

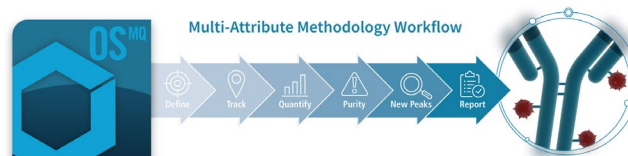
# Method Optimization of a Multiple Attribute Method for a SCIEX® X500B LC-MS system

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During the development of biopharmaceuticals, it is critical to comprehensively characterize molecules to understand product attributes and how they relate to the safety and efficacy. A common technique for this work is the use of mass spectrometry which plays a significant role in the analysis of proteins and peptides. Mass spectrometry enables the ability to characterize biotherapeutics with a high level of confidence and to localize and track potential quality attributes.

Recently the concept of a Multiple Attribute Method (MAM) using mass spectrometry has been introduced. [1] This approach enables the use of mass spectrometry to provide high resolution accurate mass (HRMS) data which provides a greater level of detail for quality attributes when compared to traditional orthogonal assays. MAM is starting to emerge as an orthogonal, and in some cases displacing, approach for definition and tracking of product quality attributes. One challenge in realizing this potential is the need for a robust method which can generate reproducible data and can be cross validated by orthogonal assays.

Presented here are optimized LC-MS parameters for a MAM assay employing a SCIEX X500B mass spectrometer coupled with ExionLC™ system. The results demonstrate an accurate glycoform profile which is cross validated by released N-glycan analysis by CE-LIF and HILIC LC analysis.



## Key Features of X500B QTOF System for MAM Assay

- Small footprint HRMS with intuitive SCIEX OS Software
- Easily defined and executed assays for biotherapeutic characterization and attribute tracking
- Accurate and reproducible data for determination of biotherapeutic post translational modifications
- Highly aligned quantitative results for quality attributes compared to orthogonal assays

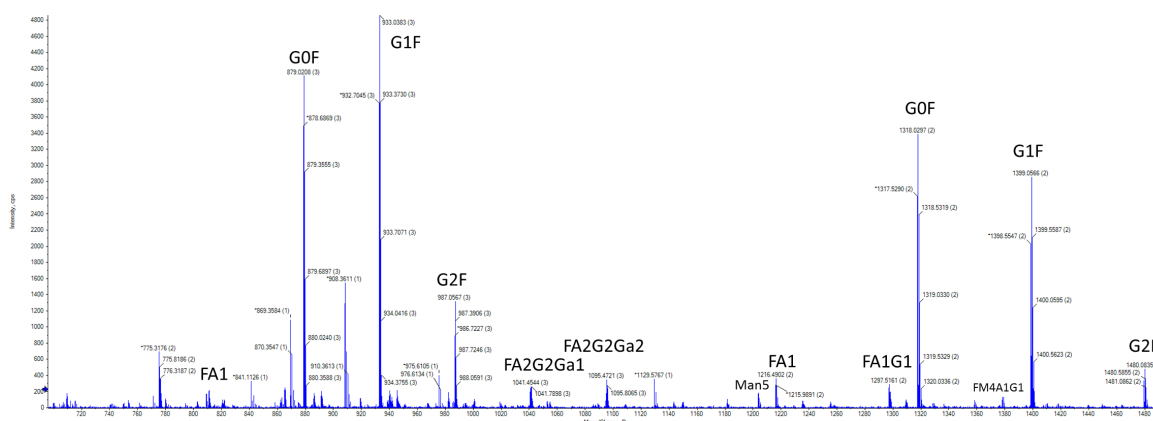


Figure 1. An Averaged TOF MS Mass Spectrum shows the Major Glycopeptide Identification.

## Methods

**Sample Preparation:** NIST mAb standard was purchased from 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 iodoacetamide at 20 mM for 20 minutes in the dark at room temperature. The sample was then digested with Trypsin (Roche, sequence grade) overnight at 37 °C. The digestion was aborted by spiking with 2 µL formic acid and the sample was ready for LC-MS analysis.

**LC-MS Analysis:** The sample was analyzed by SCIEX X500B QTOF system fitted with a Turbo V™ source with a TwinSpray probe 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.

**Table 1. Chromatographic Conditions**

Parameter	Value
Stationary phase	Agilent ZORBAX 300 SB-C18 column 1.8 µm, 2.1mm X 150 mm
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in acetonitrile
Flow rate	0.3 mL/min
Column temperature	50 °C
Injection volume	3 µL

**Table 2. Chromatographic Gradient**

Time (min)	Flow Rate (ml/min)	%A	%B
Initial	0.3	99	1
5.0	0.3	99	1
6.0	0.3	90	10
50.0	0.3	65	35
55.0	0.3	40	60
56.0	0.3	10	90
60.0	0.3	10	90
62.0	0.3	99	1
64.0	0.3	99	1
66.0	0.3	10	90
70.0	0.3	10	90
72.0	0.3	99	1
74.0	0.3	99	1
76.0	0.3	10	90
80.0	0.3	10	90
82.0	0.3	99	1
95.0	0.3	99	1

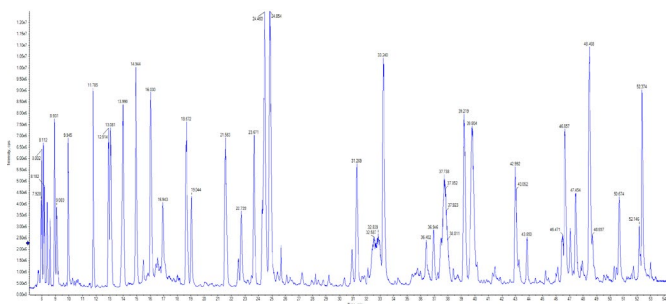
**Table 3. Mass Spectrometry Conditions**

Parameter	Value	Parameter	Value
Curtain gas:	55	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(°C):	200	Accumulation time:	0.5 sec
Scan type:	TOF MS	Declustering potential (V):	20
Polarity:	Positive	Collision energy (V):	4
Ionspray voltage:	5200	CAD gas:	7

## Discussion

Analysis of NIST mAb was employed as an example to demonstrate method optimization for a generic workflow on a SCIEX X500B QTOF system. Extensive evaluation of the appropriate MS conditions was performed on the X500B QTOF system with a particular focus on the accuracy of relative quantification of glycopeptides when compared to the released N-glycan analysis.

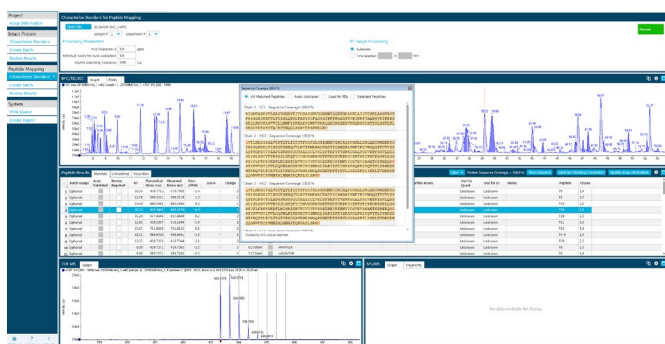
Multiple parameters, including declustering potential (DP), collision energy (CE), curtain gas, nebulizing gas (GS1), drying gas (GS2), source temperature and ion spray voltage (ISV) were evaluated. Figure 2 shows a representative Total Ion Chromatogram (TIC) of NIST trypsin digest generated on X500B system using the optimized conditions.



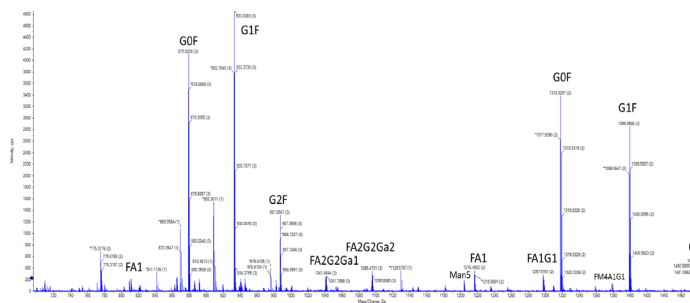
**Figure 2. Example Total Ion Chromatogram (TIC) of NIST mAb Digest.** Data was generated on X500B QTOF system coupled with ExionLC system with optimized LC-MS conditions.

BioPharmaView™ Software 3.0 was used to process data. The assay was defined by specifying the sequence, expected modifications, and digestion conditions as described in previous work [2]. Processing data collected with the optimized conditions 100% sequence coverage was achieved as shown in Figure 3.

In addition to high sequence coverage, the expected glycans were observed which were consistent with previous reports. [5] An



**Figure 3. 100% Sequence Coverage was Obtained on NIST Trypsin Digest.**



**Figure 4. Raw Mass Spectrum with Major Glycopeptide Identification.**

average raw mass spectrum from the chromatographic region containing all glycoforms is shown in Figure 4.

To monitor glycan profiles, each glycopeptide was defined as a quality attribute with pass defined as  $\pm 10\%$  of the target value from the initial characterization work. After definition, 6 replicate analyses were processed using the same search criteria following the batch analysis workflow in BioPharmaView Software. Each glycopeptide was present in the replicate analysis within our defined criteria as shown in Figure 5

View	Quality Attributes	20180406 Nist_1.wiff2	20180406 Nist_2.wiff2	20180406 Nist_3.wiff2	20180406 Nist_4.wiff2	20180406 Nist_5.wiff2	20180406 Nist_6.wiff2	Reference Value	Value or Range for Pass
1	GOF	39.80%	41.82%	42.51%	41.95%	41.33%	42.04%	39.81%	35.83% - 43.79%
2	G1F	36.83%	36.64%	36.10%	36.94%	37.03%	36.73%	38.15%	34.34% - 41.97%
3	G2F	8.65%	7.43%	7.76%	7.74%	7.80%	7.86%	7.86%	7.07% - 8.65%
4	Non-glycosylated	1.55%	1.46%	1.24%	1.34%	1.34%	1.27%	1.55%	1.00% - 2.00%
5	A1	0.73%	0.71%	0.54%	0.72%	0.55%	0.64%	0.73%	0.50% - 1.00%
6	FA2G2Ga1	0.14%	0.16%	0.11%	0.16%	0.00%	0.17%	0.14%	0.00% - 0.30%
7	MS	1.18%	1.27%	1.23%	1.16%	1.29%	1.29%	1.18%	1.00% - 1.36%
8	FA1	3.23%	3.22%	3.17%	3.20%	3.34%	3.16%	3.33%	2.83% - 3.83%
9	FA1G1	2.83%	2.91%	2.97%	2.95%	3.13%	3.14%	2.83%	2.41% - 3.25%
10	FMAA1G1	0.99%	1.20%	1.19%	1.15%	1.27%	1.14%	0.99%	0.80% - 1.30%
11	FMAA1G1	0.17%	0.00%	0.00%	0.05%	0.00%	0.00%	0.17%	0.00% - 0.30%
12	FA1G1	0.44%	0.29%	0.35%	0.00%	0.00%	0.00%	0.44%	0.00% - 0.50%
13	FA2G2Ga1	1.50%	1.32%	1.25%	1.25%	1.35%	1.38%	1.50%	1.20% - 1.80%
14	FA2G2	0.23%	0.25%	0.00%	0.18%	0.22%	0.00%	0.23%	0.00% - 0.30%
15	FA2G2Ga2	0.56%	0.51%	0.53%	0.53%	0.55%	0.55%	0.56%	0.39% - 0.73%

**Figure 5. Summary of Glycopeptides Tracking in Representative Analysis Showing Glycan Profile.**

Results from the replicate analysis of glycopeptide results were compared to previously reported data as well as an additional capillary electrophoresis assay using a SCIEX PA800 Plus system. As shown in Figure 6, the data presented in this work is consistent across all assays.

**Table 4. Comparison of Glycopeptide Results.** Released N-glycan data from CE and HILIC with fluorescence detection.

	Glycan ID	Theoretical mono m/z	Observed	Charge state	ratio (%) <sup>*</sup>	ratio from CE-LIF (%) <sup>[4]</sup>	HILIC (%) <sup>[5]</sup>
1	G0F	1633.0386	1317.5266	2	39.80	41.29	39.81
			878.6868	3			
			659.2669	4			
2	G1F	2795.0914	1398.553	2	36.83	35.81	38.15
			932.7044	3			
			699.7801	4			
3	G2F	2957.1442	1479.5794	2	8.65	6.1	7.55
			986.722	3			
			740.2933	4			
4	A1	2283.9013	1142.9579	2	0.73	0.79	0.7
			762.3077	3			
5	FA1	2429.9592	1215.9869	2	3.33	1.19	2.58 (FA1+A2)
			810.9937	3			
6	FA1G1	2592.0121	1297.0133	2	2.83	-	2.57
				3			
7	FM4A1G1	2754.0649	1378.0397	2	0.99	-	0.41
			919.0289	3			
8	FM5A1G1	2916.1177	1459.0661	2	0.17	0.12	0.36(FA3G2+FM5A1G1)
			973.0465	3			
9	FA3G1	2998.1708	1000.3975	3	0.44	-	0.41 (FA3G1+M6)
10	FA3G2	3160.2236	1054.4151	3	0.23	-	0.36 (FA3G2+FM5A1G1)
11	FA2G2Ga1	3119.1971	1560.6058	2	1.50	1.46	1.85 (FA3G2Ga2+FA2G1Gc1-b)
			1040.7396	3			
			780.8065	4			
12	FA2G2Ga2	3281.2499	1094.7572	3	0.56	0.85	1.11 (FA2G2Ga2+FA2G2Gc1)
13	FA2G1Gc1	3102.1818	1035.0679	3	0.14	-	0.14(FA2G1Gc1-a)
14	M5	2404.9276	1203.4711	2	1.18	0.36	1.17
			802.6498	3			
15	Non-glycosylated	1188.5047	595.2596	2	1.55	-	-

## Conclusions

- High quality qualitative and quantitative data for biotherapeutics
- Reproducible data across multiple injections
- Relative glycopeptide results consistent with previously reported values

## References

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