Method Optimization for a Multiple Attribute Method Using the TripleTOF® 6600 System

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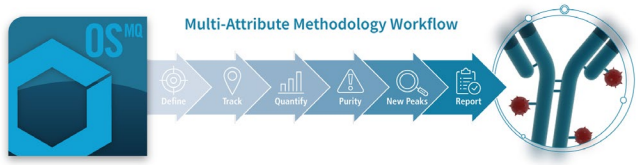
Biotherapeutics are complex and heterogeneous molecules. Their heterogenous nature arises from the presence of multiple glycoforms and other variable post translational modifications which result in a wide range of structural variants. As part of the development of a biotherapeutic it is important to characterize this variability to understand the impact on the safety and efficacy of the target molecule and to understand how to control those attributes which are identified as critical.

High resolution mass spectrometry (HRMS) is a critical tool for characterization of biotherapeutics and is rapidly emerging as a tool for tracking molecular attributes throughout the development process, particularly as molecular complexity increases. To this end, HRMS has been finding increased use in workflows targeted toward assessing a range of molecular attributes using a single LC-MS method frequently referred to as multiple attribute methodology (MAM). This approach enables assessment of multiple post-translational modifications (PTMs) including the assessment of glycosylation profiles. To realize the application of HRMS for attribute monitoring, it is critical that sample preparation and analysis parameters are well understood and controlled so an accurate and precise result is generated.



Presented here is an optimized LC-MS workflow for accurate glycopeptide characterization of monoclonoal antibody biotherapeutics by using SCIEX TripleTOF 6600 mass spectrometer coupled with ExionLC™ system.

Key Feature of TripleTOF® 6600 system for MAM assay

- Flexibility to support wide range of workflows
- Accurate correlation of HRMS data and orthogonal assay for relative glycan abundances
- Easily define custom calculations for quality attribute assessment
- Optimized conditions for execution of MAM assay using SCIEX TripleTOF® 6600 mass spectrometer

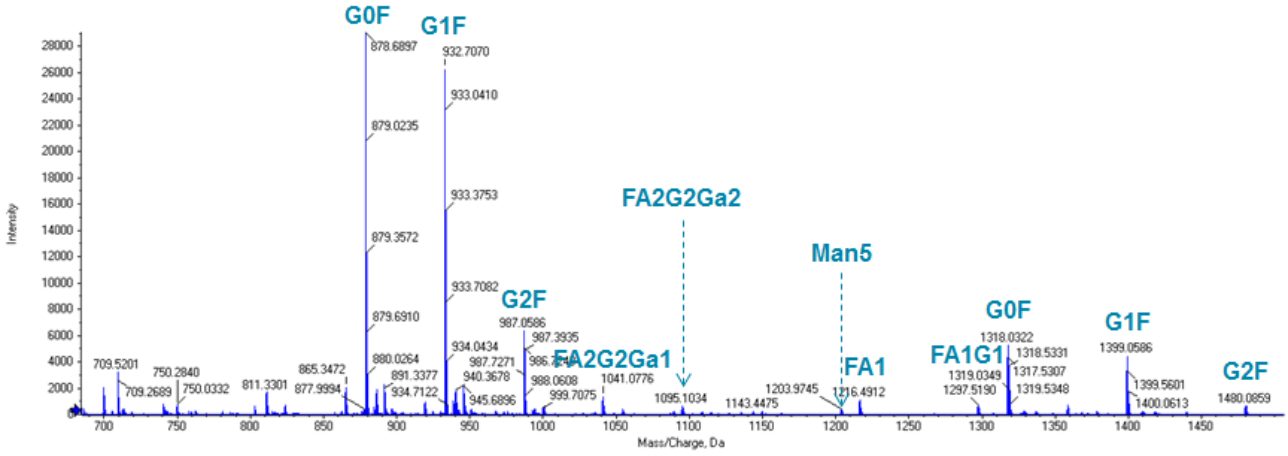


Figure 1. An Averaged TOF MS Mass Spectrum Show Major Glycopeptide Forms, with Identification Annotations.

Methods

Sample Preparation: NIST mAb standard was purchased from National Institute of Standards and Technology (NIST) and reconstituted to a concentration of 10 µg/µL according to the recommended procedure. 100 µg of NIST mAb standard was diluted to 1 g/L with 7 M guanidine hydrochloride in 100 mM Tris-HCl (pH 7.9) for protein denaturation. The guanidine hydrochloride was then removed by buffer exchange with 100mM Tris-HCl (pH 7.9) using centrifuge filter with 10kDa molecular weight cut-off (Millipore, Burlington, MA). The denatured protein was subjected to reduction with DTT at 10 mM at room temperature for 30 minutes, followed by alkylation with iodoacetomide at 20 mM for 20 minutes in the dark at room temperature. The sample was then digested with Trypsin (Roche, sequence grade) for overnight at 37 °C. The digestion was aborted by spiking with 2 µL formic acid and the sample was ready for LC-MS analysis.

Mass Spectrometry: The sample was analyzed by TripleTOF 6600 mass spectrometer coupled with ExionLC system. Table 1 describes the liquid chromatography conditions used. Table 2 describes the mass spectrometry parameters used. The data was processed using BioPharmaView™ 3.0 software.

Results

Glycoform profiles are often considered critical for overall biotherapeutic safety and efficacy. As such, the assessment of the glycoprofile is consistently assessed throughout the development, manufacture and release of products. Due to their complexity and importance, assessment of the glycoprofile is frequently accomplished using multiple orthogonal assays to ensure agreement and consistency in data. For released glycan analysis using fluorescence labels it is important to ensure consistent release and labeling. Likewise for glycopeptide analysis using HRMS is it critical to ensure glycoform assessment aligns with orthogonal assays.

Table 1. Chromatographic Conditions.

Time (min)	Flow Rate (ml/min)	%A	%B
0	*0.3*	*99*	*1*
5	*0.3*	*99*	*1*
6	*0.3*	*90*	*10*
50	*0.3*	*65*	*35*
55	*0.3*	*40*	*60*
56	*0.3*	*10*	*90*
60	*0.3*	*10*	*90*
62	*0.3*	*99*	*1*
70	*0.3*	*99*	*1*

Table 2. Mass Spectrometer Conditions.

Parameter	Value	Parameter	Value
Curtain gas:	*35*	*Time bins to sum:*	*4*
Ion source gas 1:	*60*	*TOF start mass (Da):*	*300*
Ion source gas 2:	*60*	*TOF stop mass (Da):*	*1800*
Temperature:	*250*	*Accumulation time:*	*0.25 sec*
Scan type:	*TOF MS*	*Declustering potential (V):*	*20*
Polarity:	*Positive*	*Collision energy (V):*	*6*
Ionspray voltage:	*5200*	*CAD gas:*	*7*

In this project, a very extensive MS condition optimization was performed on TripleTOF 6600 mass spectrometer to achieve accurate glycopeptide profiling for NIST mAb. Multiple parameters, including declustering potential (DP), collision energy (CE), curtain gas, nebulizing gas (GS1), drying gas (GS2), source temperature and ion spray voltage (ISV) were evaluated. The optimized method parameters were applied across multiple mass spectrometers with minimum adjustment required. An example total ion chromatogram (TIC) for a reverse phase separation of a tryptic digest is shown in Figure 2.

From the peptide map data, the TOF MS spectra was averaged for the glycopeptides identified following BioPharmaView software processing. As shown in Figure 1, each of the glycoforms was easily identified based on MS data over the

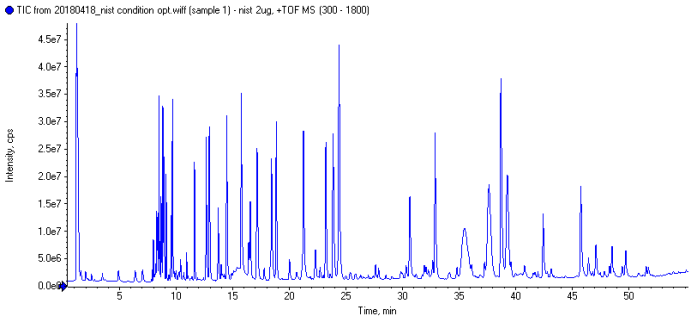


Figure 2. NIST mAb Digest. Example total ion chromatogram (TIC) for NIST mAb digest from TripleTOF 6600 mass spectrometer coupled to an ExionLC system with optimized LC MS conditions.

dynamic range required for the sample. For each identified glycoform a custom calculation was developed to accurately calculate the observed glycoform ratios based the data as shown in Figure 3. These defined calculations can be applied in ongoing studies to rapidly assess the relative ratios of each glycoform.

After defining attribute calculations for each targeted glycoform the same project to investigate the relative ratios as a function of instrument parameters for data acquisition. In this way the calculated percentages of each glycoform were easily compared to previously published values [1]. The determined values using the LC-MS approach presented here using optimized conditions are consistent with the previously reported values for the same sample as shown in Table 3.

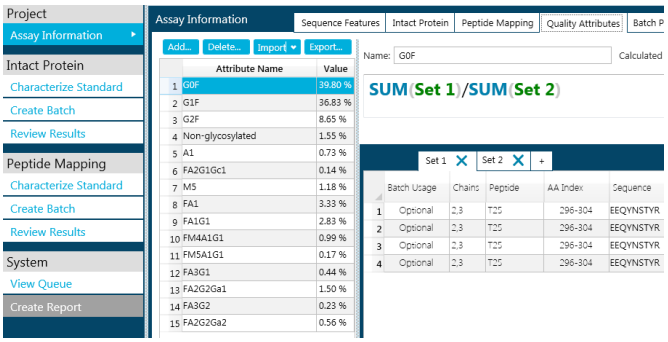


Figure 3. Set up Glycosylation Related Quality Attributes for MAM Workflow in BioPharmaView 3.0 Software.

After completing batch data processing, a close review of the calculated glycosylation levels under different MS conditions was performed. The combination of MS parameter values listed in Table 2 was found to provide the most accurate information of glycosylation levels, when comparing with the published NIST data[1]. The percentage levels of major glycoforms of NIST mAb were summarized in Table 3. In some cases direct comparison to literature reported values was challenged due to reported values representing multiple glycoforms. This highlights a significant benefit of and LC-MS approach which can reduce ambiguity in structural assignment when glycoforms with different mass may co-elute as their observed mass is different.

Conclusions

- Optimized LC-MS conditions on TripleTOF 6600 system generates consistent glycoform ratios
- LC-MS glycopeptide analysis reduces ambiguity in glycoform assignment compared to fluorescent based assays
- Consistent results obtained across multiple instruments
- Single set of parameters generally applicable for attribute assessment

Table 3: The Percentage Levels of Major Glycoforms of NIST mAb. The "Experimental Ratio" values are calculated by BioPharmaView 3.0 software.

#	Glycan ID	Theoretical mono m/z	Observed mono m/z	Charge state	Experimental Ratio (%)	Published ratio (%) [1]
		659.267	659.267	4		
		878.687	878.690	3		
1	G0F	1317.527	1317.530	2	38.16	39.81
		699.780	699.782	4		
		932.704	932.707	3		
2	G1F	1398.553	1398.556	2	37.00	38.15
		740.293	740.296	4		
		986.722	986.725	3		
3	G2F	1479.579	1479.583	2	9.46	7.55
4	A1	762.308	762.310	3	0.63	0.70
		810.994	810.996	3		
5	FA1	1215.987	1215.989	2	2.59	2.58 (FA1+A2)
		865.011	865.014	3		
6	FA1G1	1297.013	1297.017	2	2.55	2.57
		919.029	919.031	3		
7	FM4A1G1	1378.040	1378.040	2	0.66	0.41
		973.047	973.048	3		0.36
8	FM5A1G1	1459.066	1459.063	2	0.20	(FA3G2+FM5A1G
		1000.398	1000.393	3		
9	FA3G1	1500.093	1500.087	2	0.60	0.41 (FA3G1+M6)
		1054.415	1054.413	3		0.36
10	FA3G2	1581.119	1581.121	2	0.45	(FA3G2+FM5A1G
		780.807	780.809	4		1.85
		1040.740	1040.743	3		(FA3G2Ga2+FA2
11	FA2G2GA1	1560.606	1560.608	2	1.77	G2Gc1-b)
		821.320	821.319	4		1.11
		1094.757	1094.761	3		(FA2G2Ga2+FA2
12	FA2G2GA2	1641.632	1641.639	2	0.74	G2Gc1)
		776.553	776.553	4		
		1035.068	1035.070	3		0.14 (FA2G1Gc1-
13	FA2G1Gc1	1552.098	1552.102	2	0.22	a)
		802.650	802.651	3		
14	M5	1203.471	1203.473	2	0.51	1.17

References

1. State-of-the Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization The NIST mAb Case Study. John E. Schiel, Darryl L. Davis, Oleg V. Borisov.

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