

Characterization of 2-AB Labeled Released N-linked Glycans Using SCIEX TripleTOF[®] 5600+ LC-MS/MS System

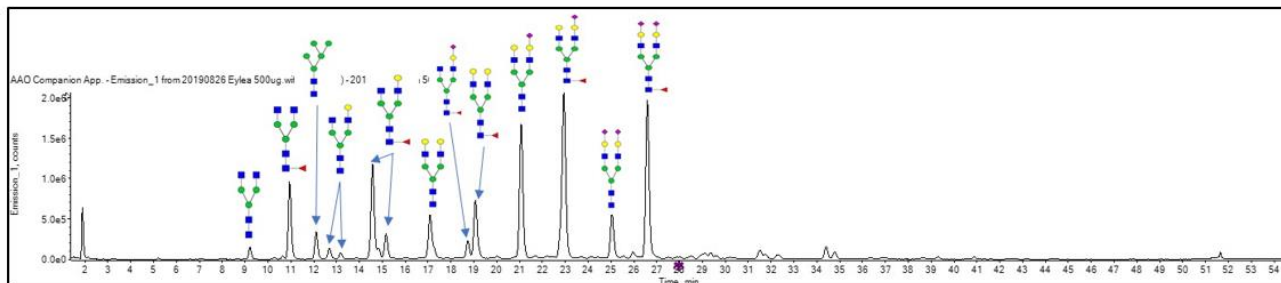
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It has been well documented that the attached N-glycans on mAbs play crucial roles in many biological and physicochemical processes such as enhancement of the structural integrity, resistance against protease, effectiveness of serum half-life in vivo, and antibody-dependent cellular cytotoxicity. For example, the sialic acids were closely related to some biological processes such as cell-cell adhesion, cell surface receptor recognition, and progression of human malignancies.

HILIC separation coupled to a fluorescence 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.



Waters Amide Column



Phenomenex Biozen Column

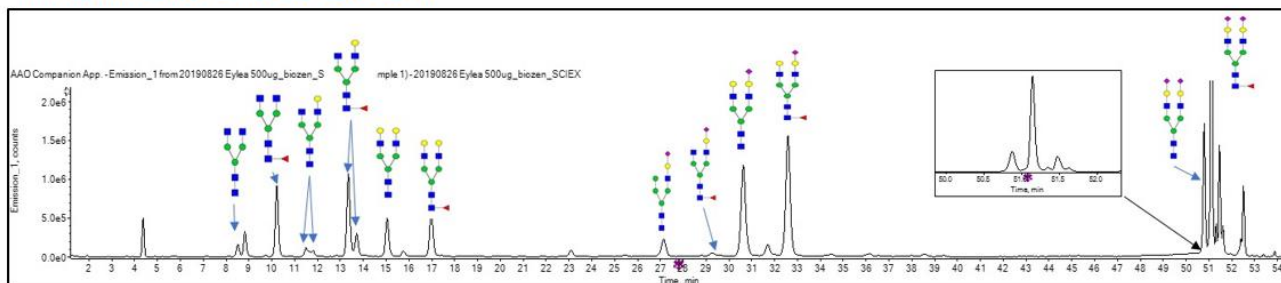


Figure 1. Separations of 2-AB labeled N-Linked Glycans. (Top) Separation using the Waters Amide column. (Bottom) Separation using the Phenomenex Biozen column.

Methods

Materials: Eylea (afibercept), 10K Amicon Ultra, PNGase F (NEB P0705L), RapiGest (Waters), Dithiothreitol, EB-10 Clean-up cartridge (Ludger LC-EB10-A6), 2-AB Glycan Labeling Kit (Ludger LT-KAB-A2), LudgerClean S Cartridge (Ludger LC-S-A6).

Sample Preparation: 500 µg of sample was diluted in 50mM Ammonium Bicarbonate and final volume becomes 400 µL. In order to remove buffer in the sample, 10K Amicon filter was used. The sample was denatured by RapiGest and reduced by DTT. Glycans were released by treating PNGase F with the manufacturer's instructions provided and the sample was cleaned up with EB10 cartridge. The released glycans were labeled with 2-AB reagent and cleaned up using S cartridge.

Chromatography: The system used mobile phase A: 100mM ammonium formate pH 4 and mobile phase B: 100 % acetonitrile. Separation was accomplished using a Dionex Ultimate3000 LC system fitted with a Waters BEH amide column (130Å, 1.7 µm, 2.1 mm X 150 mm) and Phenomenex Biozen Glycan(2.6 µm, 2.1 mm X 150 mm), using the gradient shown in Table 1. The column temperature was set at 40C. Fluorescence detector was set with excitation and emission wavelengths of 330 and 420 nm, respectively.

Table 1. Chromatographic Conditions

Time (Mins)	Flow (mL/min)	%A	%B
0	0.5	25	75
46.5	0.5	40	60
48	0.25	100	0
49	0.25	100	0
63	0.5	25	75
80	0.5	25	75

Mass Spectrometry: A SCIEX TripleTOF 5600+ System with a DuoSpray™ Source was used for data acquisition. Data was acquired using IDA in ESI positive mode. MS instrument conditions are listed in Table 2.

Data Processing: Data were processed using both MasterView™ Software 1.1 and SCIEX OS 1.6.1 in order to show the researchers how to leverage various software packages. The collected MS/MS spectra were interpreted using GlycoWorkbench software.

Table 1. MS Parameters.

	Parameter	Setting
	GS1	40
	GS2	50
Source / Gas	TEM	350
	CUR	25
	ISFV	5500
	Mass Range	250 – 2500
TOF MS	DP	60
	CE	10
	Accumulation Time	250 msec
	Mass Range	100 – 3000
TOF MS/MS	DP	60
	CE	35
	Accumulation Time	15
	With Charge State	2 - 5
	Max # of Candidate Ions	10
IDA Criteria	Mass Tolerance	50 mDa
	Dynamic Background Subtraction	On
	Exclude Former Target Ions	On
	Exclude after Occurrences	2

Results

The separation of labeled glycans is commonly completed using ammonium formate buffer and the Waters Glycan Amide column is widely used (Figure 1-1). The comparable result was found when the Phenomenex Biozen Glycan column was used. Interestingly, the eluted glycans were grouped depending on the number of sialic acids when the same gradient condition was applied to both columns (Figure 1-2). It may be beneficial to separate complex glycans such as Erythropoietin.

Analysis of the glycan data was completed using both MasterView Software (Figure 2) and SCIEX OS Analytics (Figure 3). A total of 12 N-linked glycans were identified within 5 ppm mass accuracy. The results were reviewed by selection of individual glycan species from the result table. After selection, the associated mass spectra and XIC were displayed to expedite review of results and accuracy of the identification as needed.

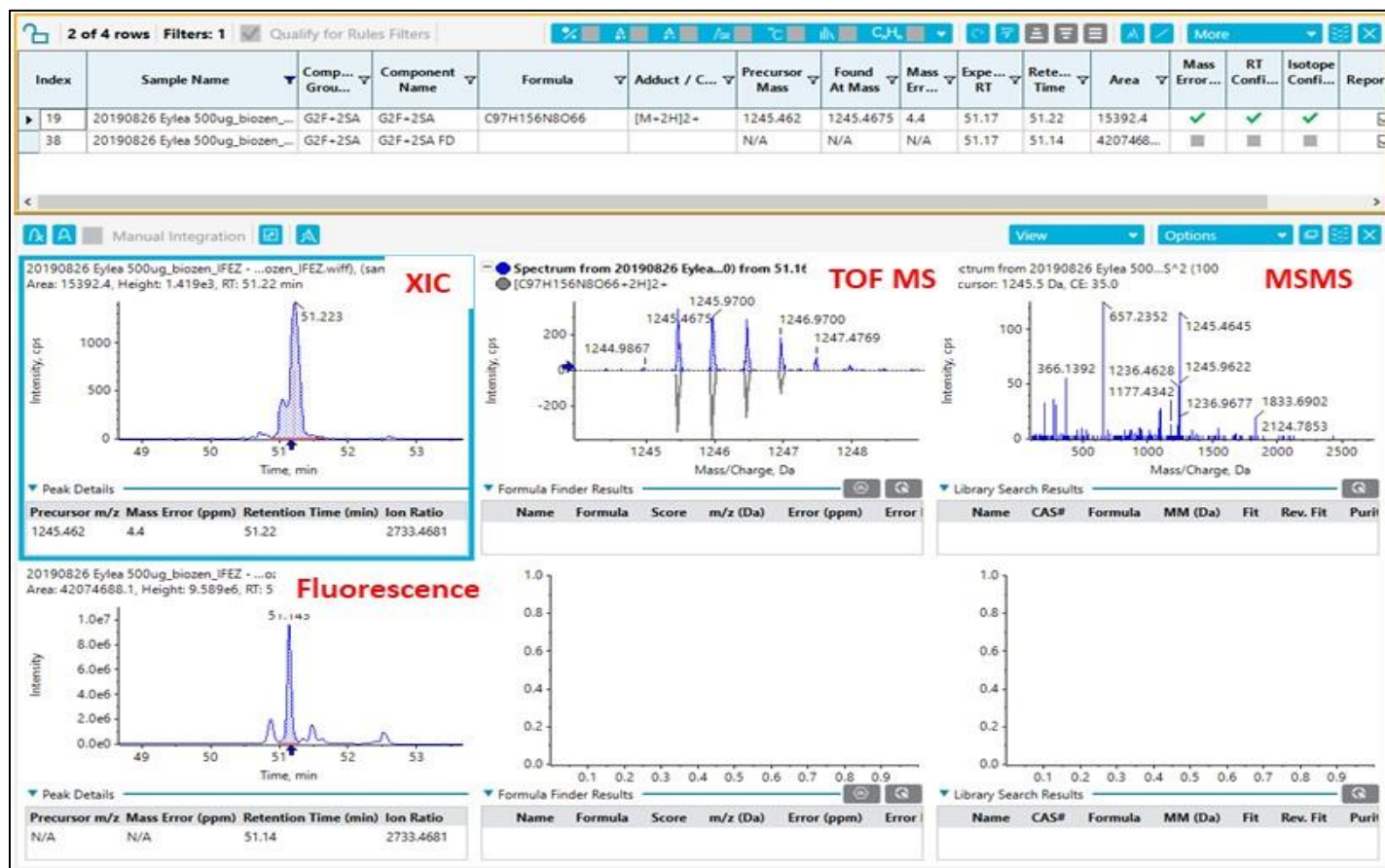


Figure 3. SCIEX OS Software Data Processing for Targeted Glycan Screening. Similar to MasterView Software, many different visuals can be plotted in a single view to better explore the data.

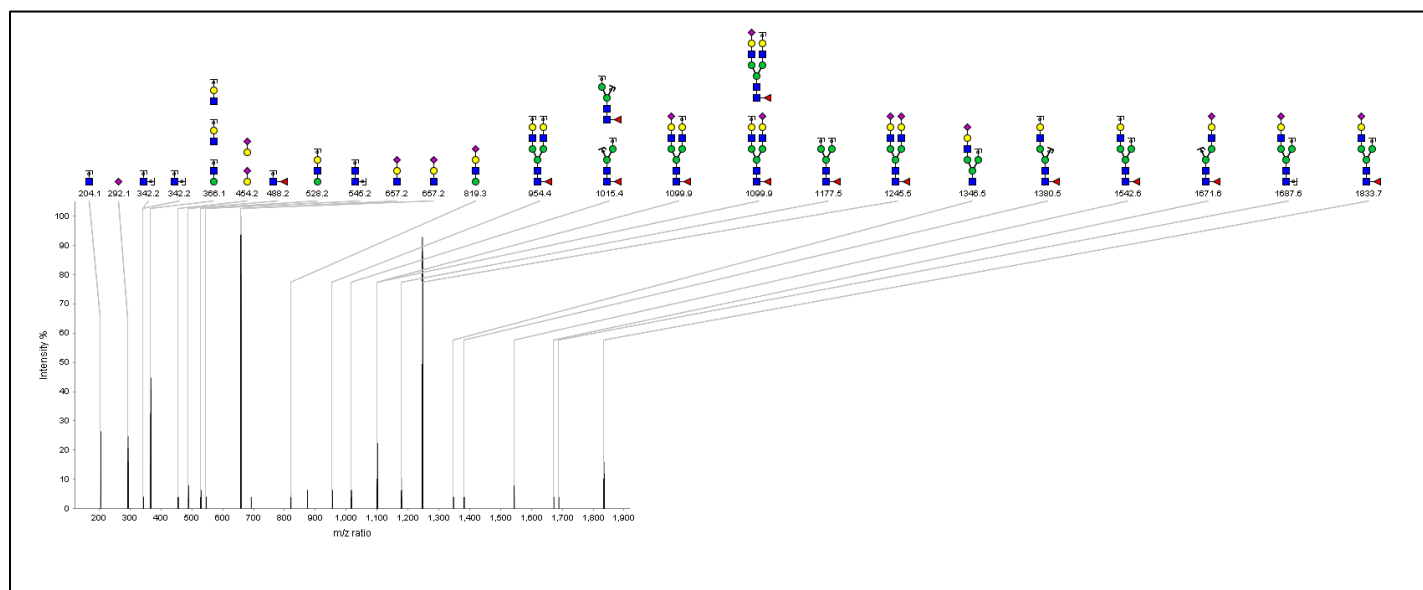


Figure 4. Example MS/MS Spectrum of the 2-AB labeled G2F+2SA Glycoform. MS/MS spectrum for Glycoform FA2G2S2, A2F is shown and labeled with GlycoWorkBench. The sequential sugar losses are readily observed.

References

1. Characterization of 2-AB Labelled Released N-linked Glycans Using the X500B QTOF System. SCIEX Technical Note RUO-MKT-02-9202-A

Acknowledgements

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