Challenges and Strategies in Glycan Analysis by MAM

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Background

Multi-Attribute Method (MAM) presents unique advantages in glycan analysis for biotherapeutics over HILIC released glycan analysis. Some of the advantages include site-specific characterization and quantitation of glycans and non-glycosylation levels in a single assay and the relative ease of sample preparation without additional labeling steps. However, it is still challenging to match the relative quantities from both techniques often due to large differences in size and ionization efficiencies of glycan containing peptides. This poster aims to describe and demonstrate the effects of various parameters on the relative quantitation results.

MS instrument setup

In-source fragmentation of glycans is a concern as some conditions can remove terminal sugars before the glycosylated peptide reaches the mass analyzer. We evaluated varying spray voltages and capillary temperatures, and the percent glycan levels of three conditions among them are summarized in Table 1. The **capillary temperature** appears to affect the removal of terminal GlcNAc the most. The difference in capillary temperatures between the conditions A and B was 100 ºC.

Glycan libraries & localization

Glycan libraries: All attribute identification requires careful consideration of modifications to be used for search. For glycan identifications, it is crucial to identify the host cell species and apply the appropriate libraries for search for both N- and O-glycans.

Figure 1: A example of a glycan structure (bisecting GlcNAcs) that is common in human cells but not in CHO cells.

Localization: Due to NX(S/T/C) motif, localization of N-glycation is relatively straight forward while O-glycans pose challenges. MS/MS data are often ambiguous when there are multiple T/S residues because of the labile nature of the O-glycans. If the possible T/S locations cannot be differentiated after enzyme digestion, ETD/ECD may be necessary in some cases.

The source conditions: Spray V & Capillary T			Spr V: A Cap T: A	Spr V: A Cap T: A	Spr V: A Cap T: A	Spr V: A Cap T: A	Spr V: A Cap T: A	Spr V: A Cap T: A	Spr V: B Cap T: A	Spr V: B Cap T: B	HILIC-FL
% De-gly (deamidated) spiked in			100% degly	80% degly	60% degly	40% degly	10% degly	0% degly	0% degly	0% degly	0% degly
Theoretical: Non-gly (rCE data) + De-gly (deamidated) spiked in			100.0	82.0	62.0	42.0	12.0	2.0	2.0	2.0	
Experimental: Non-gly + De-gly (deamidated) **MS with charge state/Averagine corrections**			99.9	80.3	62.3	44.2	13.9	2.0	2.2	2.6	
Experimental: Non-gly + De-gly (deamidated) **MS without charge state/Averagine corrections**			100.0	89.8	78.5	64.3	27.4	5.0	4.5	5.3	
Experimental: Non-gly + De-gly (deamidated) **MS with charge state/Averagine corrections**											
Non-glycosylated			1.4	1.5	1.5	1.8	1.9	2.0	2.1	2.6	N/A
Deamidation/0.9840			98.5	78.8	60.8	42.4	12.0	0.0	0.0	0.0	N/A
NGlycan/1444.5339	G0F	A2G0F	0.04	13.5	24.6	35.9	54.8	63.3	64.4	56.8	66.1
NGlycan/1606.5867	G1F	A2G1F		3.8	7.9	11.8	18.2	20.0	18.9	17.8	19.3
NGlycan/1241.4545	G0F-GlcNAc	A1G0F		0.7	1.4	1.9	3.0	3.3	2.8	9.0	2.8
NGlycan/1216.4229	Man5	M5		0.5	1.0	1.8	2.9	3.3	3.5	3.6	2.8
NGlycan/1298.4760	G0	A2G0		0.3	0.5	0.8	1.5	1.5	1.6	1.4	1.2
NGlycan/1768.6395	G2F	A2G2F		0.3	0.4	0.7	0.9	1.0	1.3	1.3	
NGlycan/1378.4757	Man6	M6		0.1	0.2	0.3	0.5	0.6	0.6	0.6	
NGlycan/1095.3966	G0-GlcNAc	A1G0			0.2	0.2	0.5	0.6	0.5	0.8	
NGlycan/1647.6132	G0F+GlcNAc	A3G0F			0.1	0.2	0.4	0.5	0.4	0.4	
NGlycan/892.3172	Man3	M3		0.1	0.1	0.3	0.4	0.5	0.5	0.7	
NGlycan/1540.5285	Man7	M7			0.2	0.3	0.4	0.5	0.5	0.5	
NGlycan/1897.6821	G1FS1	A2S1G0F		0.1	0.1	0.2	0.4	0.5	0.5	0.4	

Table 1. Relative quantities (%) of N-glycosylated and non-glycosylated peptides from Fc-region. The original % non-glycosylation level is 2% (rCE). Mixtures of deglycosylated and glycosylated samples were prepared and analyzed at various ratios. Theoretical non-glycosylation levels were compared to the experimental MS quantitation values before and after correction with charge state selection and Averagine model isotope normalization. The results from 3 different source conditions are also included in the table with effects of higher Capillary T highlighted in blue. The glycans with levels < 0.5% are not shown in the table.

Quantitation

Differences in ionization efficiency make it difficult to relatively quantify non-glycosylated and glycosylated peptides. While we cannot change the nature of peptides, we can modify quantitation parameters to minimize the difference in order to align the MS results to results from other conventional methods such as rCE-SDS for non-glycosylation and HILIC with fluorescence detection (HILIC-FL) for released glycan assays.

Charge states: For most attributes, it is ideal to use the same number of charge states among the peptides used for relative quantitation. Due to the difference in size, the available charge states for quantitation can be different particularly for the non-glycosylated Fc-peptide, EEQYNSTYR. Instead of using only the single most abundant charge state, use of multiple charge states might be more appropriate depending on the peptide.

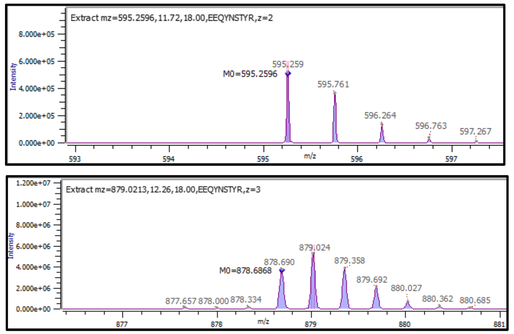

Figure 2: Isotope distributions of peptide, EEQYNSTYR, with and without a glycan, A2G0F.

Isotope distribution: With increased masses of glycosylated peptides, the number of isotopes originating from peptides increases as seen in Figure 2. While it is reasonable to only use the most abundant isotope for relative quantitation of peptides with similar masses, difference in isotope distribution also contributes to the difference in quantitation. To minimize this effect, it may be beneficial to use the sum of all isotope peaks for quantitation or isotope normalization depending on the glycosylated peptides.

Averagine model isotope normalization: A "maximum isotope peak quantitation approach" benefits from simplicity and reduced risk of background interference. However, relative quantitation accuracy can be reduced when comparing peptides with significantly different isotope peak relative abundance profiles. An "all isotope peaks quantitation approach" can reduce relative quantitation error due to differences in relative isotope abundance. The Byos® Total XIC AUC Averagine field offers an alternative approach where a single isotope peak is measured and then extrapolated to all isotopes using an Averagine model and the theoretical peptide mass (Figure 3). Total XIC AUC Averagine benefits from reduced risk of background interference of a "maximum isotope peak quantitation approach" and the improved relative quantitation accuracy of an "all isotope peaks quantitation approach".

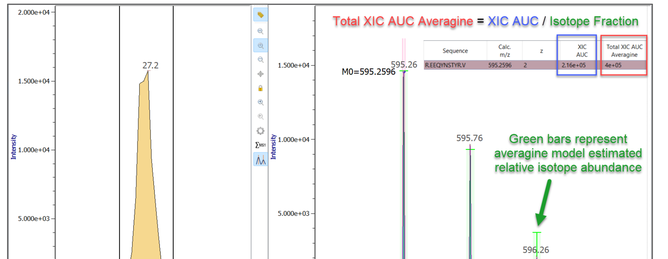

Figure 3: The left-hand XIC is generated using the monoisotopic peak 595.26. XIC AUC is calculated by integrating the area under the XIC curve. The right-hand MS1 spectrum shows the measured isotope profile for the 595.26 (+2) peptide signal, along with green bars representing theoretical isotope relative abundances estimated using an Averagine model. The Isotope Fraction is the theoretical abundance of the measured isotope (595.26, isotope 0) divided by the sum of all calculated theoretical isotope peak abundances. The XIC AUC (2.16e+05) is divided by the Isotope Fraction (0.54) to calculate the Total XIC AUC Averagine (4.0e+05), which estimates the summed XIC AUC value of an "all isotope peaks approach".

Conclusions

Multi-Attribute Method can be readily implemented for monitoring N-glycan distributions and non-glycosylated content. However, consideration of the following parameters is critical in aligning the MAM glycan results with off-line glycan analysis methods.

- Optimization of MS source conditions is critical – quantitation of major forms can vary up to 8% using non-optimized acquisition conditions
- The glycan library must be expression system specific and contain glycans found on the product
- Accurate quantitation of non-glycosylated form is best achieved by the use of all charge states and isotope peaks