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Application Note

LC-MS Optimization of a 10-Minute Released Glycan Method Using BioAccord™ HRMS

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Published on January 27, 2026

Abstract

A 10 minute chromatographic method and its optimization for the released glycan analysis using hydrophilic interaction liquid chromatography (HILIC) and BioAccord LC-MS System detection are described. This work was carried out to produce a fully optimized, fast mass spectrometry (MS)-only released glycan method suitable for routine (daily) monitoring of glycan profiles in bioreactor samples. LC gradient and MS source parameters were investigated to yield optimal and rapid detection, especially for mannose and sialic acid species. Optimization revealed that the desolvation temperature had the highest effect on signal intensity, cone voltage was mostly responsible for in-source decomposition, and the influence of capillary voltage had negligible effects.

Benefits

- Rapid 10 minute method for the released glycan analysis, with >80% analysis time reduction as compared to the traditional 55-minute method
- Optimized LC gradient conditions

- Optimized MS conditions
- Fast monitoring method based on RFMS labeled glycans

Introduction

Glycosylation refers to the enzymatic attachment of sugar molecules (glycans) to proteins as a post-translational modification (PTM) during recombinant monoclonal antibody (mAb) production. Glycosylation affects the structure, stability, and biological activity of the antibody. It is one of the critical quality attributes (CQAs) in the manufacturing process. Released glycan analysis based on GlycoWorks™ Sample Preparation Kits has been the gold standard method in which glycans are enzymatically cleaved from the antibody, labeled with a RapiFluor-MS™ (RFMS) Reagent, and analyzed by HILIC with fluorescence or MS detection (Figure 1).[1,2] A 55-minute chromatographic separation coupled with fluorescence detection has been widely used.[3] It provides excellent baseline separation of glycans and is recommended for detailed analysis and characterization. For daily (or more frequent) sampling of parallel bioreactors, especially in development, long analytical run times are undesirable. In this case, moving to an MS-only method may be appropriate to rapidly identify changes, especially the presence of undesirable glycans, such as mannose species. Several MS-based methods with shortened run times have been published to enable faster turnaround.[4] This application note describes a 10 minute chromatographic method developed on the BioAccord LC-MS Platform and specifically optimized and tuned for observing critical glycan species while maintaining fast overall run times. Methods have been thoroughly optimized and are detailed in the following sections.

Figure 1. Flow chart of sample preparation for released glycan sample preparation (figure adapted from reference 2).

Experimental

Sample Preparation

Two glycan standard samples from Waters were used: RapiFluor-MS Glycan Performance Test Standard (p/n: 186007983 <https://www.waters.com/nextgen/global/shop/standards--reagents/186007983-rapifluor-ms-glycan-performance-test-standard.html>); and RapiFluor-MS High Mannose Standard (p/n: 186008317 < https://www.waters.com/nextgen/global/shop/standards--reagents/186008317-rapifluor-ms-high-mannose-test-standard.html>). For each sample, 100 µL of H_2O was added to the sample vial prior to the analysis.

LC-MS Method Conditions

Gradient Table

Results and Discussion

I. Description of the Rapid 10 Minute Method for Released Glycan Analysis

The chromatographic separation method is described for the analysis of released glycan using the BioAccord HRMS System. Glycan identification was based on accurate mass and retention time matching, providing confident putative assignments suitable for routine monitoring. The method is intended for high-throughput released glycan profiling and routine monitoring rather than detailed isomeric or structural elucidation. The extracted ion chromatogram of glycan and high-mannose standard solutions are shown in Figure 2. For isomeric glycans G1 (peak no. 4) and G1F (peak no. 5), the results are reported as merged peaks. Figure 2 shows that the majority of glycans are detected as a combination of doubly charged species, $[M+2H]^{2+}$ and/or $[M+NH_4+H]^{2+}$. A UNIFI™ Application library was created for these glycan standards, including retention time, neutral mass, observed charge states, and structural information. Library and methods for use with the system are available upon request. A tabulation of the library entries of these standards is summarized in Table 1. Details of LC method development and source optimization are described in the following sections.

Figure 2. (A) Extracted ion chromatogram of RapiFluor-MS Glycan Performance Test Standard (p/n: 186007983). (B) Extracted ion chromatogram of RapiFluor-MS High Mannose Standard (p/n: 186008317).

IgG glycan short name	Oxford notation name	Peak No.	Formula (glycan + RFMS label)	Monoisotopic Neutral mass (Da)	+2H	+3H	+NH4+H	Expected RT (min)
G0	A2	1	C50H84N4O36 + C17H21N5O	1627.66059	814.8378	543.5615	823.3517	3.08
G0F	FA2	2	C56H94N4O40 + C17H21N5O	1773.7185	887.8668	592.2475	896.3806	3.38
G0F+GN	FA2B	3	C64H107N5O45 + C17H21N5O	1976.79787	989.4065	659.9406	997.9203	3.69
G1	A2G1	4	C56H94N4O41 + C17H21N5O	1789.71341	895.8643	597.5791	904.3781	3.80
G1F	FA2G1	5	C62H104N4O45 + C17H21N5O	1935.77132	968.8932	646.2651	977.4070	4.10
G1F+GN	FA2BG1	6	C70H117N5O50 + C17H21N5O	2138.85069	1070.4329	713.9582	1078.9467	4.30
G1F+SA	FA2G1S1	10	C73H121N5O53 + C17H21N5O	2226.86674	1114.4409	743.2969	1122.9547	5.10
G2	A2G2	7	C62H104N4O46 + C17H21N5O	1951.76623	976.8907	651.5967	985.4045	4.54
G2+1SA	A2G2S1	11	C73H121N5O54 + C17H21N5O	2242.8617	1122.4381	748.6285	1130.9522	5.46
G2+2SA	A2G2S2	14	C84H138N6O62 + C17H21N5O	2533.95707	1267.9861	845.6603	1276.4999	6.32
G2F	FA2G2	8	C68H114N4O50 + C17H21N5O	2097.82414	1049.9196	700.2827	1058.4334	4.80
G2F+GN	FA2BG2	9	C76H127N5O55 + C17H21N5O	2300.90352	1151.4593	767.9758	1159.9731	4.92
G2F+SA	FA2G2S1	12	C79H131N5O58 + C17H21N5O	2388.91956	1195.4673	797.3145	1203.9811	5.71
G2F+2SA	FA2G2S2	15	C90H148N6O66 + C17H21N5O	2680.01498	1341.0148	894.3463	1349.5288	6.53
G2F+GN+SA	FA2BG2S1	13	C87H144O63N6 + C17H21N5O	2591.99893	1297.0070	865.0076	1305.5208	5.86
G2F+GN+2SA	FA2BG2S2	16	C98H161N7O71 + C17H21N5O	2883.09435	1442.5552	962.0389	1451.0685	6.62
Man5	M5	17	C46H78N2O36 + C17H21N5O	1545.60749	773.8102	516.2105	782.3251	3.70
Man6	M6	18	C52H88N2O41 + C17H21N5O	1707.66031	854.844	570.2281	863.3515	4.46
Man7	M7	19	C58H98N2O46 + C17H21N5O	1869.71314	935.8646	624.2457	944.3779	5.18
Man8	M8	20	C64H108N2O51 + C17H21N5O	2031.76596	1016.8911	678.2633	1025.4043	5.82
Man9	M9	21	C70H118N2O56 + C17H21N5O	2193.81878	1097.9175	732.2809	1106.4307	6.30

Table 1. Glycan identification and their observed MS spectrum from RFMS glycan standard sample.

II. Optimization of LC Gradient Separation

The method uses a Premier Glycan BEH Amide 130 Å 1.7 µm 2.1 x 50 mm HILIC Column. Earlier work (not shown) showed a starting gradient of 25%B was optimal. Various end gradient conditions from 42%B (faster) to 36%B (slower) were evaluated for overall quality of separation, peak shape, and glycan retention. Using initial gradient conditions of 25-42% B between 0-7 minutes, glycans eluted between 2-6 minutes. The gradient was then systematically optimized as shown in Figure 3 to ensure the last eluting glycan (marked with arrow) has proper retention. The gradient of 25-40% B between 0 and 7 minutes was selected, providing enhanced peak resolution for reliable glycan identification.

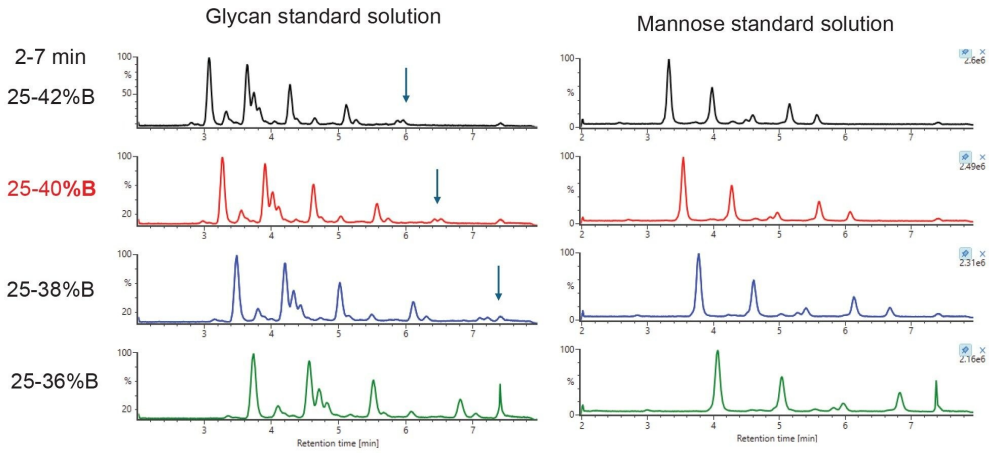

Figure 3. LC gradient optimization results. Peaks labeled with an arrow represent the last eluting glycan in corresponding standard solution.

III. Optimization of MS Source Conditions

MS source conditions— desolvation temperature, cone voltage, and capillary voltage— are optimized using the glycan and mannose released glycan standard solutions. These parameters were tested using a full factorial design, varying one parameter at a time. The range of these source parameters tested are summarized in Table 2. Data were acquired in the UNIFI Application for the samples and conditions as shown in Table 3. Three source parameters are entered as promotable parameters. This allows efficient evaluation of multiple parameters without the need to create a new acquisition method for each condition.

Table 2. Range of source parameters used in the present opmization experiment. The upper value of desolvation temperature (550 °C) and capillary voltage (1.5 kV) are instrument upper limit.

Item name	Sample Type	Sample Position	Run time (min)	Injection volume (µL)	Capillary voltage (kV)	Cone voltage (V)	Desolvation temperature (°C)	Replicates
blank	Unknown	1:F,1	10	1	1.3	40	300	1
Glycan Std Desolv temp 300	Unknown	1:F,2	10	1	1.3	40	300	2
blank	Unknown	1:F,1	10	1	1.3	40	350	1
Glycan Std Desolv temp 350	Unknown	1:F,2	10	1	1.3	40	350	2
blank	Unknown	1:F,1	10	1	1.3	40	400	1
Glycan Std Desolv temp 400	Unknown	1:F,2	10	1	1.3	40	400	2
blank	Unknown	1:F,1	10	1	1.3	40	450	1
Glycan Std Desolv temp 450	Unknown	1:F,2	10	1	1.3	40	450	2
blank	Unknown	1:F,1	10	1	1.3	40	500	1
Glycan Std Desolv temp 500	Unknown	1:F,2	10	1	1.3	40	500	2
blank	Unknown	1:F,1	10	1	1.3	40	550	1
Glycan Std Desolv temp 550	Unknown	1:F,2	10	1	1.3	40	550	2

Table 3. Sample run list for the MS glycan optimization. Desolvation temperature, cone voltage, and capillary voltage were set as promotable parameters.

Results of the optimization are summarized and overlaid in Figure 4 for glycan standard and Figure 5 for mannose standard solution, where normalized values are plotted against each of the source parameters. The data show that desolvation temperature has the greatest effect on MS response; increasing temperature from 350 °C to 550 °C resulted in greater than 150% signal enhancement. Cone voltage resulted in in-source fragmentation and differing adduct cluster formation. As cone voltage increases, there is compound-dependent signal loss consistent with increased in-source fragmentation. Simple glycans such as Man 5 and G0 showed the biggest drop in signal. More complex glycans such as G2F+2SA and Man 9 are typically more resistant to fragmentation at higher cone voltages. In addition, increasing cone voltage led to compound-dependent ratio changes between +NH4+H and +2H adducts. An example of three glycans are shown in Figure 6. Lastly, the data showed that capillary voltage has much less effect on the observed response. Increasing capillary voltage only resulted in a slight decrease in responses. Overall, desolvation temperature of 550 °C, cone voltage of 35 V, and capillary voltage of 1.2 kV were selected as the optimized parameter for this assay.

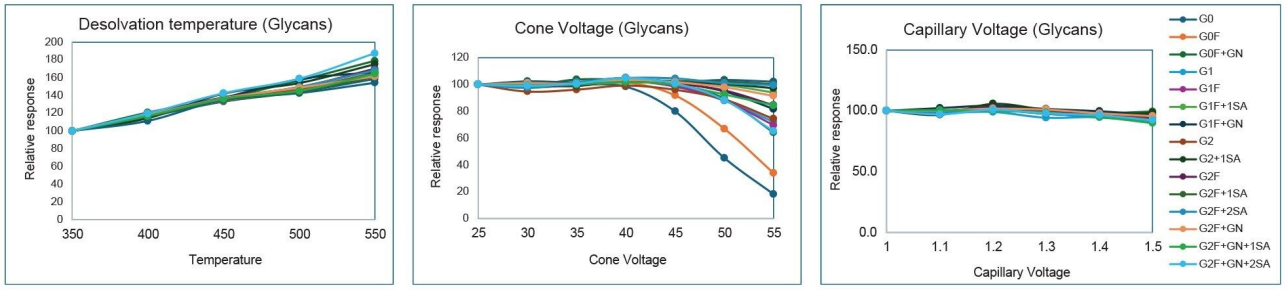

Figure 4. Normalized overlaid plot of relative response vs. each of the source parameters of glycan standard solution.

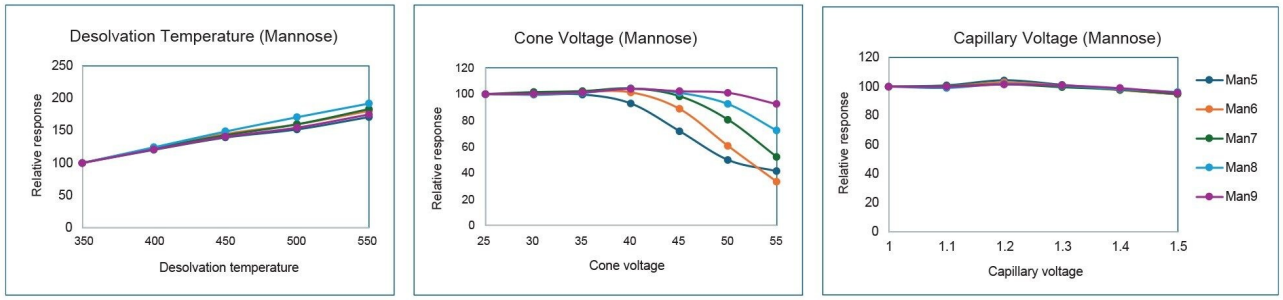

Figure 5. Normalized overlaid plot of relative response vs. each of the source parameters of mannose standard solution.

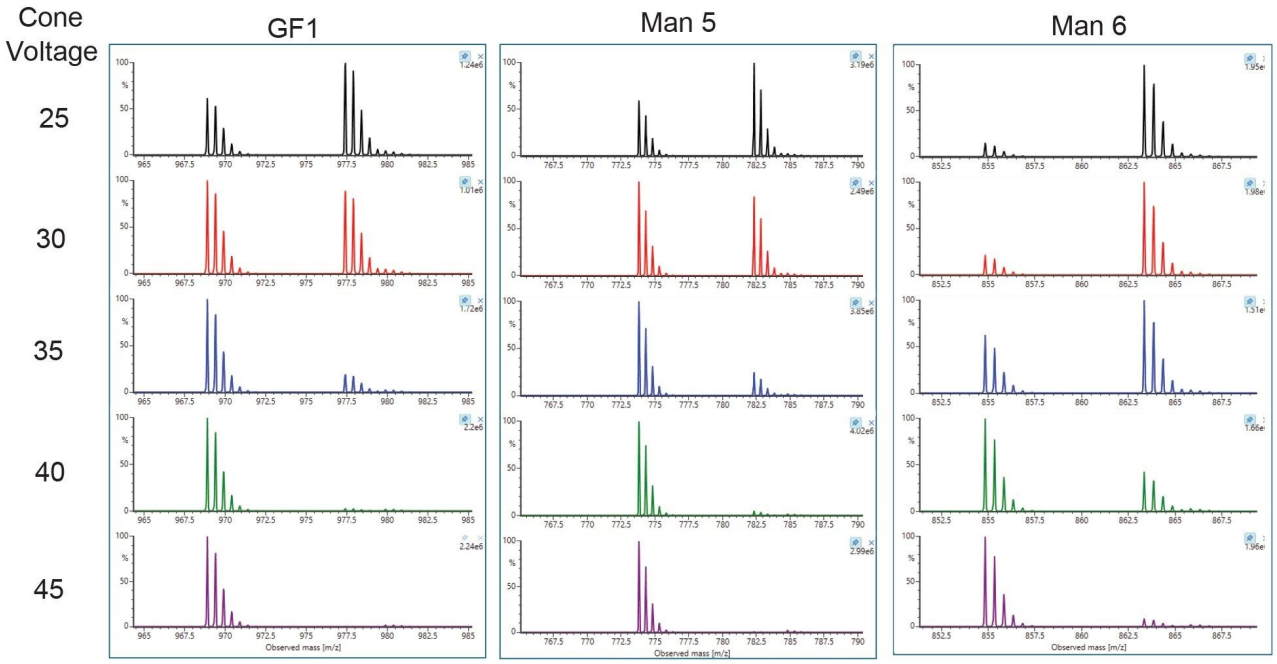

Figure 6. MS adduct at various cone voltages for representative glycans. The charge clusters on the left represent +2H adduct. The cluster on the right represents +H+NH4 adduct.

Conclusion

A 10 minute rapid released glycan analysis is described, with highlights of the method summarized as follows:

1. The 10 minute run time represents an 80% reduction in analysis time compared to the traditional 55 minute run time.
2. LC chromatographic separation was optimized to maximize separation while maintaining a total analysis time of under 10 minutes.
3. Careful optimization yielded significant signal enhancement for the BioAccord System compared with previously reported methods.

References

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720009203, January 2026