Characterization of 2-AB Labelled Released N-linked Glycans Using the X500B QTOF System

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With the rapid development of the biopharmaceutical industry, the capability to comprehensively characterize biotherapeutics has become a requirement. Glycosylation represents one of the most important post-translational modifications of biotherapeutics since the extent and nature of glycosylation plays a significant role in drug affinity, safety and efficacy. Given their importance, the characterization and control of glycan profiles is frequently a requirement during biopharmaceutical development and commercialization.

HILIC separation coupled to florescence detector is frequently used for the analysis of labeled N-linked glycan analysis. The identifications of structures were traditionally assigned using HILIC fluorescence methods and use glucose units (GU) or relative retention times. The compatibility of HILIC with mass spectrometry has enabled wide use of this combined approach to supplement GU identification. The added specificity provided by MS allows unambiguous assignment of glycan structures. In addition, use of MS/MS fragment data supplements accurate mass data of the intact glycans to assist with specific structure determination.

Presented in this technical note is the use of the SCIEX X500B QTOF System for characterization released N-linked glycans. The separation was accomplished using HILIC chromatography

SCIEX X500B QTOF System

with fluoresce detection. Data acquisition and processing was accomplished using SCIEX OS for compliance and ease of use. In this study we characterized the glycoprofile of a humanized IgG however the approach presented is applicable across a wide range of biotherapeutics.

Key Feature of X500B QTOF Solution

- High-resolution mass spectrometer for a wide range of biopharmaceutical applications
- A complete solution for biopharmaceutical characterization
- Integrated compliant software for both acquisition and characterization
- Easy to use hardware and software accessible for a wide range of users

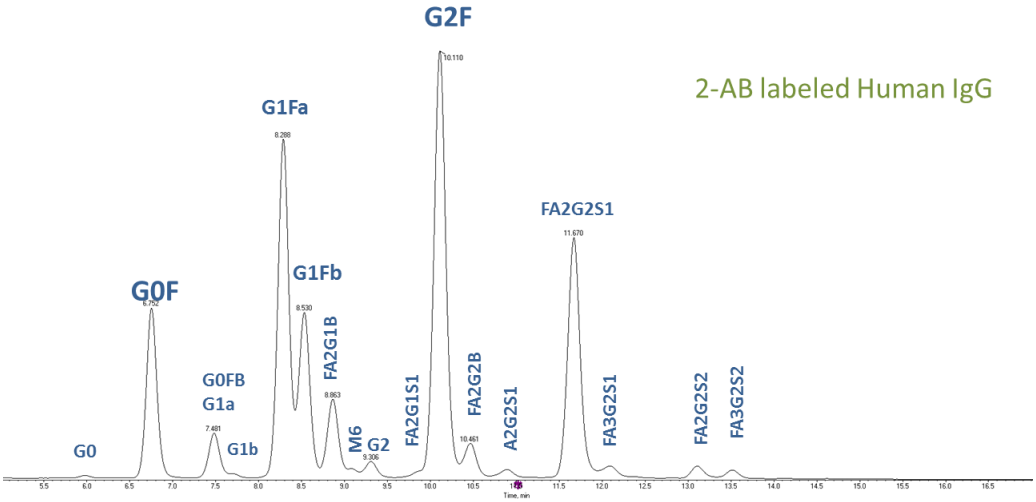

Figure 1. Florescence trace of a 2-AB labeled released N-glycans.

Table 1. LC Gradient Conditions for TFA mobile phase

Time (min	%A	%B	Flow Rate ml/min
Initial	*28*	*72*	*0.3*
27	*50*	*50*	*0.3*
28	*90*	*10*	*0.2*
30	*90*	*10*	*0.2*
31	*28*	*72*	*0.3*
45	*28*	*72*	*0.3*

Table 2. LC Gradient Conditions for ammonium formate mobile phase.

Time (min	%A	%B	Flow Rate ml/min
Initial	*32*	*68*	*0.4*
26.7	*56*	*44*	*0.4*
27.7	*100*	*0*	*0.2*
29.7	*100*	*0*	*0.2*
32.1	*32*	*68*	*0.2*
35	*32*	*68*	*0.2*
45	*32*	*68*	*0.4*

Methods

Sample Preparation:

N-linked glycans were released from Human IgG (Sigma I4506) using N-glycanase (Prozyme GKE-5016A). The experiment follows the protocol provided by Prozyme. After release, the glycans were labeled with 2-AB using 2-AB labeling kit (Prozyme GKK-404) and following the protocol provided in the kit. After sample clean up (acetone precipitation) and dry, labelled and released glycans were re-suspended with 100 µL of LC/MS water. 30 µL of the sample is mixed with 70 µL of acetonitrile and inject into LC/MS.

Chromatography:

Separation was accomplished using a Shimadzu LC system fitted with a Waters BEH amide column (130Å, 1.7 µm, 2.1 mm X 100 mm), using the gradient shown in Table 1. Two different mobile phases were used for comparison in this study. The First system used mobile phase A: 0.05% Trifluoroacetic acid in water and mobile phase B: 0.05% Trifluoroacetic in acetonitrile. The second system was mobile phase A: 50mM ammonium formate pH 4.2; mobile phase B: 100% acetonitrile.

Mass Spectrometry

A SCIEX X500B Mass Spectrometer fitted with a Turbo V™ source with a TwinSpray probe was used for data acquisition. Data was acquired using IDA mode. MS instrument conditions are listed in Table 3.

Table 3. MS Parameters

Parameter	Setting
Scan Mode	*Positive*
Scan Type	*IDA*
Workflow:	*Peptide*
GS1	*60*
GS2	*60*
Curtain Gas	*45*
Temperature	*350°C*
Ion Spray Voltage	*5000 V*
Time Bins to Sum	*4*
Accumulation Time (ms)	*0.25 sec*
TOF Start Mas (Da)	*200*
TOF Stop Mas (Da)	*1800*
Declustering Potential	*20*
Collision Energy	*7*
Intensity threshold exceeds (counts/s):	*100*
Maximum candidate ions	*8*
Charge state	*From 2 to 3*
CE equation	*CE = (0.01) * (m/z) + (2.5)*
Collision Energy	*7*

Florescence detector

A Shimadzu RF-20xs florescent detector was online with the LC system for florescent trace acquisition. FLD instrument conditions are listed in Table 4.

Data processing:

Data was processed using SCIEX OS software 1.5.0.23389.

Table 4. Florescence detector Parameters

Parameter	Setting
Chanel Ex Wavelength (nm)	*360*
Chanel Em Wavelength (nm)	*450*
Data acquisition	*Yes*
Lamp on	*Yes*
Sampling	*100ms*
Response	*0.5s*
Gain	*X1*
Sensitivity	*Medium*
Cell temperature	*30°C*
Auto Zero	*Yes*

Results and Discussion

N-linked glycans are easily released from proteins using PNGaseF. Upon release, glycans do not contain a chromophore so they must be derivatized prior to analysis with a suitable label which provides fluoresce and mass spectrometric response. The use of 2-AB is the most common label for HILIC-FLR-MS analysis and was selected for this study. Additional labeling approaches have been introduced recently which enhance fluoresce and/or mass spectrometric response, however these labels are typically more expensive than traditional labels such as 2-AB. If these labels are preferred the approach presented here is applicable.

The separation of labeled glycans is commonly completed using ammonium formate buffers. Recently there have been reports using alternative approaches with trifluoroacetic acid as biopharmaceutical development labs seek greater utilization of instrumentation for a greater number of workflows. Here we explored the use of both mobile phase systems, TFA based as shown in Figure 1 and the traditional ammonium formate as shown in Figure 2. Overall the separations are comparable with similar selectivity and relative peak areas. Interestingly our data indicates that TFA additives provide greater signal sensitivity for sialic acid containing species.

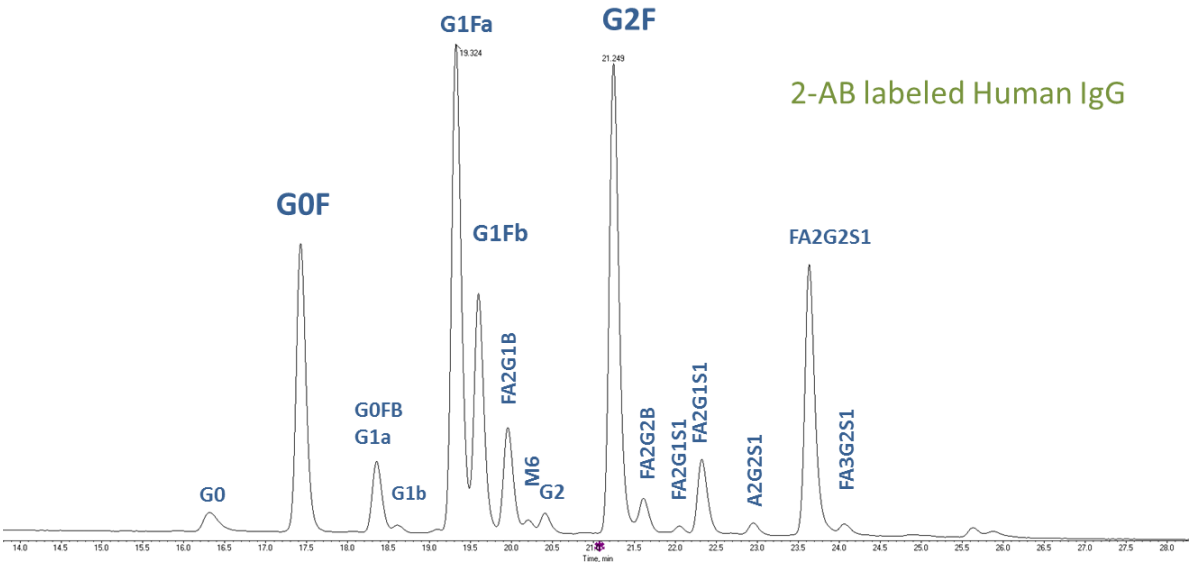

Figure 2. Florescence trace of a 2-AB labeled release N-linked glycan with ammonium formate in the mobile phase.

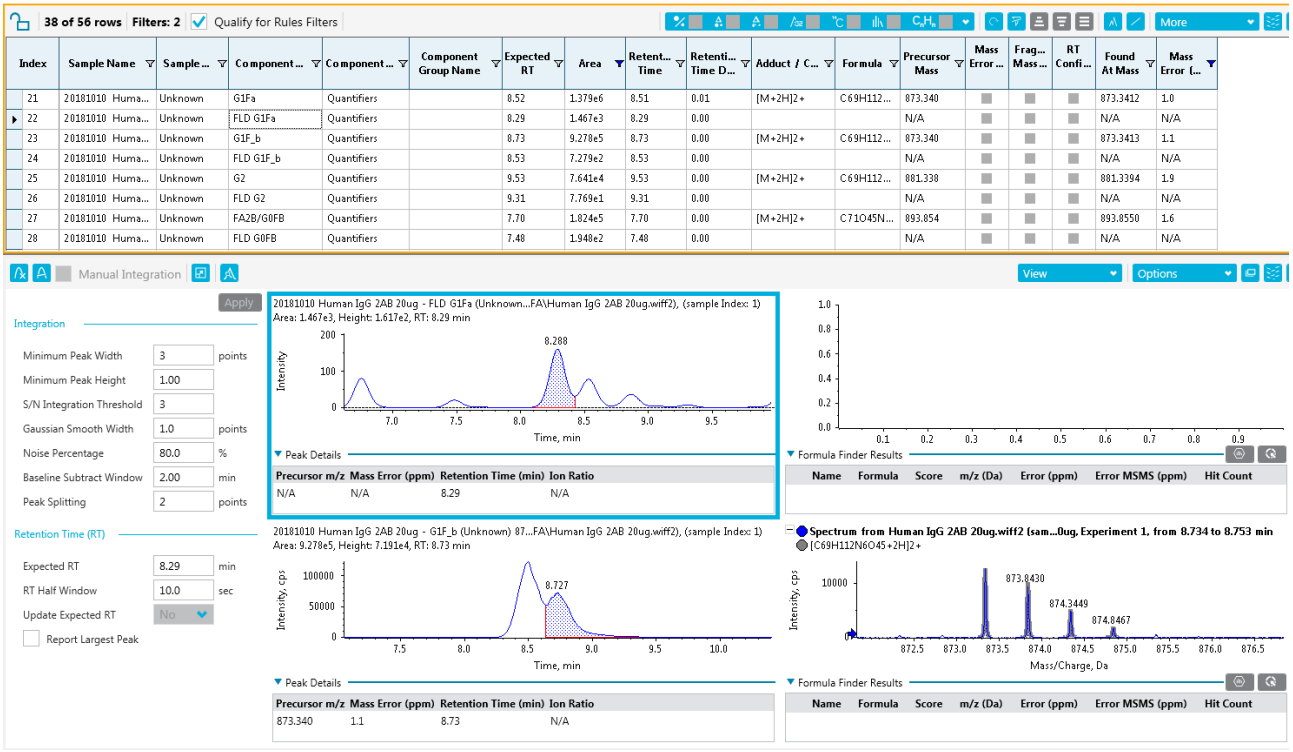

Figure 4. Integration of florescent trace in SCIEX OS Analytics for quantitation.

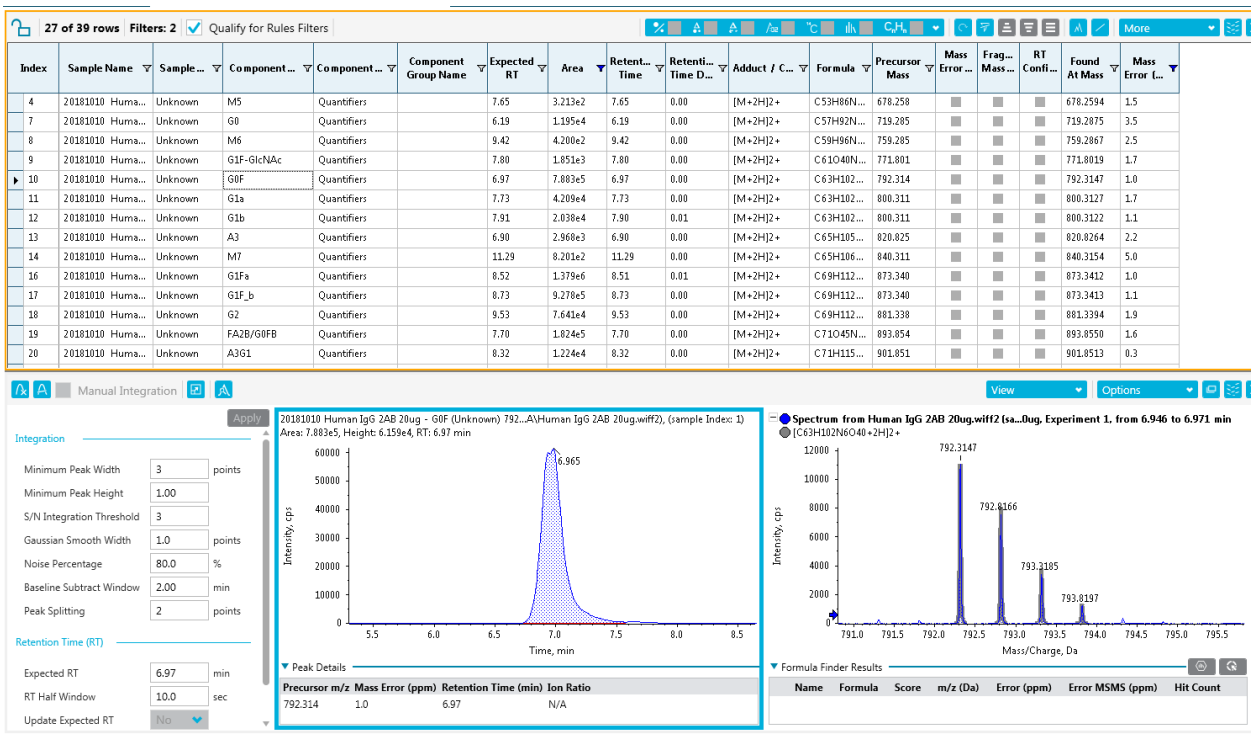

Figure 3. Characterization of 2-AB labeled glycan in 0.05%TFA mobile phase by SCIEX OS Analytics. .

Analysis of the glycan data was completed using SCIEX OS Analytics (Figure 3). A total of 27 N-linked glycans were identified within 5 ppm mass accuracy. The results are reviewed by selection of individual glycan species from the result table. After selection, the associated mass spectra and XIC are displayed to expedite review of results and accuracy of the identification as needed. In addition, the florescence trace can be added to SCIEX OS Analytics for workflow to enable relative quantitation (Figure 4). The SCIEX OS Analytics workflow provides the capability to integrate the fluorescence trace for each glycan component based on retention time.

MS/MS data was collected during acquisition for all detected species. The analysis was accomplished using an Information Dependent acquisition mode (IDA). This approach provided high quality fragment data for each of the identified species to confirm assignments based on FLR and MS data.

Figure 5 shows an example of the MSMS fragment ion spectrum of the G0F glycoform. As shown in the figure, sequential sugar loss is readily identified, and the fragment data clearly indicates that the structure is core fucosylated.

Another example of MS/MS spectrum is G2F with one sialic acid (FA2G2S1) shown in Figure 6. Sequential sugar loss clearly shows that the glycan structure is a bi-antenna core fucosylated glycan, with two galactoses and one sialic acid.

Conclusions

- Glycan analysis on the X500B QTOF system provides accurate identification of glycan profile with high mass accuracy
- SCIEX OS Analytics offers single integrated software for both acquisition and data analysis
- Comprehensive fragmentation on X500B QTOF provides structure information for glycan

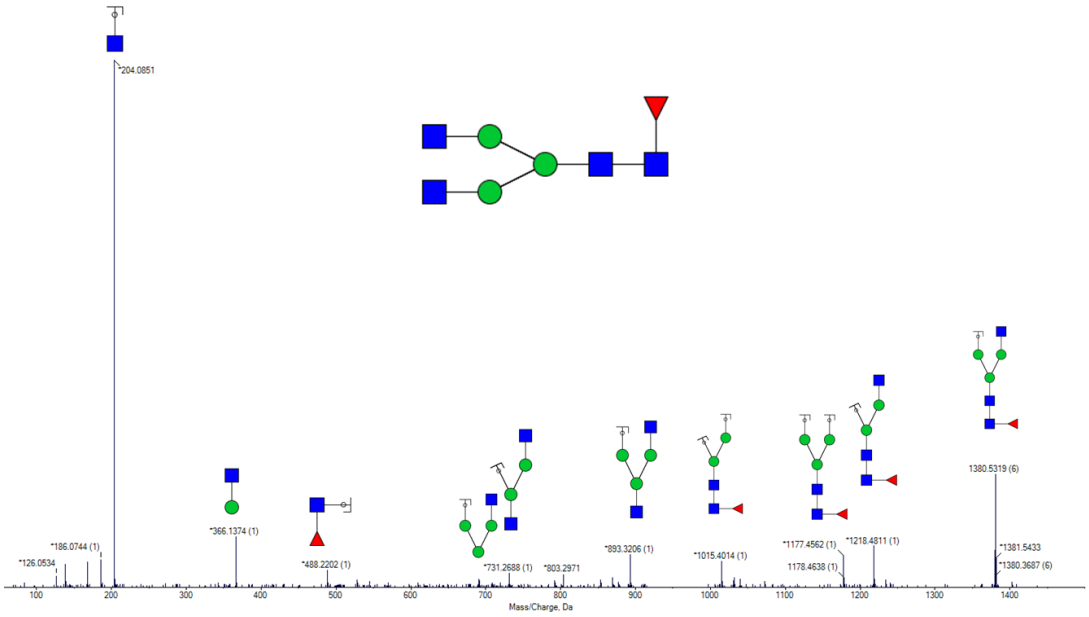

Figure 5. MS/MS spectrum of 2-AB labeled G0F from IDA experiment.

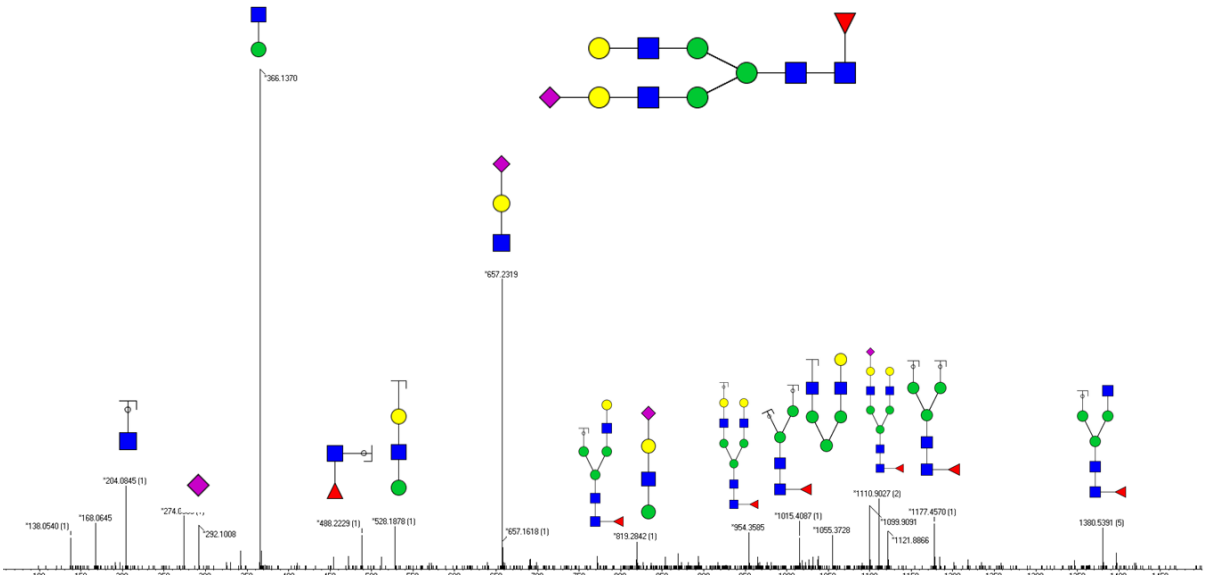

Figure 6. MS/MS spectrum of 2-AB labeled FA2G1S1 from IDA experiment.

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