

## Increasing Productivity and Confidence for N-linked Glycan Analysis of Biosimilars Using the BioAccord System

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### Abstract

The objective of this work is to demonstrate that the BioAccord System can increase confidence and productivity in released glycan analysis for the development of biosimilars.

The SmartMS-enabled BioAccord System is an easy-to-use LC-MS platform solution that was purposefully designed for comprehensive analysis of biopharmaceutical drug products with built-in analytical workflows for specific analysis such as N-Glycan identification and profiling. Within the BioAccord System, highly robust chromatographic separation and accurate mass information can be obtained using the ACQUITY UPLC I-Class PLUS System and the ACQUITY RDa Detector controlled by UNIFI Scientific Information Software. The ACQUITY RDa Detector is a compact bench top time-of-flight mass detector with built-in self-calibration function and straightforward instrument method setup (Figure 1B and 1C), which substantially reduces the complexity of operating MS instruments. Together, high quality LC-FLR and LC-MS data can be acquired and transformed into meaningful results for released glycan analysis using an all-encompassing workflow, reducing the cost and time for biosimilar development without compromising product quality.

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## Benefits

- A compliance ready all-encompassing LC-FLR-MS solution for comprehensive N-linked glycan analysis
- Automated workflow from sample preparation to data reporting for improved productivity in biosimilar development
- Robust and specific platform solution that improves the confidence of released glycan analysis

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## Introduction

Monoclonal antibody (mAb) based therapeutics have been well established in the effective treatment of various diseases due to their high efficacy and specificity. With the up coming patent expiration of several commercially available mAb-based drugs in the next few years, growing efforts are being devoted to developing biosimilars as less expensive alternatives of innovator mAbs. To reduce the need for and size of expensive clinical trials and need to expedite the drug commercialization process, manufacturers must demonstrate high similarity in analytical properties of the biosimilar and its reference product through comprehensive analyses.<sup>1</sup> Due to the impact on drug efficacy and safety, glycosylation is one of the critical quality attributes of mAb based therapeutics that needs to be well characterized for similarity assessment and quality assurance of biosimilars.<sup>2</sup> With the high complexity of glycosylation profile of mAbs, orthogonal technologies such as mass spectrometry (MS) are commonly used to complement conventional LC-fluorescence (FLR) based methods for increased specificity. However, high resolution MS often requires experienced scientists for instrument operation, data processing and interpretation, which can be time and resource consuming. To this end, scalable technologies and methods that can add confidence and are easy to deploy for comprehensive glycan analysis in biosimilar development are highly desirable.

The SmartMS-enabled BioAccord System is an easy-to-use LC-MS platform solution that was purposefully designed for comprehensive analysis of biotherapeutic drug products with built-in analytical workflows for specific analysis such as N-Glycan identification and profiling. Within the BioAccord System, highly robust chromatographic separation and accurate mass information can be obtained using the ACQUITY UPLC I-Class PLUS System and the ACQUITY RDa Detector controlled by UNIFI Scientific Information Software (Figure 1A). The ACQUITY RDa Detector is a compact bench top time-of-flight mass detector with built-in self-calibration function and straightforward instrument method setup (Figure 1B and 1C), which substantially reduces the

complexity of operating MS instruments. Together, high quality LC-FLR and LC-MS data can be acquired and transformed into meaningful results for released glycan analysis using an all-encompassing workflow, reducing the cost and time for biosimilar development without compromising product quality.

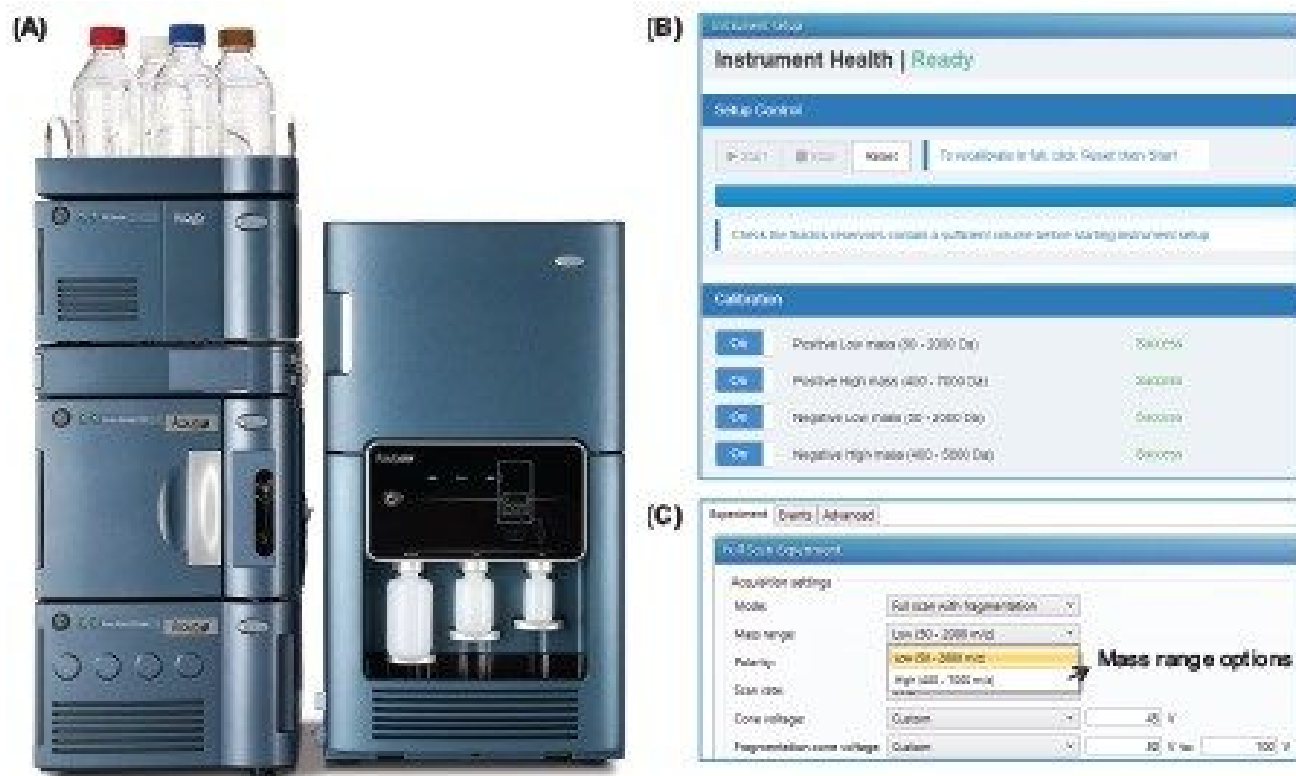


Figure 1. The BioAccord System. (A) Integrated instrument configuration for streamlined released glycan analysis. (B) ACQUITY RDa Detector setup page showing SmartMS™-enabled auto-calibration function for efficient instrument operation. (C) Instrument method page for MS data acquisition. For released glycan analysis, the low mass range option (50–2000 m/z) was selected.

The objective of this work is to demonstrate that the BioAccord System can increase confidence and productivity in released glycan analysis for the development of biosimilars. As an example of assessing the similarity of glycosylation, released glycans from innovator and biosimilar infliximab were analyzed using the BioAccord System.

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## Experimental

### Chemical and reagents

Innovator and biosimilar infliximab samples were donated by external collaborators. LC-MS grade water and acetonitrile were purchased from Honeywell and used as received. Concentrated ammonium formate (p/n 186007081) was used as the additive to prepare mobile phase.

### Sample preparation

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)<sup>3</sup> via an Andrew Alliance automated sample preparation platform.<sup>4</sup> An amount of 2.5 pmol released glycan sample was injected for each analysis.

### LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS
Detectors:	ACQUITY FLR Detector, $\lambda_{ex}=265$ nm, $\lambda_{em}=425$ nm, ACQUITY RDa MS Detector
LC column:	ACQUITY Glycan BEH Amide, 1.7 µm, 130 Å, 2.1 × 150 mm, (p/n 186004742)
Column temp.:	60 °C
Sample vial:	12 × 32 mm glass vial Total Recovery, (p/n 600000750cv)
Mobile phase A:	Water with 50 mM Ammonium formate, pH = 4.4

Mobile phase B: Acetonitrile

### Gradient table:

Time (min)	Flow rate (mL/min)	%A	%B
Initial	0.4	25	75
35	0.4	46	54
36.5	0.2	80	0
39.5	0.2	80	0
43.1	0.2	25	75
47.6	0.4	25	75
55	0.4	25	75

### ACQUITY RDa Detector settings

Mass range:	50–2000 <i>m/z</i>
Mode:	ESI+
Collection mode:	Continuum
Sample rate:	2 Hz
Cone voltage:	45 V for full scan 80–100 V for fragmentation

Desolvation temp.:	300 °C
Capillary voltage:	1.5 kV
Lock mass:	Leu-enkephalin at 50 fmol/μL in 50/50 water/acetonitrile with 0.1% formic acid
Informatics:	Glycan Application Solution within UNIFI 1.9.4

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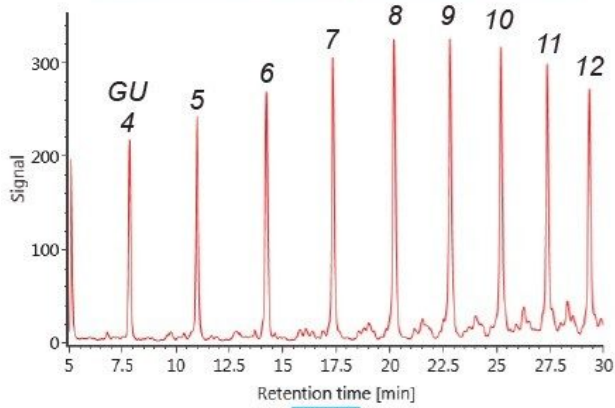
## Results and Discussion

As an integrated solution, the established glycan analysis workflow supported by the BioAccord System can be used to streamline the identification and comparison of glycosylation on biosimilars.<sup>5</sup> To demonstrate this workflow, *RapiFluor*-MS labeled N-linked glycans released from innovator and biosimilar infliximab were analyzed via HILIC separation followed by inline FLR and MS detection using the BioAccord System. With the self-calibration and self-tuning function of the RDa mass detector, high quality MS data were obtained in an efficient manner. The “Glycan FLR with MS confirmation” workflow within UNIFI was used for data interpretation, allowing automated data processing using the retention time and accurate mass information of separated glycans.<sup>6</sup> As shown in Figure 2, 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 Glycan Scientific Library search within the UNIFI software for peak identification. With the use of GU values for library search, high confidence of peak assignment is ensured by minimizing the potential variation from retention times across analyses.

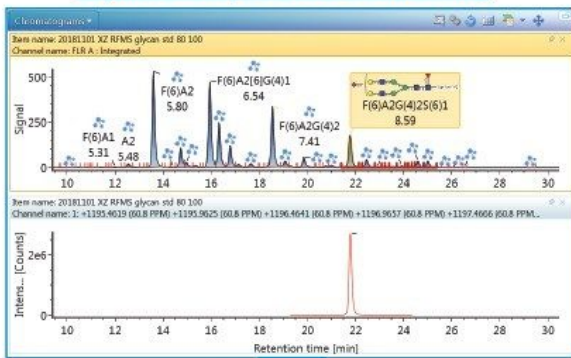
**HILIC-FLR-MS**  
**RT and Accurate Mass**



**RT Calibration Using Dextran Ladder**



**Glycan Sample Data Processing**



**Library Search Results**

Component name	Structure	Expected GU	Δ GU	GU Std Dev	Expected m/z	Δ m/z	Mass Confirmed
F(6)A1A2(5)G(4)S(6)1		8.5800	0.0068	0.0000	734.9525	-75.0063	<input type="checkbox"/>
A2G(4)2S(6)1		8.5700	0.0168	0.0140	1267.9861	33.0218	<input type="checkbox"/>
F(6)A2G(4)2S(6)1		8.5500	0.0368	0.0000	1196.4673	0.0054	<input checked="" type="checkbox"/>
A2G(4)2S(6)1		8.5500	0.0368	0.0000	1223.0781	0.0045	<input checked="" type="checkbox"/>
A2G(4)2S(6)1		8.6000	0.1032	0.0000	694.6504	-115.3484	<input type="checkbox"/>

Figure 2. The integrated "Glycan FLR with MS Confirmation" workflow for

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*automated released glycan analysis within the UNIFI Informatics platform.*

Figure 3 shows the processed FLR trace of innovator infliximab with each peak automatically assigned and annotated with its glycan name and associated structural information. As a result of the automated data processing, a total of 26 N-linked glycans were identified for both innovator infliximab samples and 27 for the biosimilar infliximab within a 10-ppm mass tolerance, demonstrating the high sensitivity and accuracy afforded by the ACQUITY RDa Detector. For the glycans identified, the similarities of glycan profiles between the innovator and biosimilar mAbs can be automatically assessed within the workflow.

During the development of biosimilar products, critical glycan structures often rank as high-risk quality attributes and need equivalence testing (Tier 1) for analytical similarity assessment according to the Three-tier approach recommended by FDA.<sup>7</sup> To this end, a straightforward approach to quantitatively compare glycan profiles is of particular importance to facilitate the biosimilar mAb development. Within the BioAccord System workflow, a direct comparison can be made in both qualitative and quantitative mode. Figure 4A shows the overlaid FLR chromatograms for the two innovator infliximab samples (black and red trace) and one biosimilar sample (blue trace), suggesting high similarity of glycan profiles between the two innovator mAb samples. Meanwhile, differences in relative abundance were observed for multiple sialylated glycan species between innovator and biosimilar mAb, as shown in the zoom-in chromatogram in Figure 4A (the inset figure). To confirm the differences, a Summary Plot can be accessed from the clickable workflow steps (Figure 4B) to quantitatively compare the FLR response or relative abundance of selected glycan species. As shown on the right side of Figure 4B, the Summary Plot for %Amount confirmed a consistent relative abundance of FA2G1Sg1 (F= Fucose, A2= biantennary, G=Galactose, Sg= NeuGc) in the two samples of the innovator mAb, and an elevated abundance in the biosimilar mAb. Collectively, the above results demonstrated the BioAccord System's ability to efficiently transform LC-MS data into meaningful results for biosimilarity assessment.



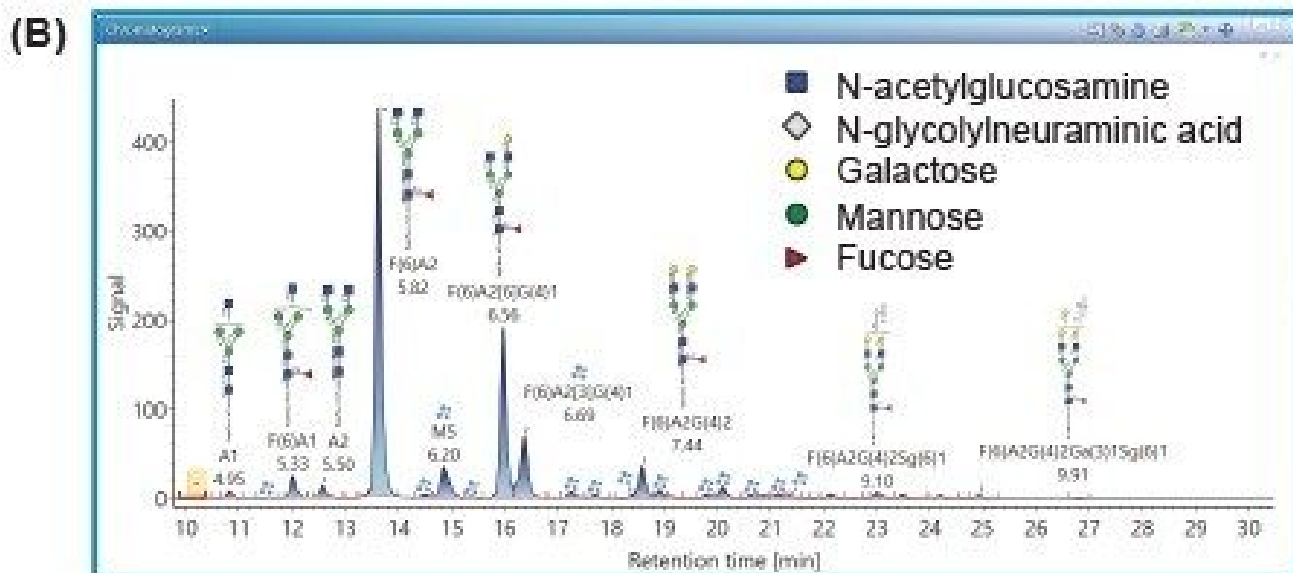
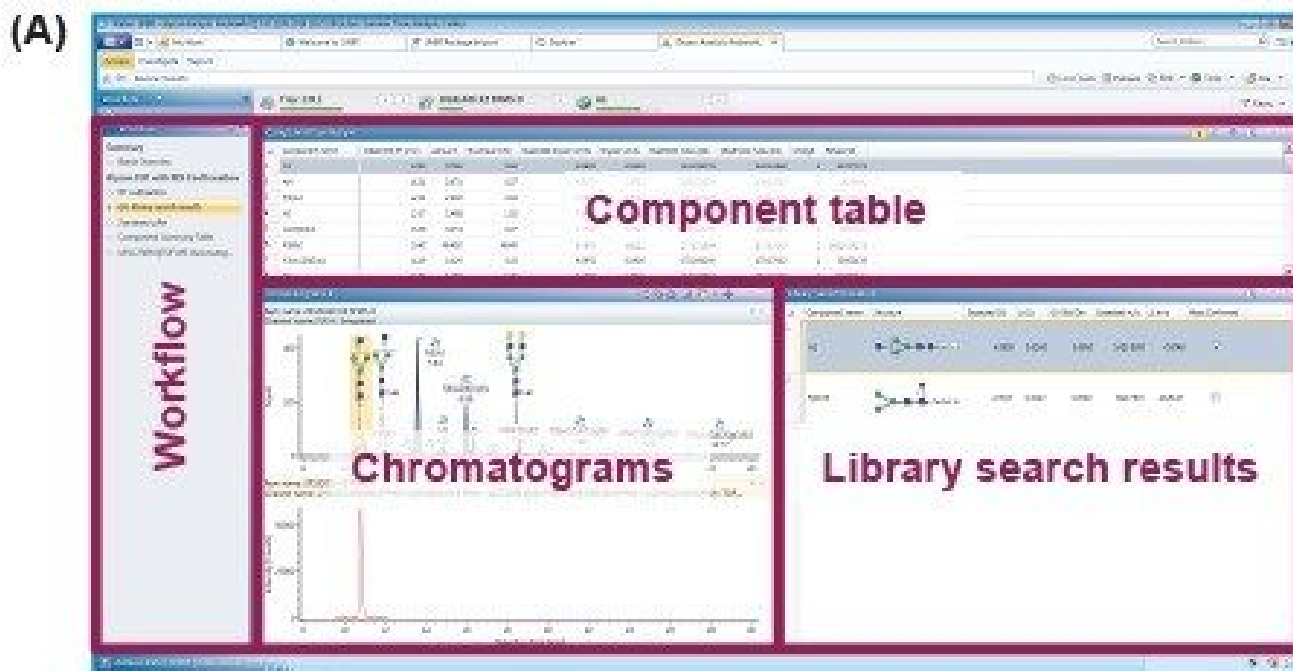


Figure 3. Review of the processed results. (A) The Review tab showing the clickable workflow steps, processed chromatograms, and library search results for identified peaks. (B) Processed FLR trace from the Review tab showing identified peaks annotated with glycan name, GU value, and associated structure information. To avoid over crowded structure display, detailed glycan structures can be viewed by simply mousing over the IBM connection icon. User has the option to choose between CFG or Oxford structure nomenclature to display (CFG

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*is used for figures in this Application Note).*

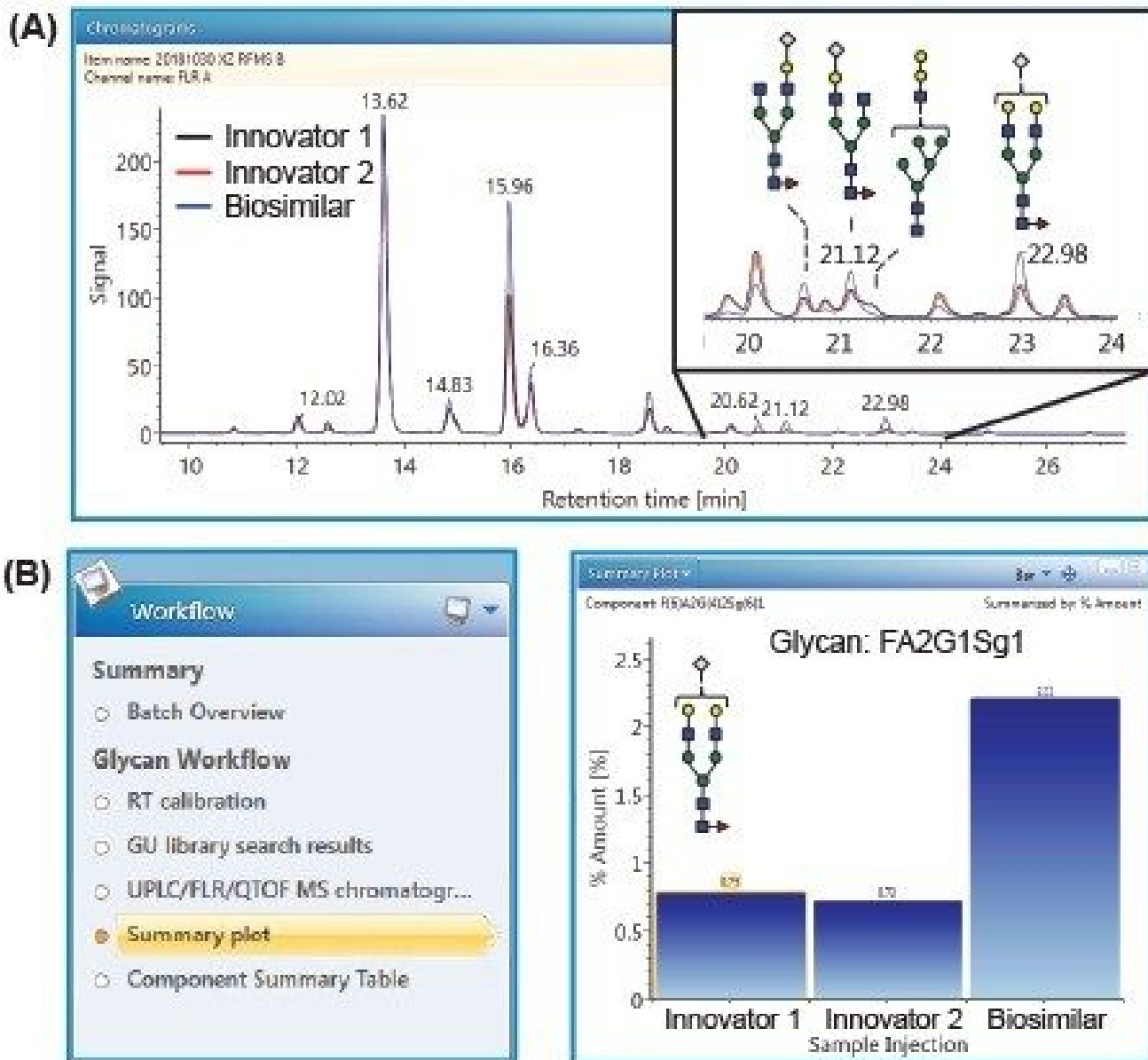


Figure 4. Comparison of glycan profiles between innovator infliximab samples and biosimilar sample. (A) Overlaid FLR chromatograms. Zoom-in chromatogram shows the region of low abundant glycan species. (B) An example of the Summary Plot as part of the clickable workflow steps showing the elevated abundance of a glycan, FA2G1Sg1 (Sg stands for *n*-glycolylneuraminic acid), in the biosimilar mAb.

When performing released glycan analysis of biosimilar mAbs, it is quite common to come across glycans with challenging MS spectra for correct assignment, such as isobaric glycans, which might lead to inconsistency in

glycan analysis results. High collisional energy fragmentation is the common MS based method to help differentiating isobaric glycans with similar structures. The ACQUITY RDa Detector can acquire low collisional energy and high collisional energy MS fragmentation spectra in an alternative scan mode without pre-selecting the precursor ions, which provides additional diagnostic fragment ions to improve the confidence in structure assignment. (Note: The low energy MS channel is used for MW confirmation using the Scientific Glycan GU library by default). Figure 5 shows an example of how MS fragmentation data can be used to aid in the identification of a critical pair of glycans. As shown in Figure 5A, glycan FA2G2 eluted very close to its immunogenic isomer, FA2G1Ga1 (G1Ga1 = Gal- $\alpha$ (1,3)-Gal) resulting in highly similar GU values. The simultaneously acquired MS fragmentation data offers an opportunity to structurally differentiate the isobaric glycans. As shown in Figure 5B, high quality MS full scan and fragmentation data were obtained for both peaks, where a diagnostic ion ( $m/z$  528) for  $\alpha$ -1,3 Galactose was observed as the most dominant fragment ion in the MS fragmentation spectra of the shoulder peak confirming its identity as FA2G1Ga1.<sup>3</sup> To this end, the BioAccord System offers the flexibility to interrogate results to improve the robustness and confidence for released glycan analysis in biosimilar development.

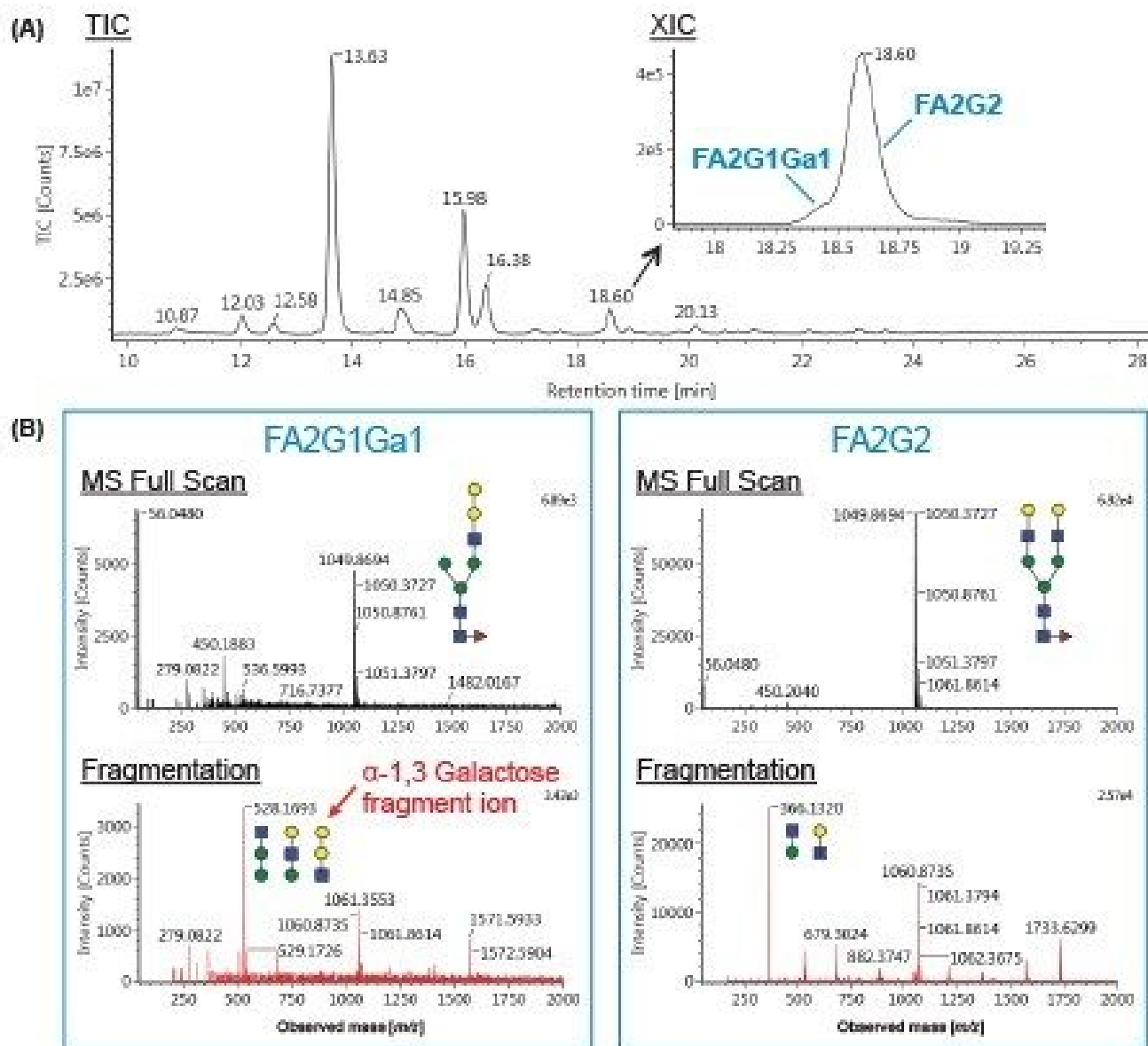


Figure 5. Additional details under Investigate tab for manual data interrogation. (A) MS (TIC) chromatogram of released glycans from innovator infliximab. Zoom-in chromatogram shows the XIC of the two isobaric glycans, FA2G1Ga1 and FA2G2. (B) MS full scan and fragmentation data of FA2G1Ga1 and FA2G2, showing a diagnostic ion ( $m/z$  528) of  $\alpha$ -1,3 Galactose.

To further accelerate the glycan analysis, UNIFI Software can automatically generate analysis reports to simplify the review, sharing, and filing of data. To demonstrate the reporting function of UNIFI Software, a report was

created using a customizable template to summarize the glycan analysis results. As shown in Figure 6, the report was formatted to include Analysis Information, Chromatograms, and Summary Tables, showing the direct comparison for relative abundance of identified N-glycans across samples. The comparison based on different groups of glycans was also reported. To meet requirements in different laboratories, the report can be customized to include other desired information for simplified review of analysis results.

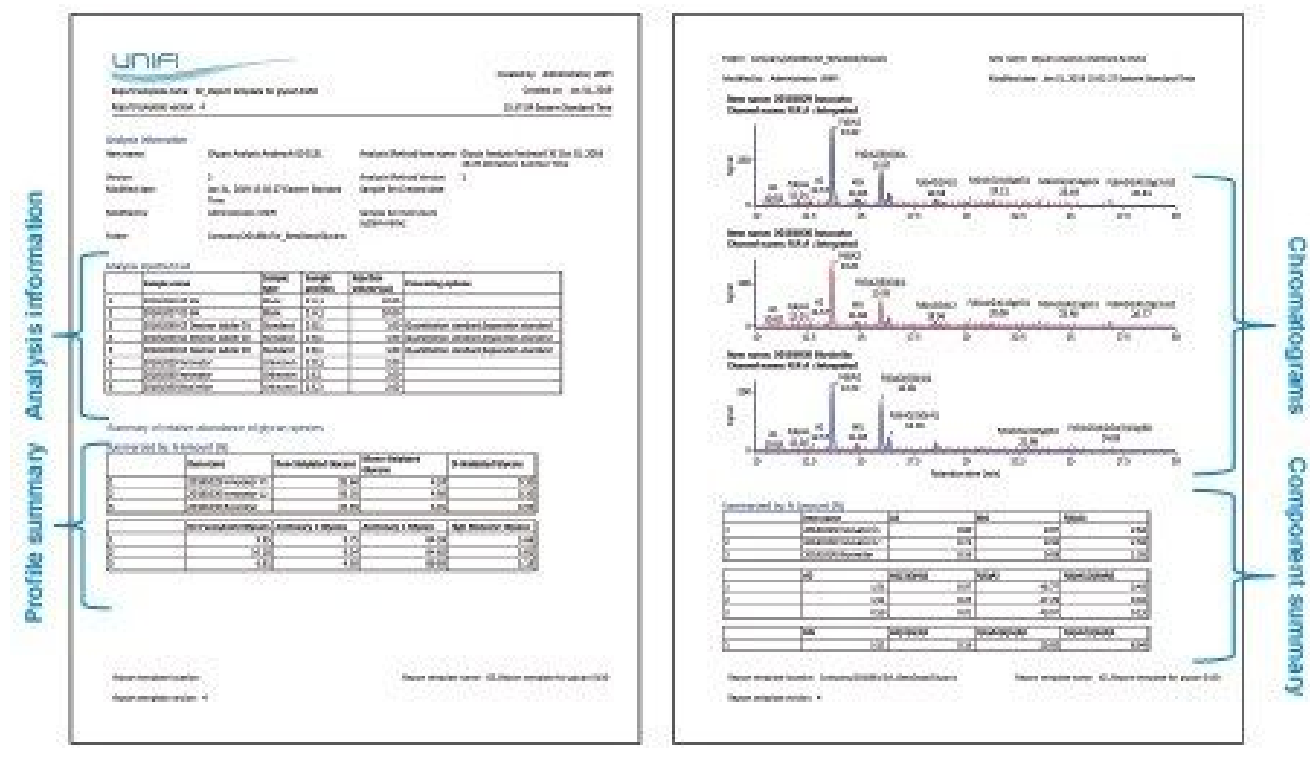


Figure 6. Report of analysis results (selected pages). The customizable report shows Sample List, Chromatograms, and Summary Table for easily reviewing the differences across samples.

## Conclusion

Through this work, an efficient workflow has been demonstrated for comprehensive analysis of fluorescent labeled released glycans using the BioAccord System. Within the integrated workflow, highly robust separation and accurate mass measurement of N-linked glycans were obtained and transformed automatically to analysis

results, allowing for fast determination of differences in identity and abundance of glycans. In summary, the BioAccord System simplifies the workflow of biosimilarity assessment and can be used to improve the productivity and confidence of released glycan analysis in biosimilar mAb development.

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## References

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