APPLICATION NOTE

A Complete Analytical and Bioinformatics Solution for High-Throughput Biotherapeutic Glycosylation Profiling Using High-Resolution Ion Mobility-Mass Spectrometry (HRIM-MS)

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Introduction

As mass spectrometry instrumentation becomes faster and more sensitive, overall method time, resolution and depth of analysis are now limited by separation methods. To enhance overall front-end separatory techniques, post-ionization gas-phase separations have become more common in conjunction with traditional liquid separations prior to mass analysis.[1] The use of ion mobility-mass spectrometry (IM-MS) can significantly reduce analysis times and improve resolution of glycan isomers compared to LC-MS alone.[2] IM-MS platforms have become commercially available and the importance of this additional dimension of separation is now being realized in industry settings. The High-Resolution

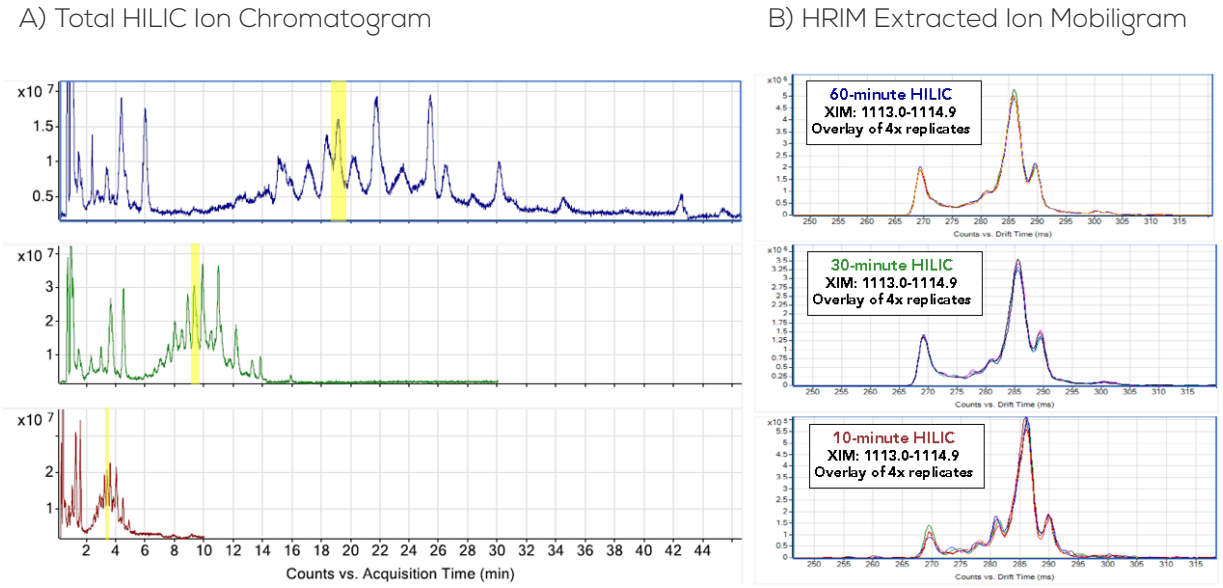

Figure 1: Consistent ion mobility separations were achieved for all LC gradients assessed. A) Example total ion current chromatograms for the 60- (blue), 30- (green), and 10-minute (red) HILIC gradients used to analyze the Aranesp® released *N*-glycan samples. Highlighted areas represent the elution regions corresponding to the extracted HRIM arrival time distributions in (B). B) Overlay of extracted ion mobiligrams (XIM) for Aranesp® glycan $(HexNAc)_5(NeuAc)_3(Fuc)_1(Hex)_6$ (*m/z* =1113.09, +3 charge state) observed with 60-, 30-, and 10-minute HILIC gradients. Four replicate XIMs plotted of the LC-HRIM-MS runs collected for each HILIC gradient for the same released *N*-glycan signal. "Extracted ion mobiligram" represents the ion mobility arrival time trace for a single *m/z*.

Takeaways

- Addition of HRIM to a traditional LC method provides a solution for rapid fingerprinting of glycosylation profiles in biotherapeutics.
- Comparable glycan feature identification, relative quantitation, and mobility profiles were obtained for LC gradients ranging from 60 to 10 minutes.
- 4D data analysis resulting in glycan feature detection, determination of glycan composition and relative quantification of glycan species are easily obtained using the automated HRIM-Byos software.

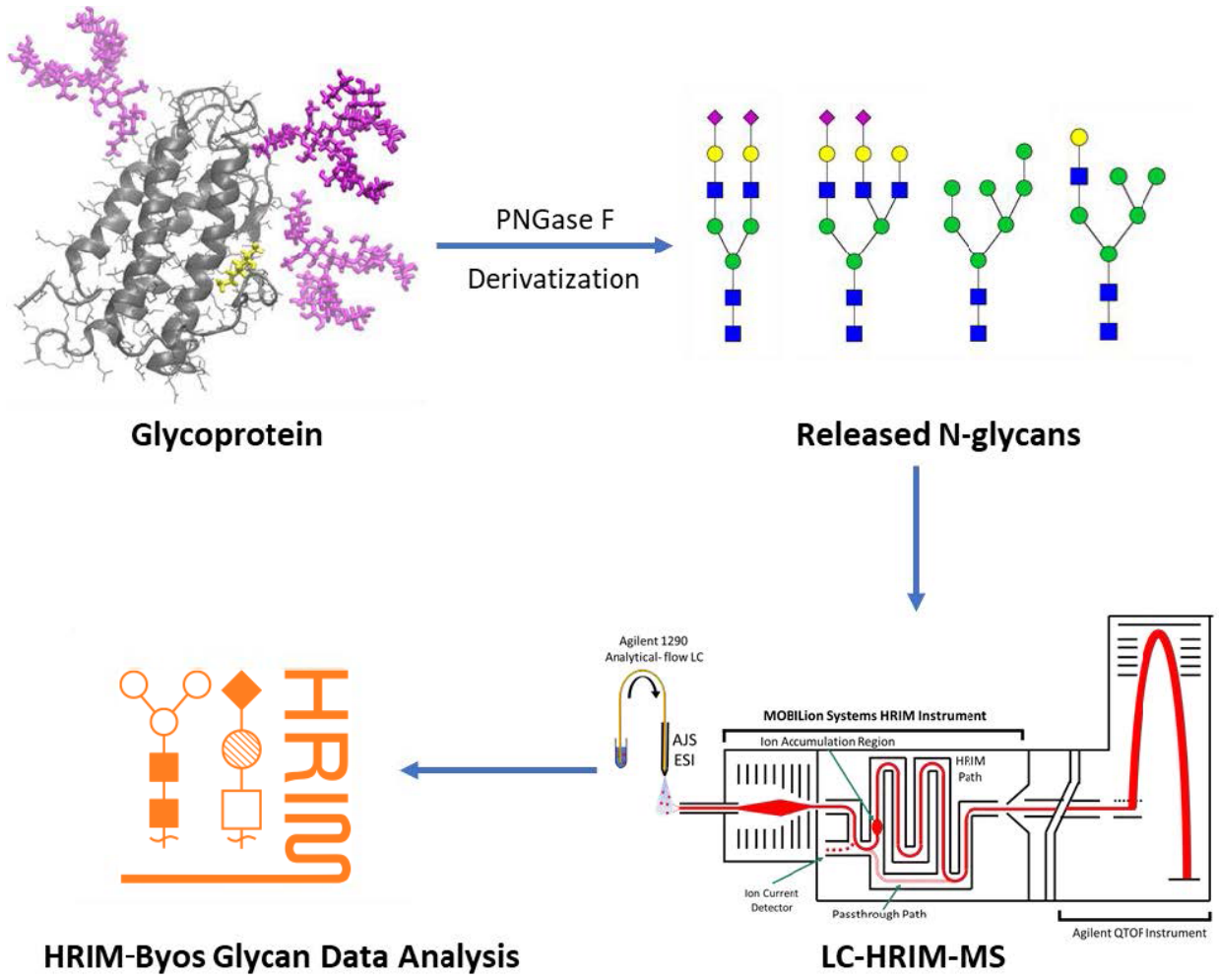

Figure 2: Overview of workflow for LC-HRIM-MS analysis of released *N*-glycans from glycoprotein standards and biotherapeutics.

Ion Mobility-Mass Spectrometry (HRIM-MS) platform MOBIE™ demonstrates great potential for revealing complex glycoform profiles[3] and, in combination with LC, yields multiple dimensions of information for feature determination and quantitation, an example of which is shown in Figure 1.

In the pharmaceutical manufacturing space, protein glycosylation is a critical quality attribute (CQA) for biotherapeutics that is regularly characterized and monitored due to glycosylation's direct impact on product safety and efficacy. The non-template driven nature of glycan biosynthesis results in high structural heterogeneity, with even minor changes to the manufacturing process potentially causing significant alteration in glycosylation profiles. Glycoform characterization typically involves liquid chromatography (LC) separations followed by detection with either laser-induced fluorescence of fluorescently labeled glycan species or mass spectrometry (MS). However, liquid-phase separations alone are limited in capacity for resolving glycan isomers, and efforts at separating isomeric species can require methods with extended separation times.

We have employed LC and HRIM-MS for comprehensive characterization of *N*-linked glycan species released from a protein biotherapeutic. LC methods of varying run times were implemented to assess the ability of HRIM to resolve glycans in the gas phase while LC separation times were gradually reduced (Figure 1). The data collected were then analyzed using a novel, automated workflow designed to enable seamless processing and reporting of LC-HRIM-MS released glycan data. Combined with data analysis using Protein Metrics Byos® HRIM-MS workflows,[4] we present a solution for high-throughput glycan profiling for biotherapeutics (Figure 2).

Methods

Glycan samples were prepared from Aranesp® (Darbepoetin alfa, Amgen Inc.). *N*-glycans were released using PNGase F (New England Biolabs, Ipswitch, MA) and then labeled using GlycoWorks RapiFluor-MS *N*-Glycan Kit (Waters Corp., Milford, MA), following the manufacturer's protocols. Samples were analyzed by LC-HRIM-MS utilizing a 1290 Infinity UHPLC system and AdvanceBio Glycan Mapping Hydrophilic Interaction Liquid Chromatography (HILIC) column (Agilent Technologies, Santa Clara, CA), followed by HRIM separation and detection using a MOBIE (MOBILion Systems, Inc. Chadds Ford, PA) commercial equivalent unit coupled to an Agilent 6545-XT QTOF. All parameters were optimized for transmission, separation, and detection of Aranesp® *N*-glycans. HILIC gradients of 10-, 30-, and 60-minutes were used for analysis of Aranesp® released *N*-glycans, (n=4 replicate injections). The HRIM-Byos Released Glycan workflow (Protein Metrics, Cupertino, CA) was used for data processing and report generation of released glycan LC-HRIM-MS data.

Results & Discussion

The Aranesp® released *N*-glycans were analyzed by LC-HRIM-MS with 60-, 30-, and 10-minute HILIC gradients (Total Ion Current (TIC) plots for the HILIC separations are shown in Figure 1a). All data were analyzed using the Protein Metrics HRIM-Byos Released Glycan workflow. Images taken from the Protein Metrics workflow are shown in Figures 3 and 4. Automated detection and relative quantitation of 52 glycan compositions were performed

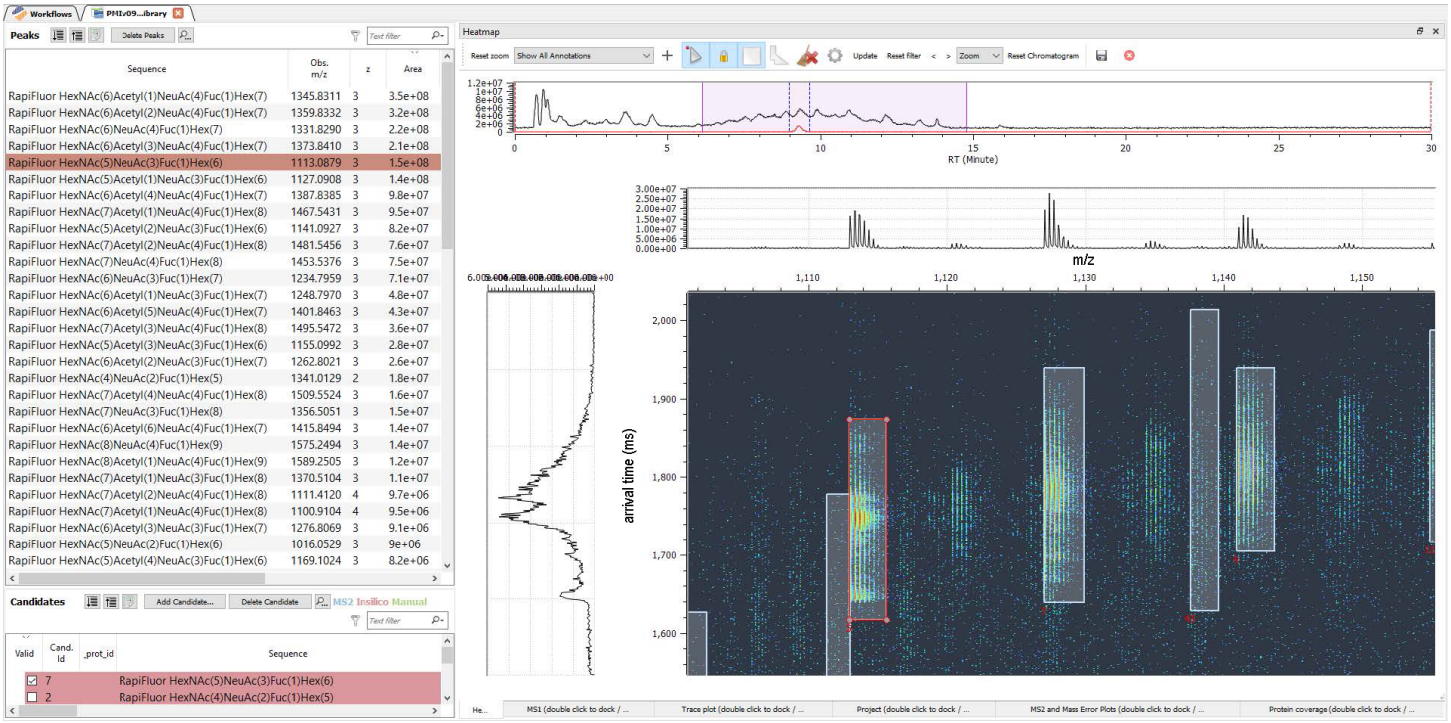

Figure 3: The Protein Metrics HRIM-Byos Released Glycan Project view. A Peaks Table on the left includes a list of identified and assigned 4D features. An interactive heatmap on the right can be used to easily inspect, adjust, and add feature assignments. The heatmap can also be used to plot extracted ion chromatograms (see red trace above heatmap).

(see Table 1 and Figure 4 for the top 15 most abundant glycans represented) using the Protein Metrics HRIM-Byos Released Glycan workflow. Figure 3 displays a view of the user interface for the Byos software when processing a sample HRIM-MS dataset, while Figure 4 shows a representative ion arrival time heatmap produced by Byos.

All three HILIC gradients performed resulted in comparable relative glycan quantitation and species coverage. The relative peak areas obtained for each gradient were similar across all three separation timespans. Presented in Figure 5 is a visual demonstration of the similarity of relative peak areas across the three gradients.

Figure 1a shows an example where an extracted HILIC peak corresponding to the glycan composition $(HexNAc)_5(NeuAc)_3(Fuc)_1(Hex)_6$ is resolved into multiple ion mobility peaks, which suggests the possible separation of glycan isomer species in the gas-phase. Furthermore, the consistent HRIM peak profiles of Aranesp® released *N*-glycans suggests HRIM can serve as a robust approach for achieving a deeper characterization and monitoring of complicated glycoprofiles (Figure 1b). LC-HRIM-MS analysis combined with the Byos HRIM Glycan workflow allows for deep coverage of the Aranesp® glycosylation profile with a total of 52 *N*-glycan compositions identified. The novel Protein Metrics HRIM-Byos workflows, including a 4D feature finder, offers a high throughput, automated solution for LC-HRIM-MS data processing saving significant time and effort.

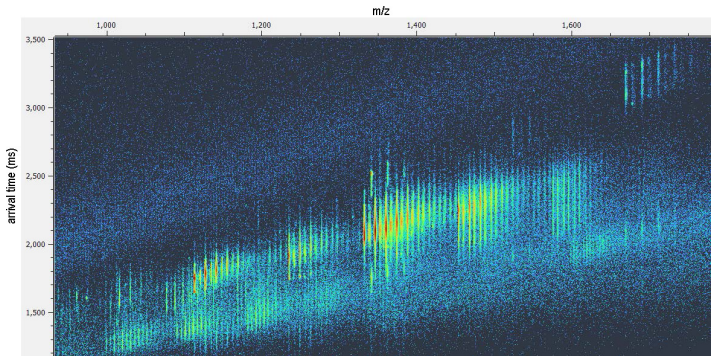

Figure 4: The Protein Metrics heatmap showing the Aranesp® released *N*-glycans signals. The x-axis is *m/z* and the y-axis is Ion Mobility arrival time. Different glycan compositions and gas-phase conformations result in unique arrival time profiles.

Table 1: Relative % peak area for the top 15 of 52 assigned Aranesp® *N*-Glycans (n=4).

Glycan	60-Minute		30-Minute		10-Minute	
HexNAc:Acetyl: NeuAc:Fuc:Hex	Avg	%RSD	Avg	%RSD	Avg	%RSD
6:1:4:1:7	15.6	0.4%	15.0	0.6%	15.2	0.9%
6:2:4:1:7	14.5	0.7%	13.9	0.2%	13.9	1.7%
6:0:4:1:7	10.3	0.6%	9.4	1.0%	9.0	1.8%
6:3:4:1:7	8.4	0.8%	9.1	0.3%	9.8	2.3%
5:0:3:1:6	7.0	0.9%	6.7	1.4%	4.9	2.5%
5:1:3:1:6	5.8	1.3%	6.3	2.5%	5.4	2.1%
7:1:4:1:8	4.6	0.7%	4.4	1.4%	4.5	2.2%
6:4:4:1:7	4.1	1.0%	4.4	0.9%	4.9	1.1%
7:2:4:1:8	3.7	0.9%	3.7	1.5%	3.9	0.8%
7:0:4:1:8	3.4	0.5%	3.3	1.1%	3.7	1.7%
5:2:3:1:6	3.1	0.5%	3.6	2.0%	3.4	3.2%
6:0:3:1:7	3.0	1.2%	3.0	0.8%	2.7	1.6%
6:1:3:1:7	2.3	0.2%	2.1	1.8%	2.0	3.0%
7:3:4:1:8	2.0	1.6%	1.5	1.1%	1.8	3.5%
6:5:4:1:7	1.5	1.6%	1.8	1.0%	2.1	5.1%

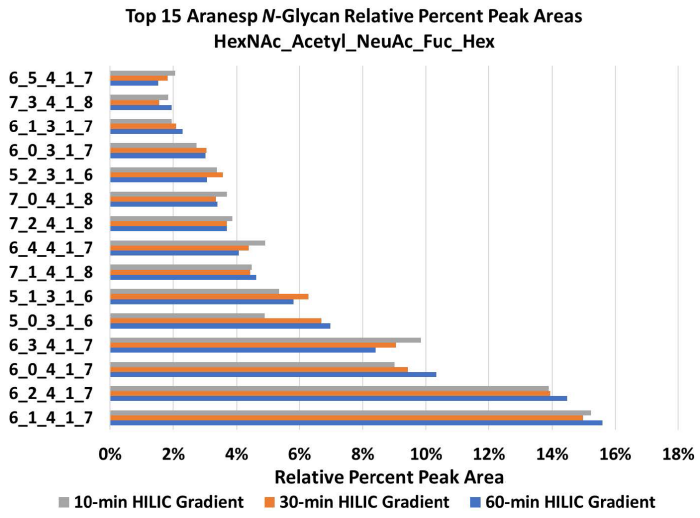

Figure 5: Average (n=4) relative peak areas for the top 15 Aranesp® *N*-glycans.

Conclusion

LC-HRIM-MS analysis of released *N*-glycans from Aranesp across multiple LC gradients ranging from 60- to 10-minutes provided consistent and reproducible relative quantitation results. With the addition of HRIM, separation of potential glycan isomers was achieved. This separation was consistent across all LC gradients used leading to the potential for HRIM to provide more in-depth high throughput glycan profiling. Given the data-rich results provided by LC-HRIM-MS, the tedious nature of high-dimensional data analysis for this technique can be a major challenge facing the larger adoption of high-resolution ion mobility separations. HRIM-Byos workflows alleviate this bottleneck and enable the complex 4D data to be processed rapidly and efficiently with automatically generated reports. The MOBIE platform combined with automated data analysis can provide a solution for rapid fingerprinting of glycosylation profiles for biotherapeutics. HRIM-MS combined with LC separations offers a greater depth of sample information than can be achieved from LC-MS alone. Overall arrival time distributions from HRIM-MS data provide additional glycan identification metrics for increased confidence in establishing a robust set of measures for biopharma workflows. In the future, these arrival times may be converted to collision cross section values providing for even further increased confidence in measurements.

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