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

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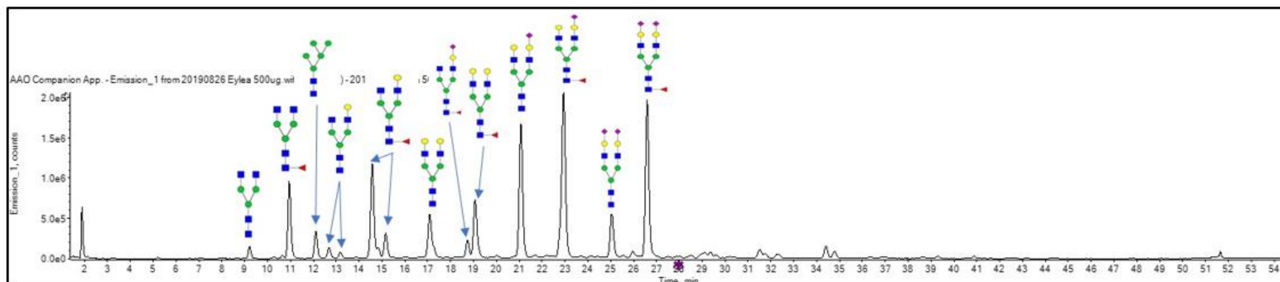
It has been well documented that the attached N-glycans on mAbs play crucial roles in many biological and physicochemical processes such as enhancement of the structural integrity, resistance against protease, effectiveness of serum half-life in vivo, and antibody-dependent cellular cytotoxicity. For 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

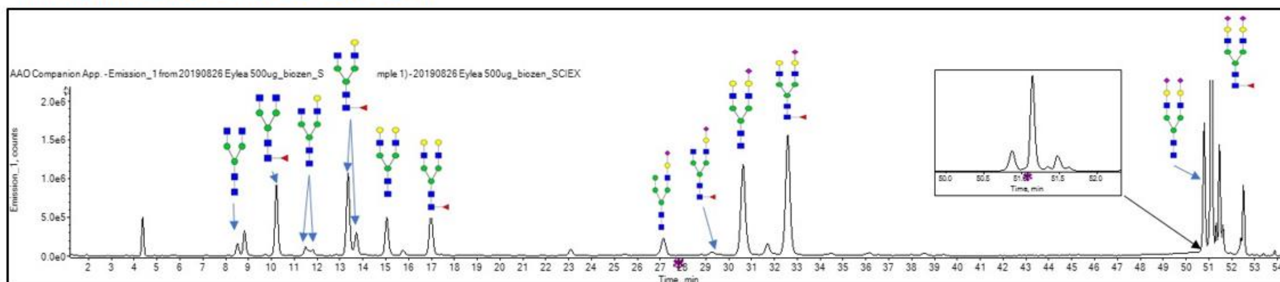


Figure 1. Separations of 2-AB labeled N-Linked Glycans. (Top) Separation using the Waters Amide column. (Bottom) Separation using the Phenomenex Biozen column.

Methods

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

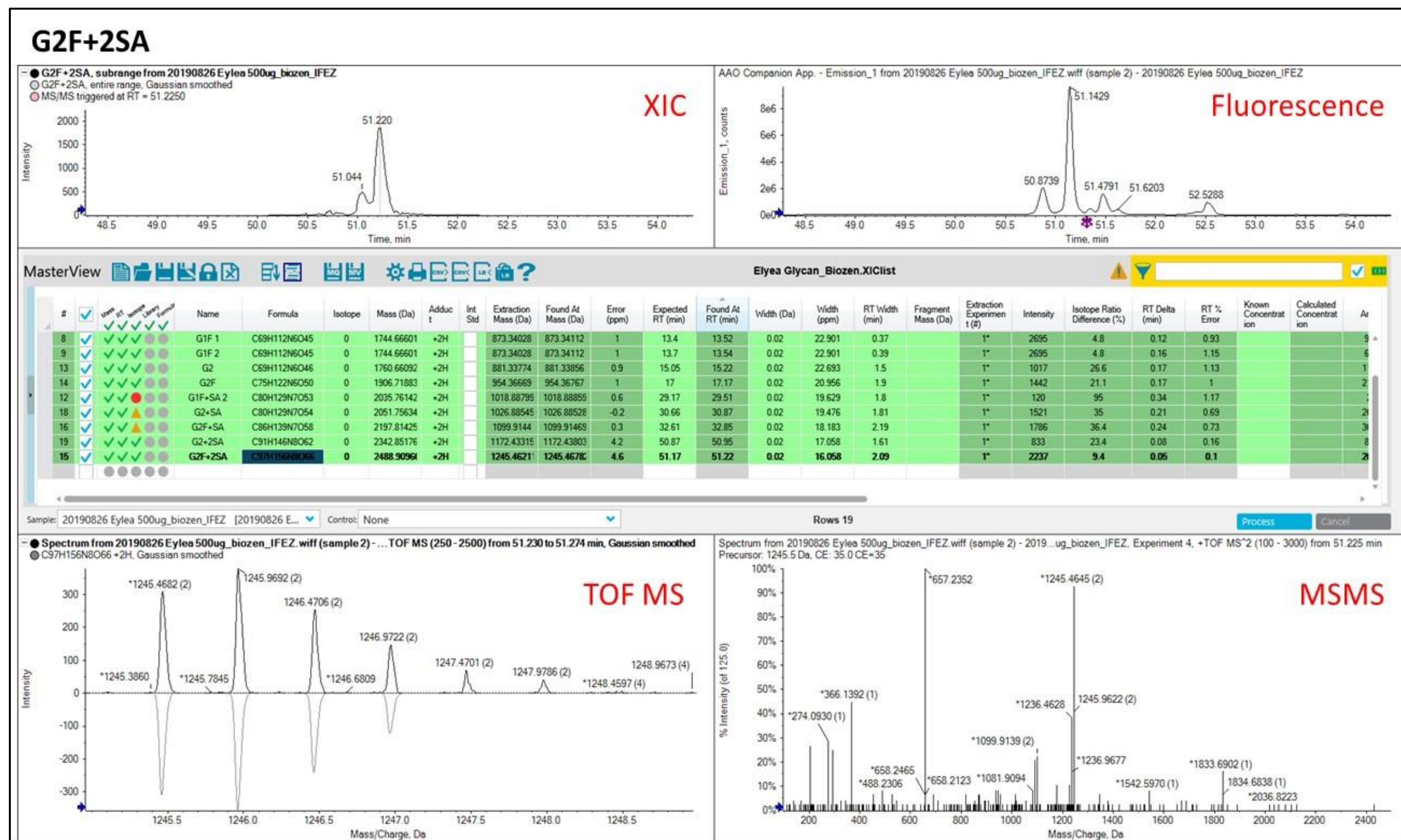


Figure 2. MasterView Software Data Processing for Targeted Glycan Screening. Numerous different visuals can be plotted in a single view including the Extracted Ion Chromatogram, TOF MS and MS/MS information corresponding to the selected targets from the list.

Because the analysis was accomplished using an Information Dependent acquisition mode (IDA), MS/MS data were

simultaneously collected during acquisition for all detected species. This approach provided high-quality fragment data for each of the identified species to confirm assignments based on FLR and MS data. Figure 4 shows an example of the MS/MS fragment ion spectrum of the G2F+2SA glycoform. As shown in the figure, sequential sugar loss is readily identified, and the fragment data clearly indicates that the structure is core fucosylated.

Conclusions

- The chromatographic comparison between the two columns tested showed very different patterns in the fluorescence traces. Sialylated glycans can be well-separated with non-sialylated glycans without any sensitivity loss on the Phenomenex column.
- MasterView Software and SCIEX OS Software provide the targeted data extraction which is useful to easily find out the target masses. SCIEX OS has better performance for the quantitation with less data processing steps.
- Comprehensive fragmentation information provides structure information for glycans.

2 of 4 rows Filters: 1 Qualify for Rules Filters

Index	Sample Name	Comp... Grou...	Component Name	Formula	Adduct / C...	Precursor Mass	Found At Mass	Mass Err...	Expe... RT	Rete... Time	Area	Mass Error...	RT Confi...	Isotope Confi...
19	20190826 Eylea 500ug_biozen_...	G2F+2SA	G2F+2SA	C97H156N8O66	[M+2H]2+	1245.462	1245.4675	4.4	51.17	51.22	15392.4	✓	✓	✓
38	20190826 Eylea 500ug_biozen_...	G2F+2SA	G2F+2SA FD			N/A	N/A	N/A	51.17	51.14	4207468...			

XIC

Precursor m/z	Mass Error (ppm)	Retention Time (min)	Ion Ratio
1245.462	4.4	51.22	2733.4681

TOF MS

MSMS

Fluorescence

Precursor m/z	Mass Error (ppm)	Retention Time (min)	Ion Ratio
N/A	N/A	51.14	2733.4681

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.

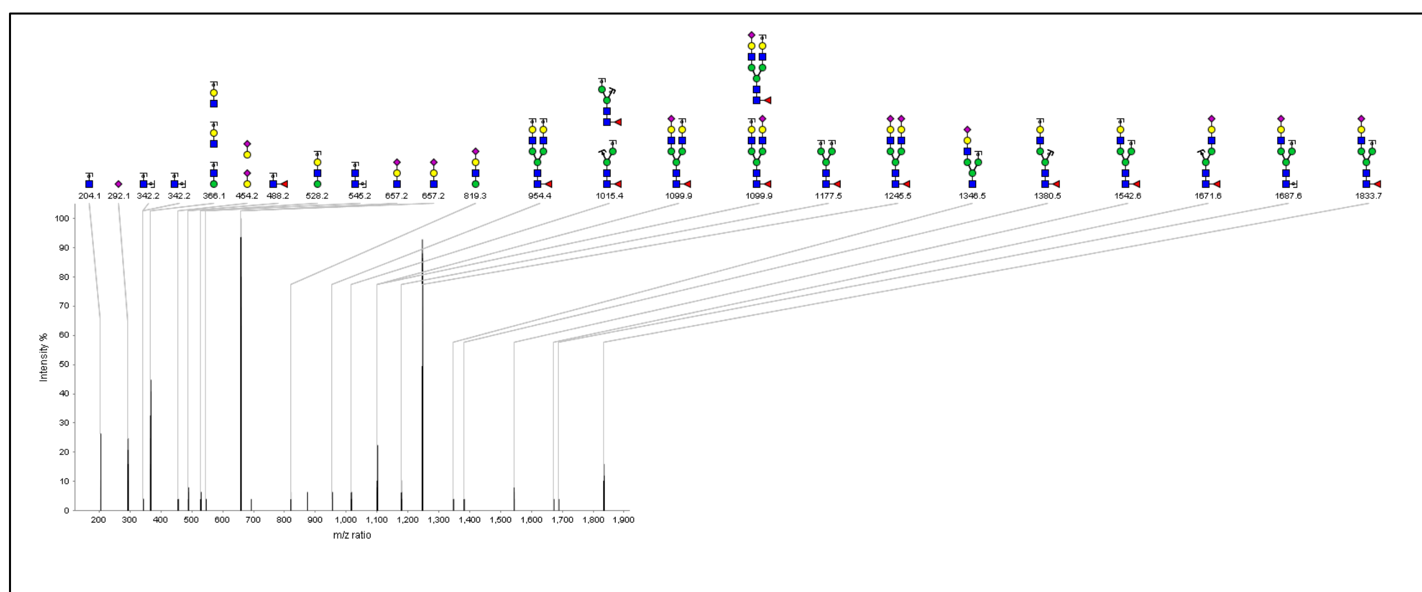


Figure 4. Example MS/MS Spectrum of the 2-AB labeled G2F+2SA Glycoform. MS/MS spectrum for Glycoform FA2G2S2, A2F is shown and labeled with GlycoWorkBench. The sequential sugar losses are readily observed.

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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