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Note d'application

Trastuzumab Glycan Batch-to-Batch Profiling using a UPLC-FLR/Mass Spectrometry Platform

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Abstract

This Application note demonstrates UPLC-FLR/MS system as a robust tool for separation and analysis of minor glycoforms or isomers that are otherwise difficult to assign.

Benefits

Used together, UPLC with detection by FLR and time-of-flight MS comprise a powerful solution for producing required analytical data for batch-to-batch glycan profiling of a recombinant mAb, Trastuzumab. The chromatographic resolution, reproducibility, and mass spectrometry sensitivity enable glycoprofiling of therapeutic antibodies mandated by regulatory agencies. This UPLC-FLR/MS system represents a robust tool for separation and analysis of minor glycoforms or isomers that are otherwise difficult to assign.

Introduction

Glycosylation plays a vital role in the safety and efficacy of many therapeutic proteins such as recombinant monoclonal antibody (rmAb). Glycosylation of rmAb occurs at the Fc region on the heavy chain (Figure 1). Several studies have shown the correlation between glycosylation variations caused by cell-line selection and changes in culture-medium parameters.[1] These variations can have a profound effect on the biological activities of the rmAb drugs, which leads to changes in drug potency in the final product. Regulatory agencies require monitoring of batch-to-batch rmAb drug-production quality, and mandate detailed assessment of the protein glycosylation's micro-heterogeneity and consistency.

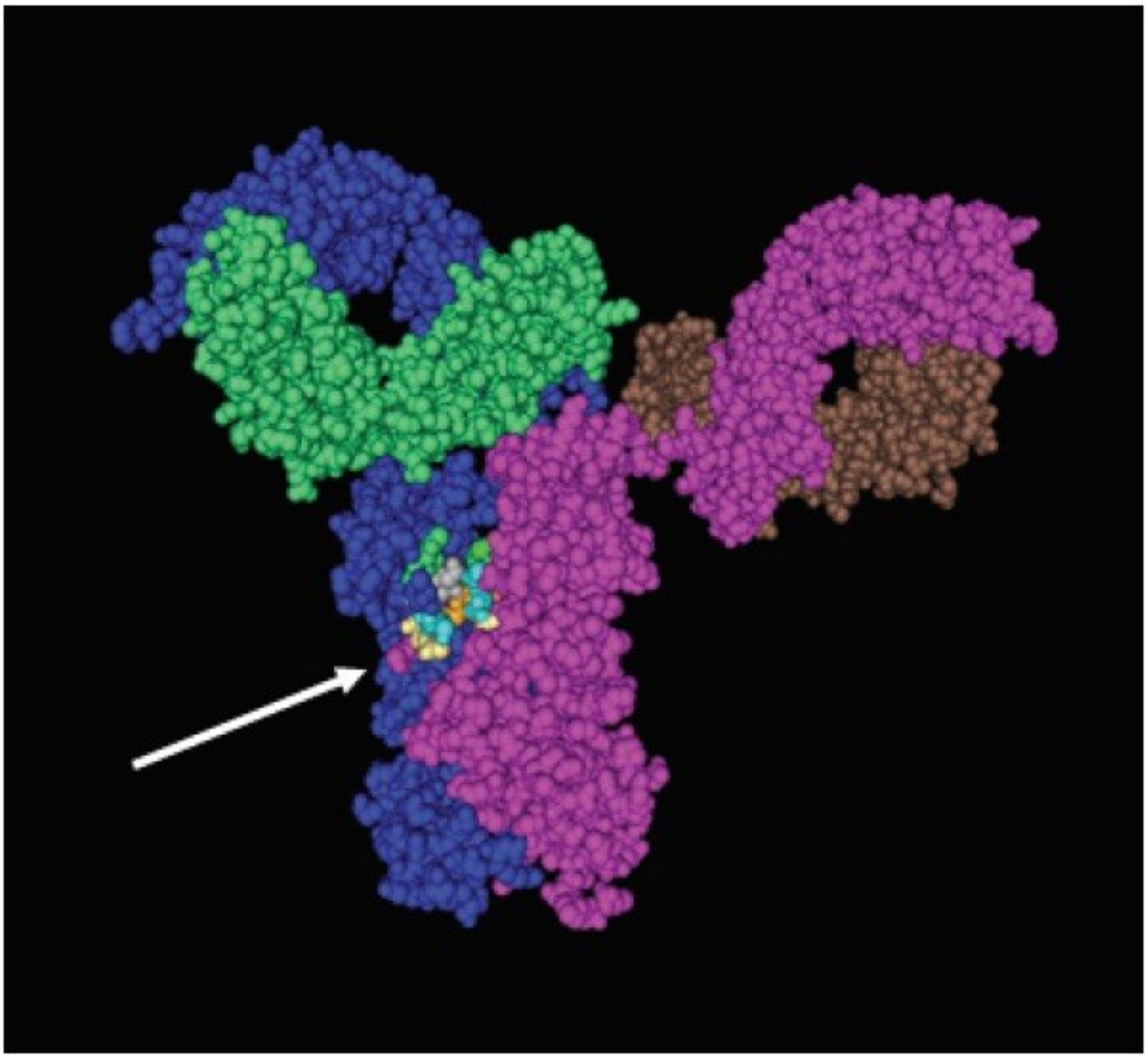

Figure 1. A crystal structure of Trastuzumab. The arrow points to the oligosaccharides that are located in the Fc region of the heavy chain.

In this study, we applied a robust, sensitive, and reproducible analytical platform that comprises a UltraPerformance LC (UPLC), a fluorescence (FLR) detector, and a XevoTM QTof Mass Spectrometer (MS) for batch-to-batch glycan profiling of an rmAb, Trastuzumab.

Trastuzumab is a therapeutic rmAb (IgG1 subclass) that is widely used for breast cancer treatment. N-linked glycans were released from three batches of Trastuzumab enzymatically, and labeled with a fluorescent tag, 2-aminobenzamide (2-AB). An ACQUITY UPLC HILIC Column was used to separate the released and labeled glycans; the LC was interfaced with the Xevo QTof MS via electrospray ionization. Peak areas from the FLR detector were utilized for glycan quantitation; MS was used for peak assignment using an accurate molecular weight of corresponding glycans.

Experimental

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH Glycan Column 2.1 x 150 mm, 1.7 µm
Column temp.:	40 °C
Flow rate:	400 µL/min
Mobile phase A:	100 mM ammonium formate, pH 4.5
Mobile phase B:	Acetonitrile
Gradient:	72% to 62% B in 45 min
Weak wash:	75% acetonitrile
Strong wash:	20% acetonitrile
Injection:	5.0 µL partial loop

FLR Conditions

FLR:	Waters ACQUITY UPLC Fluorescence Detector
Excitation:	330 nm
Emission:	420 nm
Data Rate:	1 pts/s
PMT Gain:	1.00
Time Constant:	Normal

MS Conditions

MS System:	Waters Xevo QTof MS
Ionization Mode:	ESI +
Capillary Voltage:	3200 V
Cone Voltage:	35 V
Desolvation Temp.:	350 °C
Desolvation Gas:	800 L/Hr
Source Temp.:	120 °C
Acquisition Range:	800 to 2000 *m/z*
Collision Energies:	6 V

Lock Mass:	Cesium iodide, CSI, (1 µg/µL in 50% isopropanol)

Method

- Protein solubilization: 20 µL of Trastuzumab (21 µg/µL) was solubilized with 172 µL, 0.1% RapiGest in 50 mM ammonium bicarbonate.
- Reduction/alkylation: 1 µL Dithiothreitol (DTT, 1M) was added to the sample to a final concentration of 5 mM. The sample was heated at 60 °C for 30 min, and cooled down to room temperature; 2 µL iodoacetamide (IAM, 1M) was added to alkylate the free cysteine (final concentration was 10 mM); incubation time was 30 min in the dark at room temperature.
- Deglycosylation: PNGase F (New England Biolab, 50,000 unit/mL, 5 µL) was added to the sample for overnight incubation. The final protein concentration was about 2.1 µg/µL.
- Extraction of released glycans: 50 µL of the deglycosylated protein sample was reconstituted with 450 µL pure acetonitrile prior to HILIC µElution-plate extraction. For details see the extraction protocol.
- FLR labeling using 2-AB: The labeling was performed using a modified protocol.2 50 µL (instead of 5 µL) of a 2-AB/DMSO/acetic acid/sodium cyanoborohydrate mixture of defined composition was used for the labeling.
- 2-AB Glycan extraction: The same protocol was used as for extraction on unlabeled glycans. The eluted glycans were lyophilized and reconstituted in 40 µL of 50% acetonitrile in water prior to UPLC-FLR/MS analysis.

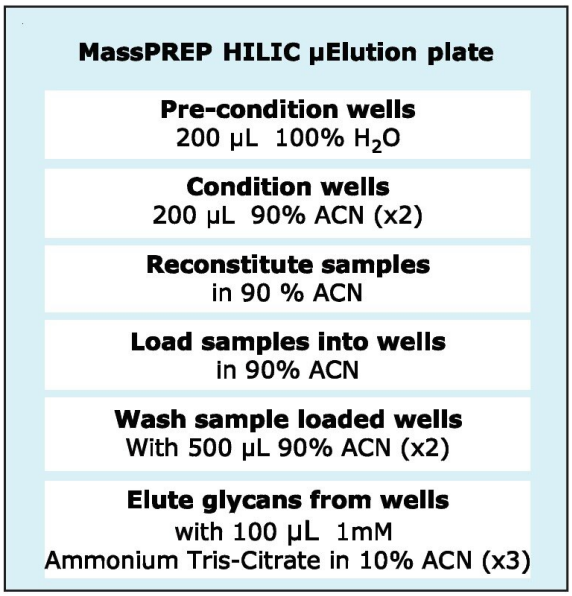

Figure 2. Released Glycan Extraction Protocol. (MassPREP HILIC µElution plate was operated using a vacuum manifold.)

Results and Discussion

UPLC/FLR detection sensitivity

The rmAb N-linked glycans present in the sample are biantennary and high mannose type. They exhibit considerable heterogeneity and wide dynamic range. Identification and quantification of low-abundant glycans requires sensitive fluorescence detectors. About 5 pmol of sample is typically injected on the column in order to detect minor glycans. The limit of detection for FLR was estimated to lie between 1 to 5 femtomoles.

UPLC resolution of glycans

UPLC HILIC separation provides significantly greater resolution compared to conventional HPLC methods.[3] UPLC better resolves isomeric glycans, such as G1 and G1F isomers, makes the data interpretation less ambiguous, and improves quality of quantitation (peak integration).

UPLC/FLR injection-to-injection reproducibility

The main purpose of LC/FLR glycans analysis is its relative quantitation. Injection-to-injection variability of UPLC/FLR system was evaluated as shown in Figure 3. The variation (RSD) in peak areas of three injections of the same sample was less than 2% even for minor peaks.

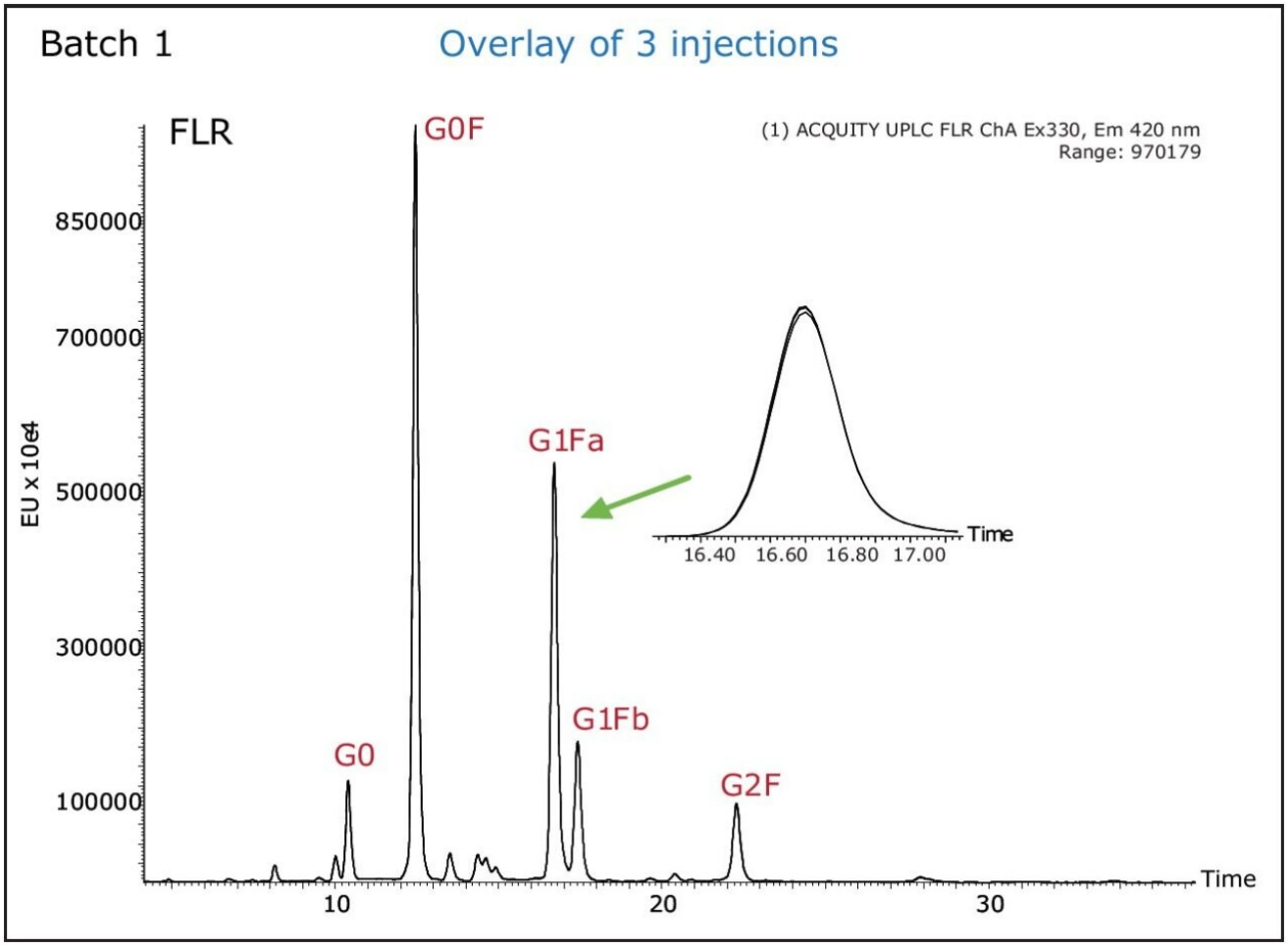

Figure 3. UPLC/FLR chromatograms of triplicate injections of 2-AB labeled glycans released from the same Trastuzumab batch. Overlay of three injections showed perfect alignment of chromatograms. The peak area response variation was less than 2% (RSD). The most abundant glycans were labeled at the peak top.

Glycan mass profiling using Xevo QTof MS

While FLR data are useful for glycan quantitation, MS provides information in addition to chromatographic

retention times. Accurate mass data permit the assignment of glycans present in mAb with high confidence (Figures 4A, B). Proposed structures for the 2-AB labeled Trastuzumab glycans are shown in Figure 5.

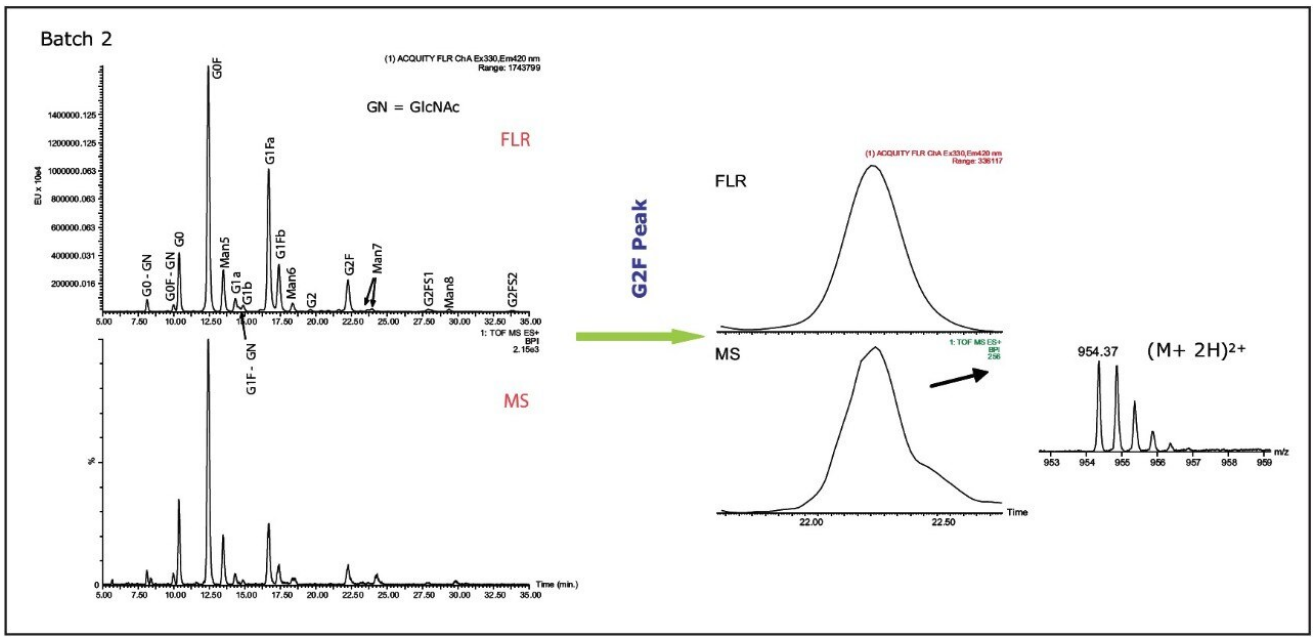

Figure 4A (left). UPLC/FLR/MS analysis of 2-AB labeled glycans from Trastuzumab (Batch 2). The top chromatogram is the FLR chromatogram; the bottom is the MS chromatogram. The glycan identified were confirmed by their accurate mass. Glycan structures are listed in Figure 5.

Figure 4B (right). 2-AB labeled glycan assignment was made by aligning the FLR chromatogram peak with the BPI MS peak. The summed BPI MS scans for G2F peak are shown on the right. The mass error was 20 ppm.

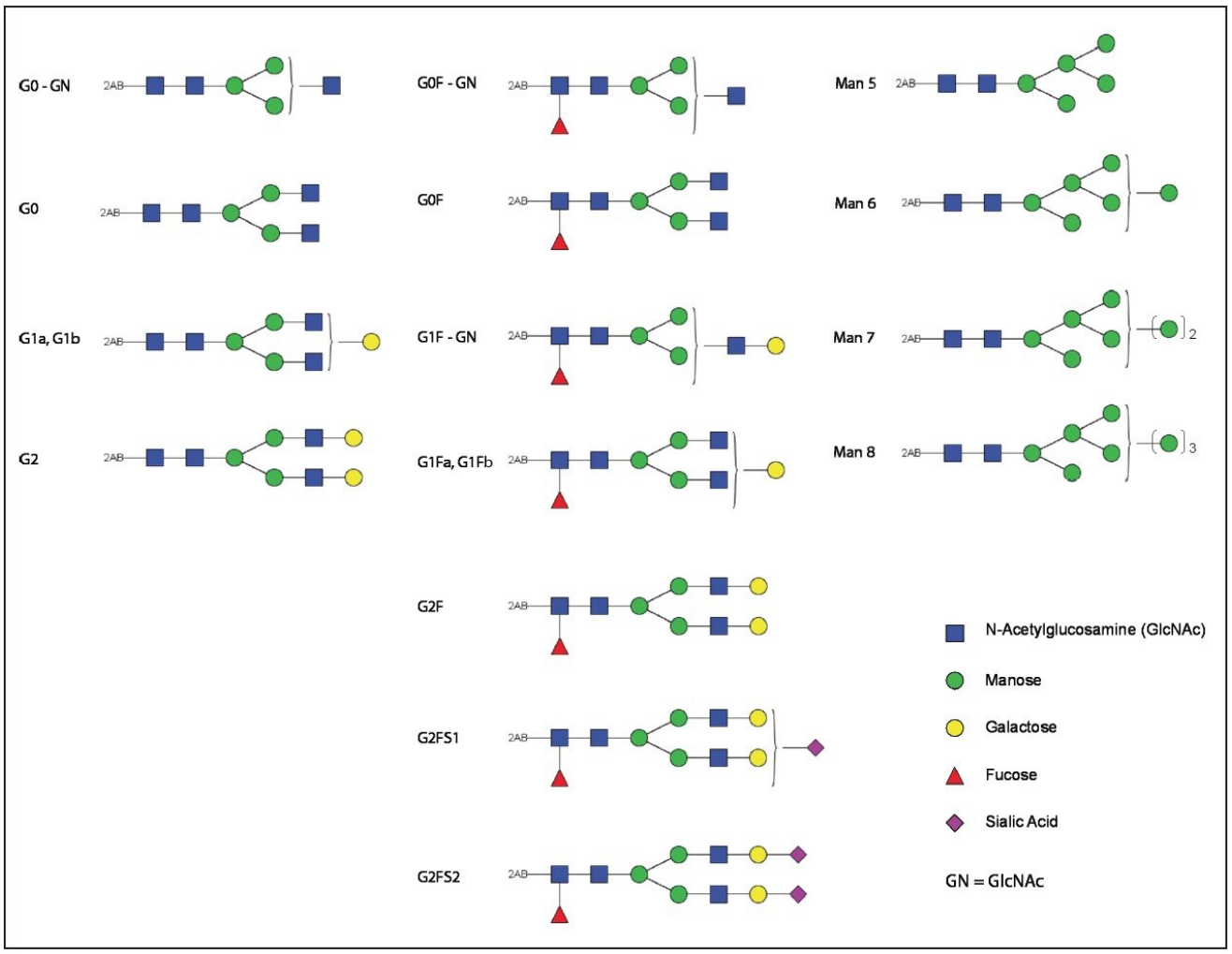

Figure 5. Proposed structures for the 2-AB labeled Trastuzumab glycans. The left panel lists the non-fucosylated complex type, the middle panel shows the fucosylated type, and the right panel shows the high mannose type.

An example of FLR and MS data for G2F peak along with MS spectrum is shown in Figure 3B. MS/MS fragmentation was used to elucidate glycan structure (data not shown). The sensitivity of Xevo QTof MS was sufficient to assign even the minor components. For example, the two sialylated glycans, G2FS1 and G2FS2 with a low fluorescence signal, did not show peaks in the MS base peak ion (BPI) chromatogram. However, the extracted ion chromatograms (XIC) of the doubly charged ions of G2FS1 and G2FS2 glycans clearly confirm the presumed identities (Figure 6).

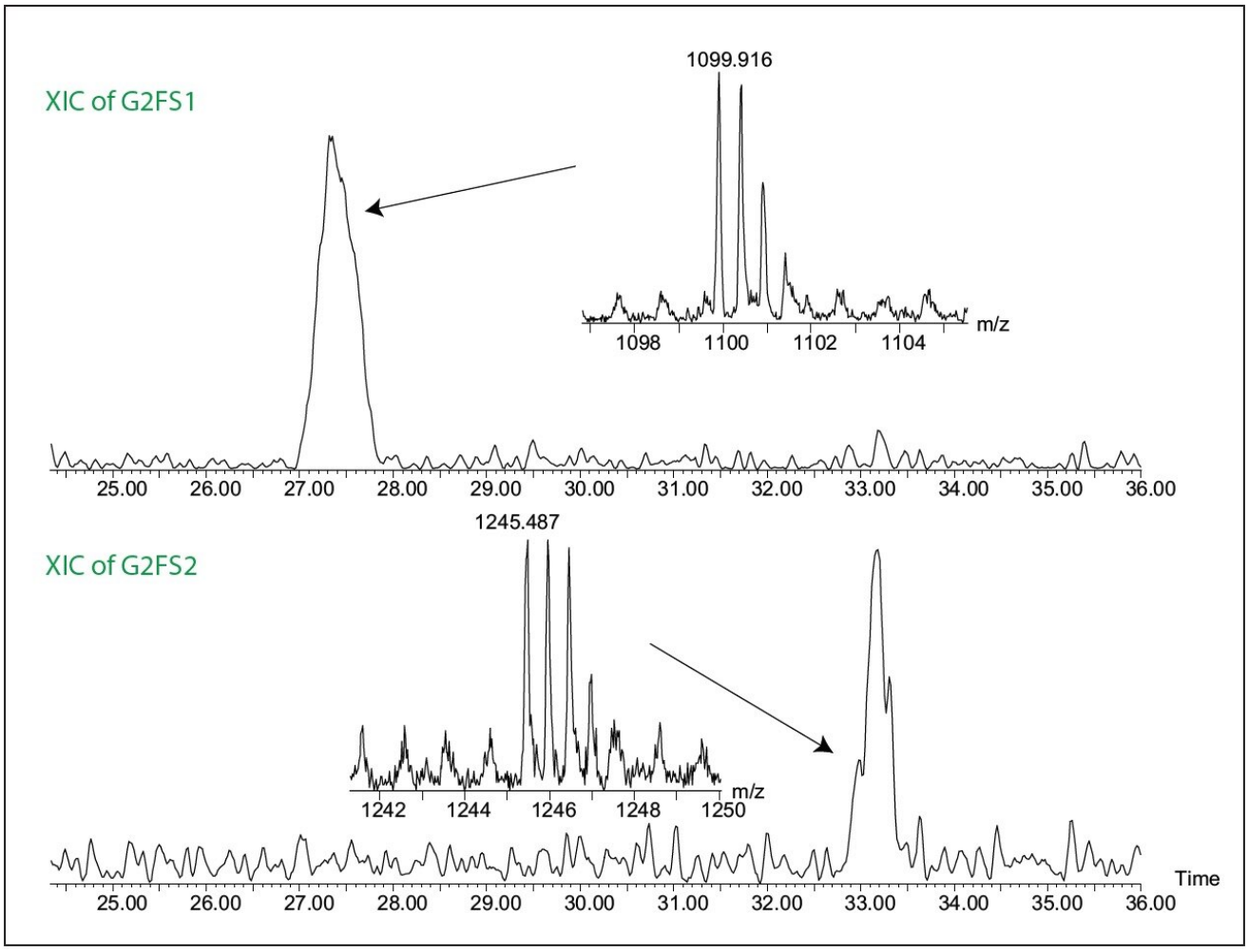

Figure 6. Extracted ion chromatogram (XIC) for G2FS1 and G2FS2. The extracted mass is the doubly charged ions $(M+2H)^{2+}$ for both components.

Batch-to-batch glycoprofiling comparison

Three different Trastuzumab batches were analyzed (Batch 1 through 3). In order to accurately compare the glycan profiles, the robustness of sample preparation (sample preparation and extraction step using HILIC µ Elution plate), its variability was also evaluated. Figure 7 shows the overlay of the FLR chromatograms of glycans released from these three Trastuzumab batches, while the graph in Figure 8 compares both the relative glycan abundance and the sample preparation variability. The narrow error bars in confirm that glycan relative quantitation is highly repeatable. The differences between batches of glycan profiles were significantly greater than variability introduced by sample preparation.

Among other differences, we observed significantly higher G0F content in Batch 3 than the other two batches. Man6, Man7, and Man8 were observed only in Batch 2.

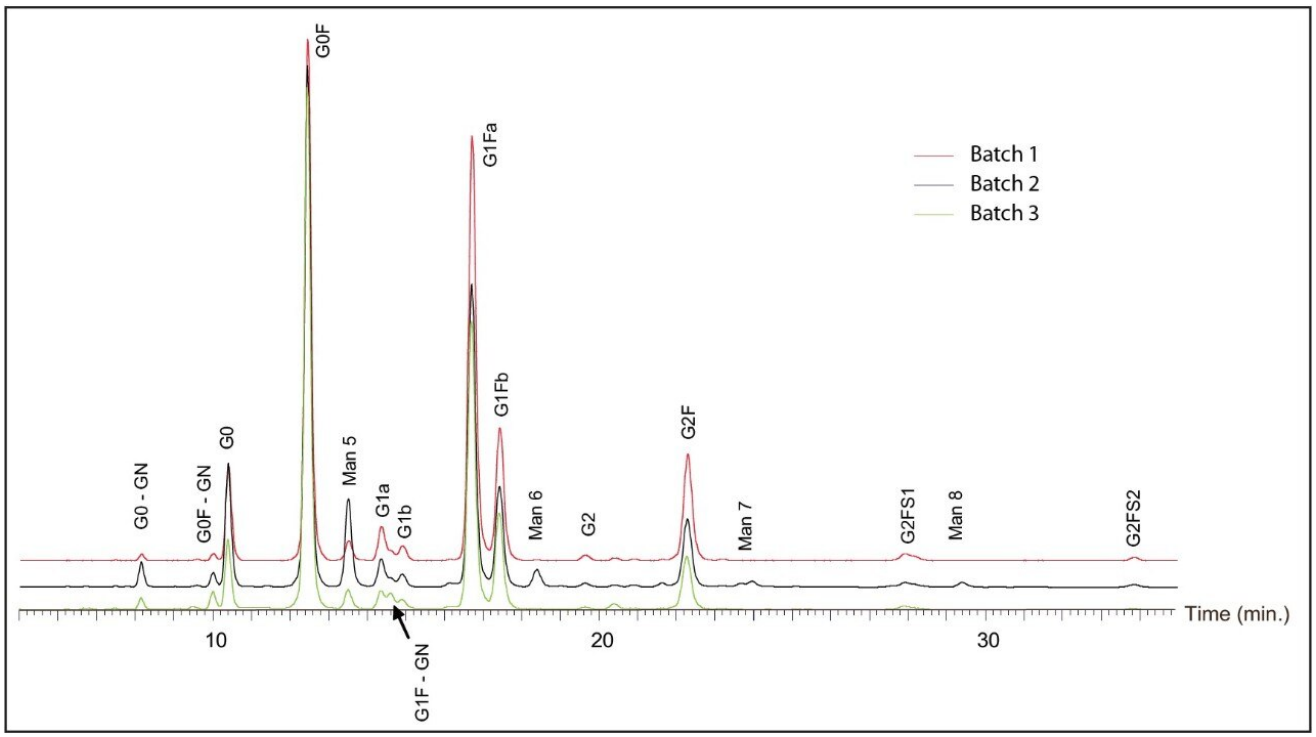

Figure 7. UPLC/FLR chromatograms of 2-AB labeled glycans released from three Trastuzumab batches.

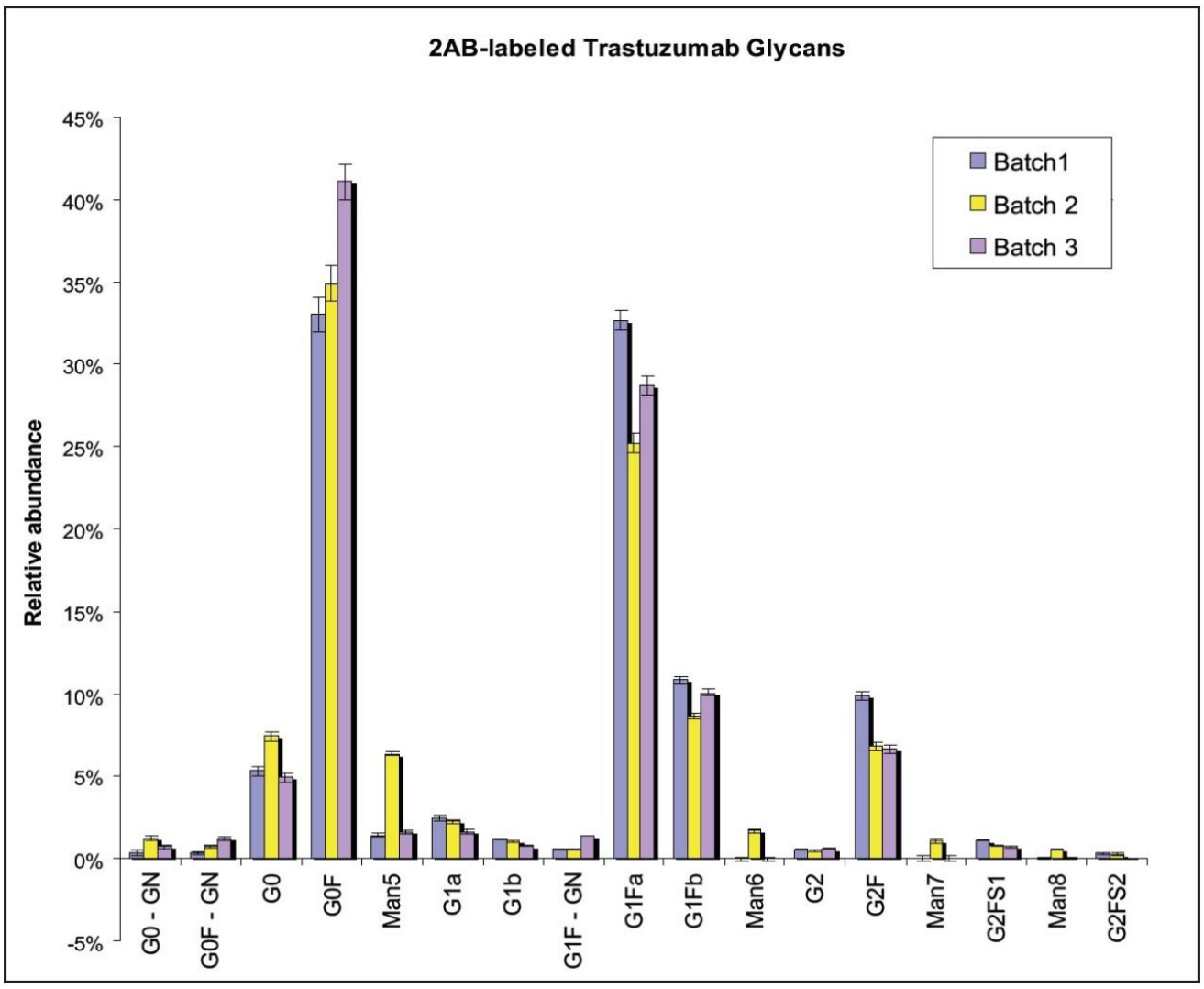

Figure 8. Relative abundance of 2-AB labeled glycans from three batches of Trastuzumab. Each relative abundance value has error bars based on triplicate analyses (three aliquots of deglycosylated Trastuzumab samples have undergone µElution extraction, FLR labeling, and additional µElution SPE cleanup). Error bars were calculated from sample preparation replicates. For details see Table 1.

Xevo QTof MS sensitivity was sufficient to confirm the glycan identity for peaks at 0.3% relative intensity (FLR data) of overall glycan content. Table 1 summarizes identified glycans with their relative abundance (%), standard deviation, and %RSD of the integrated FLR peaks (N = 3).

	Batch 1		Batch 2		Batch 3	
	Rela. Conc. (%)	RSD (%)	Rela. Conc. (%)	RSD (%)	Rela. Conc. (%)	RSD (%)
G0 - GN	0.34 ± 0.01	3.78	1.22 ± 0.14	11.50	0.71 ± 0.01	1.69
G0F - GN	0.36 ± 0.04	11.96	0.74 ± 0.09	11.94	1.19 ± 0.16	13.00
G0	5.35 ± 0.22	4.14	7.43 ± 0.28	3.86	4.95 ± 0.35	7.10
G0F	33.03 ± 0.85	2.58	34.90 ± 1.1	3.11	41.09 ± 1.49	3.63
Man5	1.41 ± 0.05	3.80	6.35 ± 0.11	1.75	1.61 ± 0.02	1.03
G1a	2.45 ± 0.04	1.70	2.25 ± 0.15	6.62	1.61 ± 0.15	9.00
G1b	1.20 ± 0.06	5.04	1.06 ± 0.03	2.98	0.77 ± 0.02	2.81
G1F - GN	0.55 ± 0.003	0.49	0.55 ± 0.02	3.16	1.38 ± 0.04	2.64
G1Fa	32.65 ± 0.59	1.80	25.22 ± 0.62	2.44	28.72 ± 0.77	2.68
G1Fb	10.83 ± 0.30	2.75	8.68 ± 0.2	2.25	10.06 ± 0.44	4.37
Man6	—	—	1.68 ± 0.13	7.89	—	—
G2	0.54 ± 0.04	7.55	0.42 ± 0.04	10.03	0.59 ± 0.03	5.10
G2F	9.89 ± 0.31	3.10	6.82 ± 0.23	3.44	6.63 ± 0.47	7.14
Man7	—	—	1.08 ± 0.17	16.11	—	—
G2FS1	1.09 ± 0.08	7.62	0.79 ± 0.02	2.59	0.71 ± 0.03	3.55
Man8	—	—	0.55 ± 0.07	12.90	—	—
G2FS2	0.31 ± 0.04	14.31	0.26 ± 0.05	18.20	—	—

Table 1. Summary of the relative abundance of the identified 2-AB labeled glycans (N=3).

Conclusion

- UPLC HILIC/FLR analysis provides sensitive and accurate methods for quantification of glycans.
- Injection repeatability is well below 1% for major peaks and better than 2% RSD for minor components. Sample cleanup introduces only minor variability into the quantitative glycoprofiling.
- UPLC HILIC/FLR/MS analysis revealed significant differences in glycan profiles between three batches of Trastuzumab.

Used together, ACQUITY UPLC with detection by FLR and Xevo QTof MS comprise a powerful system for producing required analytical data. The chromatographic resolution, reproducibility, and mass spectrometry sensitivity enable glycoprofiling of therapeutic antibodies mandated by regulatory agencies. The UPLC-FLR/MS system represents a robust tool for separation and analysis of minor glycoforms or isomers that are otherwise difficult to assign.

This UPLC-FLR/MS platform improves the overall quality of the rmAb-carbohydrate characterization assay and the batch-to-batch consistency test, which are components of drug release tests. The proposed method enables

a routine and robust rmAb glycan analysis and may become a tool of choice for biopharmaceuticalrmAb characterization.

References

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