IMPROVING CONFIDENCE AND PRODUCTIVITY FOR N-LINKED GLYCAN ANALYSIS IN BIOTHERAPEUTICS DEVELOPMENT USING AN INTEGRATED UPLC-FLR-HRMS SYSTEM

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INTRODUCTION

Due to the impact on drug efficacy and safety, glycosylation has been identified as a critical quality attribute that requires characterization and monitoring throughout the product lifecycle of biotherapeutics. Glycosylation on biotherapeutics is often highly complex with broad dynamic range. Therefore it is generally assessed at glycan level using Fluorescence (FLR) detection. While sensitive, optical detection alone lacks specificity and provides limited structural information. Orthogonal technologies, such as high-resolution mass spectrometry (HRMS), provide additional structural information and have been increasingly used in released glycan analysis. Previously, we developed a streamlined analytical workflow from sample preparation to LC/FLR/HRMS data collection and data processing using a glycan structure library. Such workflow has been demonstrated on many Waters LC/QTOF MS systems with UNIFI software control and data processing.

In this work, we are introducing a BioAccord System comprised with AQUITY I-Class PLUS UPLC, ACQUITY FLR detector and a new compact TOF MS (ACQUITY RDa detector). With reduced instrumentation and analytical methodology complexity, this system can be consistently operated and deployed in process development and manufacturing environments for glycan analysis. A case study, comparing innovator and biosimilar infliximab glycan profile, is used to illustrate the complete analytical workflow incorporating the bench top RDa detector as part of the integrated system.

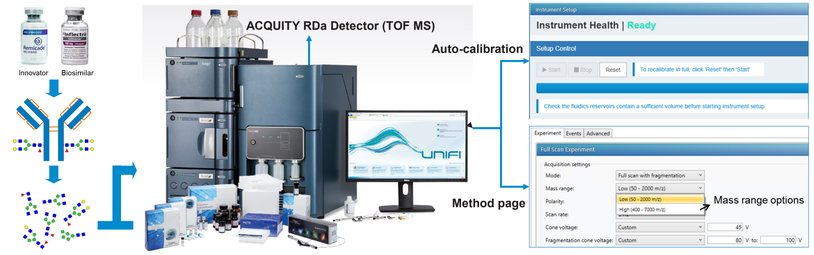

Figure 1, Released glycan analysis of innovator and biosimilar infliximab using the BioAccord System. The integrated instrument configuration and simple MS operation improves the productivity of released glycan analysis workflow. ACQUITY RDa Detector setup page and instrument method page showing SmartMS™ enabled auto-calibration function and MS data acquisition.

METHODS

LC conditions

LC system	ACQUITY UPLC I-Class PLUS
FLR set up	ACQUITY FLR detector ($\lambda_{excitation}$=265 nm, $\lambda_{emission}$=425 nm, 2Hz)
LC column	ACQUITY Glycan BEH Amide column, 1.7 µm, 130 Å, 2.1 × 150 mm
Column temp.	60 °C
Mobile phase A	H_2O with 50 mM Ammonium Formate
Mobile phase B	ACN

ACQUITY RDa MS detector

Mass range	50-2000 m/z
Polarity	ESI Positive
Capillary voltage	1.5 kV
Mode	Full scan with Fragmentation
Cone voltage (CV)	45 V
Fragmentation CV	70-90 V
Probe temp	300 °C
Sample rate	2 Hz
Lock mass	Leu-enkephalin at 50 fmol/µL in 50/50 H_2O/ACN with 0.1% formic acid

Gradient table

Time (min)	Flow (mL/min)	%A	%B
Initial	0.400	25.0	75.0
35.00	0.400	46.0	54.0
36.50	0.200	80.0	0.0
39.50	0.200	80.0	0.0
43.10	0.200	25.0	75.0
47.60	0.400	25.0	75.0
55.00	0.400	25.0	75.0

Informatics

Software	UNIFI 1.9.4
Workflow	Glycan FLR with MS confirmation
Mass tolerance	10 ppm
Library	RFMS glycan GU library
GU tolerance	±0.2 GU

Sample Prep

Two infliximab samples from innovator Remicade™) and one sample from biosimilar (Inflectra™) were diluted with water to a final concentration of 1.5 µg/µL. N-glycans from infliximab were released from 15 µg of diluted mAb samples and labeled using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176004082)$_b$ via an Andrew Alliance automated sample preparation platform. An amount of 2.5 pmol released glycan sample was injected for each analysis.

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RESULTS AND DISCUSSION

Automated Glycan Identification

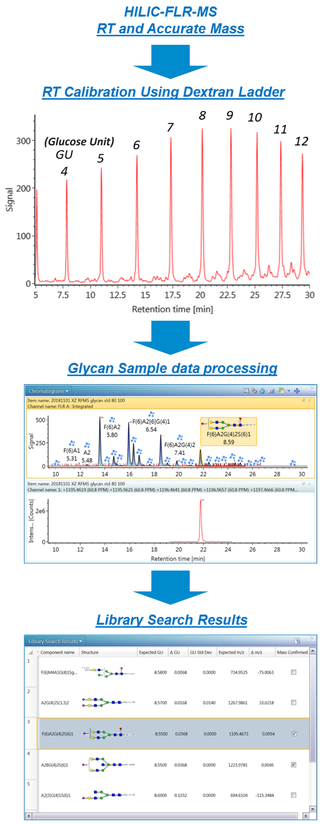


Figure 2. The integrated "Glycan FLR with MS confirmation" workflow. Retention times of glycans were calibrated against a dextran ladder standard and converted to Glucose Units (GU) values, and then used along with accurate mass information to conduct a library search for peak identification. The use of GU values ensures robust peak assignment by minimizing the potential variation from retention times across analyses.

Review of Analysis Results

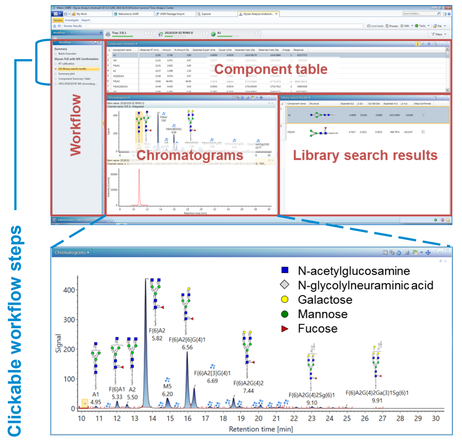
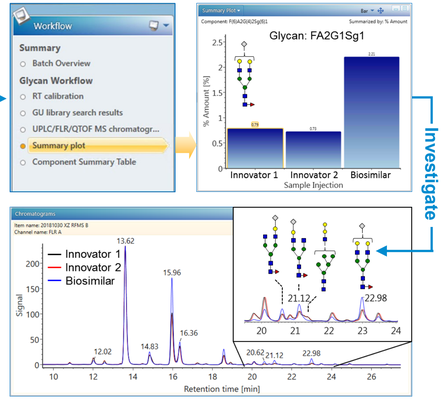


Figure 3. The review of the processed results, showing the clickable workflow steps, processed chromatograms, and library search results for identified peaks. The processed FLR trace shows identified peaks annotated with glycan name, GU value, and associated structure information. To avoid over crowed structure display, detailed glycan structures can be viewed by simply mousing over the IBM connection icon.



Fragmentation Assisted Data Interrogation

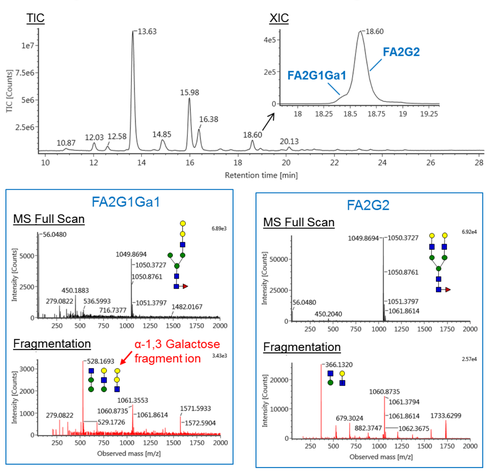


Figure 5. Zoom-in chromatogram shows the XIC of FA2G2 and its immunogenic isomer, FA2G1Ga1. Full MS scan with fragmentation data acquisition allows high energy data channel (MS2) to be acquired in parallel with the low energy MS data channel. MS1 is used for the accurate mass measurement of glycans, MS2 can be used manually to inspect questionable structure assignments. The MS full scan and fragmentation data of FA2G1Ga1 and FA2G2 showed a diagnostic ion (m/z 528) of α-1,3 Galactose.

Figure 4 (left). Comparison of glycan profiles between two innovator infliximab samples and biosimilar sample. As part of the workflow steps, an example of Summary Plot shows the elevated abundance of a glycan, FA2G1Sg1 (Sg stands for n-glycolylneuraminic acid), in the biosimilar mAb. The results can also be investigated through the overlaid FLR chromatograms. Zoom-in chromatogram shows the region of low abundant glycan species.

CONCLUSION

- **Introducing a new compact BioAccord system for comprehensive released glycan profiling assay using streamlined analytical workflow.**
- **Simplified HILIC-FLR-HRMS workflow to obtain and transform data into meaningful results for released glycan analysis in biosimilarity assessment**
- **Fast determination of differences in identity and abundance of glycans**
- **MS fragmentation data can be used for manual data interrogation**
- **Improved productivity and confidence of released glycan analysis**

References

1. Alley, W. R. J. and Yu, Y. Q. Combining RapiFluor-MS and UNIFI Scientific Information System for a total N-linked glycan solution for innovator vs. biosimilar infliximab comparisons. Waters Application Note. 720005753EN. 2016
2. Yu, Y. Q. A holistic workflow for acquiring, processing, and reporting fluorescent-labeled glycans. Waters Application Note. 720004619EN. 2016

Auto-generated Report

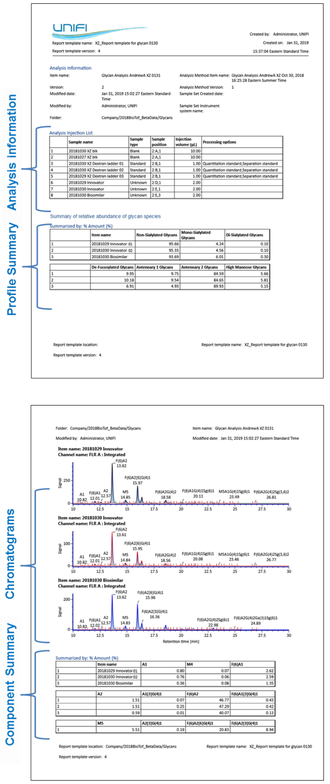


Figure 6. Report of analysis results (selected pages). The customizable report shows Sample List, Chromatograms, and Summary Table for straightforward inspection of the comparison data across samples. To meet requirements in different laboratories, the report can be customized to include desired information for simplified review of analysis results.