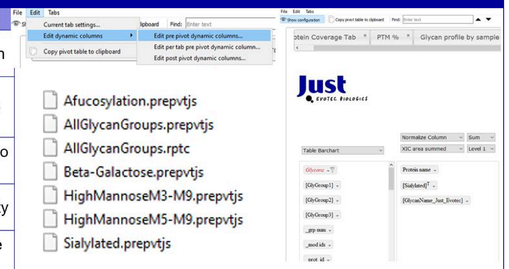
Analysis of Glycosylated Proteins

Description	Released Glycan Analysis	Multi-Attribute Method (MAM)
Separation	HILIC Column	RP Column
Detector	Fluorescence	High Resolution Mass Spectrometry
Species detected	Detached glycans separated on a column	Digested peptides separated on a column
Information from analysis	Overall glycan profile and diversity in the form of broad glycan groups	Glycan occupancy, site specific information
Data Output	Sialylation: 2.331; Beta Galactose: 63.772; Afucosylated: 1.863; Total High Mannose: 0.778 (%)	Glycans / Modification / PTM (%): HexNAc(3)Hex(3)Fuc(1) / NGlycan/1241.4545 / 0.9; HexNAc(3)Hex(4)Fuc(1) / NGlycan/1403.5073 / 0.2; HexNAc(4)Hex(3) / NGlycan/1298.4760 / 2.3; HexNAc(4)Hex(3)Fuc(1) / NGlycan/1444.5339 / 49.2; HexNAc(4)Hex(4) / NGlycan/1460.5288 / 0.7; HexNAc(4)Hex(4)Fuc(1) / NGlycan/1606.5867 / 38.6

HILIC-FLR data output comprised of summarized glycan grouping and individual glycan reporting. Glycan grouping helps us identify broad glycan types that can have an impact on mAb safety and efficacy. While this reporting feature us available on our analytical platform, we must perform this task manually with MAM data which can be error prone.

Glycan Grouping and Customization with Protein Metrics™

Glycan Type	Glycan Cartoon	Details
High Mannose		Common in early stages of glycosylation
Complex		Involved in more specific biological interactions and are common in mature stages of N-glycan processing
Hybrid		Often found in glycoproteins that undergo partial modification
Fucosylated		Can impact Ab-dependent cellular toxicity
Sialylated		Can influence stability, half-life, immune responses



- A custom reporting tool was created in collaboration with Protein Metrics that can help consolidate MAM data
- The tool can be applied in the reporting section as a pre-pivot column option
- Scripts defining each glycan group can be used to customize reporting as needed

Use Case 1 – Bridging HILIC and MAM data

HILIC –FLR Data	Sialylation	Beta Galactose	Afucosylated	Total High Mannose	⟷	MAM data after glycan grouping	Sialylation	Beta Galactose	Afucosylated	Total High Mannose
	2.331	63.772	1.863	0.778			2.2	59.7	2.7	0.7

Use Case 2 – Comparison between day of culture samples

Experimental Setup:

- Number of bioreactors: 12 (R1-R12) - Number of days of culture: 3 (D8, D9, D10) - Molecule with multiple glycan sites and therefore HILIC-FL analysis would not give us enough information

- MAM analysis was performed and raw files were searched against a custom glycan database for PTMs to generate large data files
- MAM data without glycan grouping can be complex to visualize (Figure 1). Glycan grouping helps us track trends for e.g. sialyation across bioreactors and days of culture (Figure 2)

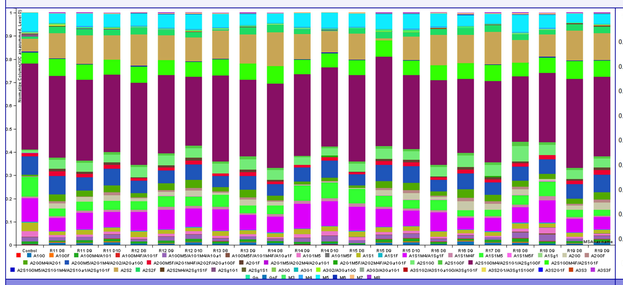


Figure 1. Typical MAM data from day of culture samples

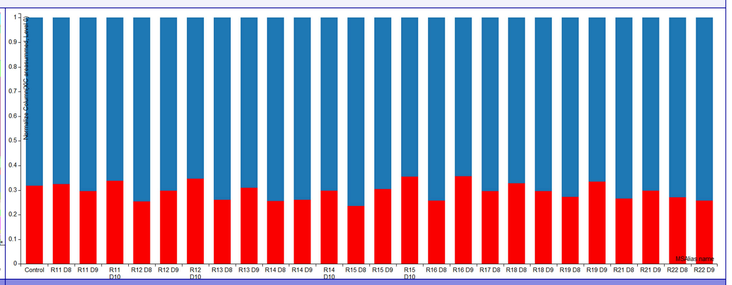


Figure 2. MAM data from day of culture samples with glycan grouping – red bar (a-sialylated) and blue bar (sialylated)

Similarly, changes can be observed and tracked specific to glycosylation sites. Figure 3 shows that for the day of culture samples, changes can be observed in the fusion protein glycan sites. Fc-glycans remain largely consistent with sialylation (blue bar) and a-sialyation (yellow bar)

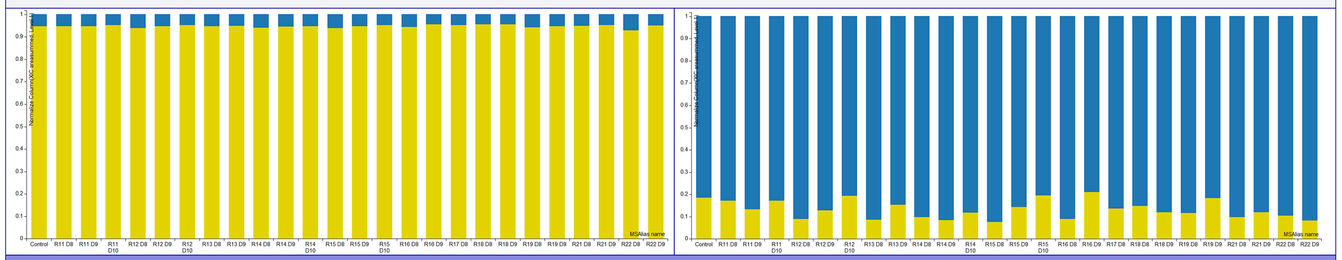
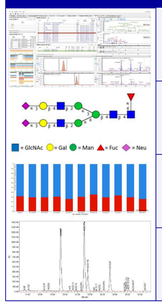


Figure 3. Site specific MAM data after applying glycan grouping

A Sweet Solution!



- High throughput analysis – multiple sets of data allowing easy comparison across multiple samples in one analysis
- Identification and quantitation with possible customization – customizable databases, site specific information
- Visualization tools – easy visualization using the glycan grouping tool making data more comprehendible for stakeholders
- Integration with other analytical platforms allowing comparison and bridging with other analytical methods that may not use mass spectrometry

Conclusion

- Presented here is a glycan grouping tool to streamline mass spectrometry-based glycan analysis using automated glycan grouping with software
- Protein Metrics provides high throughput analysis, peptide level information, easy visualization of data an aids in the correlation of data between multiple analytical methods

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The authors declare no competing financial interest.