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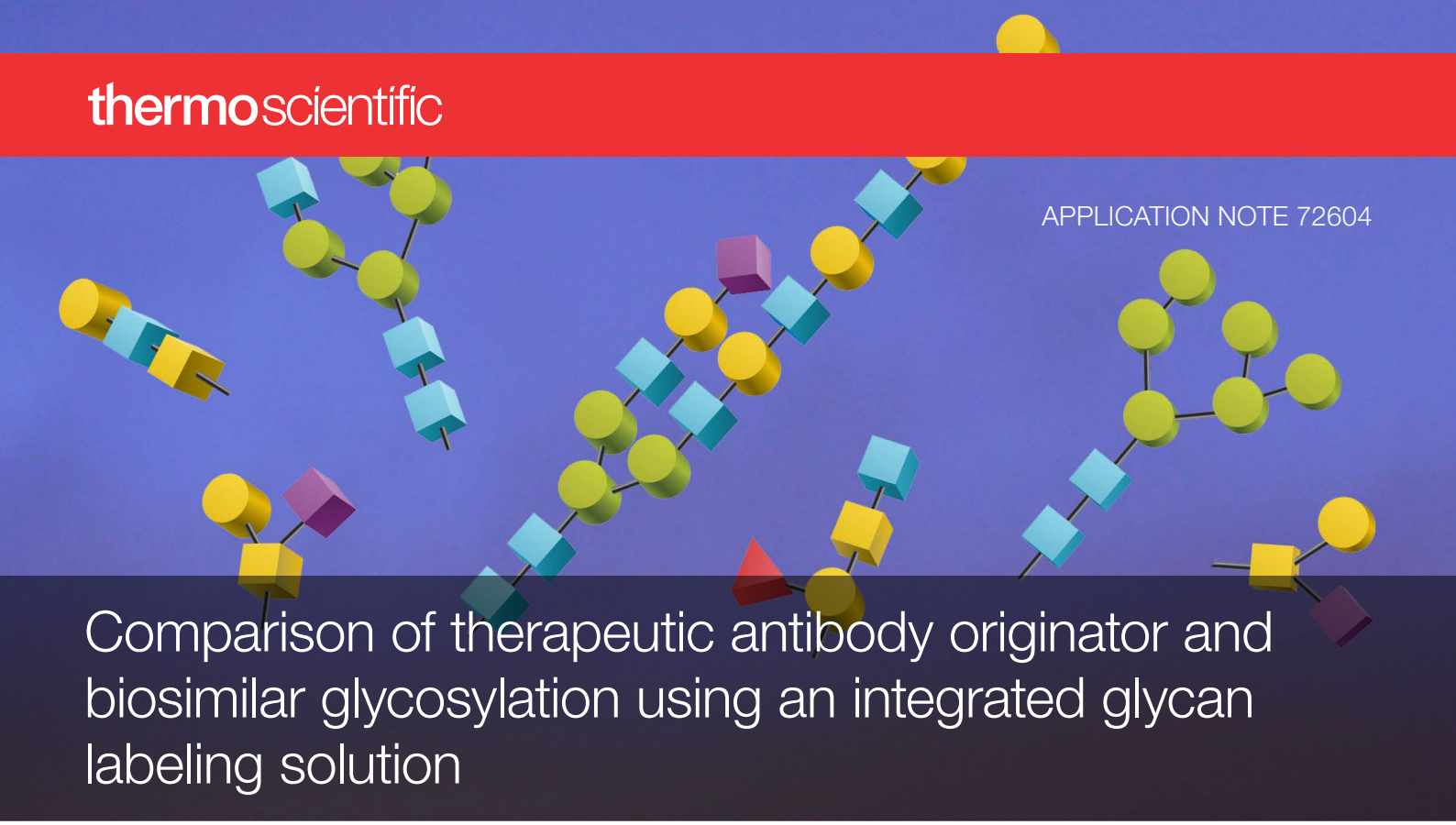

Comparison of therapeutic antibody originator and biosimilar glycosylation using an integrated glycan labeling solution

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Keywords

Monoclonal Antibodies, Biosimilar, Biocompatible UHPLC, Biotherapeutic Characterization, Biopharma, Vanquish Flex UHPLC, NIST mAb, GlycanAssure HyPerformance APTS kit, APTS, HILIC, Amide HILIC, Glycoprofiling, Accucore, Glycoform, Glycosylation, *N*-glycan, glycan analysis

Goal

Demonstrate the suitability of the Applied Biosystems™ GlycanAssure™ HyPerformance APTS labeling kit with the Thermo Scientific™ Vanquish™ Flex UHPLC system and Thermo Scientific™ Accucore™ 150 Amide HILIC column for the glycosylation comparison of a biosimilar against an originator.

Introduction

Remsima™ and Inflectra® were the first monoclonal antibody (mAb) biosimilars to be approved in the European Union. Remsima and Inflectra are both infliximab biosimilars to the originator Remicade™ (Janssen).[1] During their evaluation, as per the ICH Q6B and EMA guidelines,[2] these mAbs had to meet a significant number of strict criteria to be granted biosimilarity by the EMA (European Medicines Agency).[3] The evaluation of the glycosylated sites found on the heavy chain of mAbs are among the critical quality attributes (CQAs) to be characterized. Many conditions during the up- and downstream processing affect the glycosylation, and thus it is essential to monitor and ensure that the nature of the glycans remain within expectations, preventing undesired effects on efficacy, toxicity, and immunogenicity. Glycosylation is characterized with an array of chromatographic techniques. Depending on the scope of the analysis, glycans can be analyzed at the glycopeptide level, normally with MS detection, after enzymatic digestion of the protein.[4]

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Alternatively, glycans may be cleaved from the protein and then analyzed either natively (with MS) or after fluorescent labeling (with fluorescence detection) as shown here. An integrated glycan solution simplifies the sample preparation process significantly and reduces error in analysis.

In this work, the glycosylation profile of one originator (Remicade) and one of its biosimilar (Inflectra) mAbs were compared using the integrated *N*-glycan solution composed of a glycan labeling kit, the GlycanAssure HyPerformance APTS kit, an ultra-high-performance liquid chromatography (UHPLC) system, and separation column. The workflow additionally included a system suitability test (SST) based on a mAb standard, and assignment of a glucose unit (GU) number to the unknown glycans released from the samples. The released *N*-glycan profiles of the infliximab originator and biosimilar were evaluated on the Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. The separation was achieved using hydrophilic interaction liquid chromatography (HILIC) with the Thermo Scientific™ Accucore™ 150 Amide HILIC column.

This integrated solution is a globally applicable platform method for glycosylation profiling of many biotherapeutics. The GlycanAssure HyPerformance APTS kit allows a very wide range of sample protein concentration to be used without the need for prior buffer exchange—resulting in a simplified and streamlined sample preparation procedure. The HILIC separation was powered by the Vanquish Flex Binary UHPLC system, which features a high-pressure mixing binary pump for lowest gradient delay volume and most precise gradient composition. In addition, all Vanquish UHPLC systems feature Thermo Scientific™ SmartInject technology. SmartInject technology significantly improves the retention time precision, thereby increasing the confidence in peak assignments.[5]

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Accucore Amide HILIC column, 2.6 µm, 150 Å, 2.1 × 150 mm (P/ N 16726-1520130)
- GlycanAssure HyPerformance APTS kit, 24 sample kit (P/N A33953)
- Fisher Scientific™ ammonium formate (Optima™ LC-MS) (P/N A11550)
- Thermo Scientific™ formic acid, LC-MS grade (for pH adjustment) (P/N 85178)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Fisher Scientific™ Fisherbrand™ Premium Microcentrifuge Tubes 1.5 mL (P/N 05-408-129)
- Thermo Scientific™ 9 mm MS certified clear screw thread kit (P/N C4000-LV1W): Sample vials, with insert and 9 mm vial screw caps with pre-assembled septa

Recommended lab equipment

- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)
- Virtuoso 9 mm Wide Opening SureStop Screw Thread Vial Convenience Kit (P/N 60180-VT405)
- Applied Biosystems™ DynaMag™-2 Magnet (16-position Magnetic Stand) (P/N 4457858)
- Thermo Scientific™ Digital Heating Shaking Drybath (P/N 88880028)
- Thermo Scientific™ Orion Star™ A211 pH Benchtop Meter (P/N 13-645-519)
- Fisher Scientific™ Microcentrifuge (benchtop) (P/N 3722L)
- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)

Sample preparation

Two commercially available mAb infliximab drug products (Janssen UK Limited, High Wycombe, United Kingdom and Hospira UK Limited, Leamington Spa, United Kingdom) were supplied at a concentration of 10 mg/mL in formulation buffer. NIST mAb at 10 mg/mL (#8671, lot# 14HB-D-001) was purchased from The National Institute of Standards and Technology. The *N*-glycan samples were prepared as outlined in the GlycanAssure HyPerformance kit manual (MAN0016959). The *N*-glycan samples were denatured using a proprietary denaturant at 80 °C for 5 minutes before the glycans were enzymatically released using PNGaseF at 50 °C for 10 minutes. The digested samples were then directly reduced and labeled with APTS at 55 °C for 60 minutes. All heating steps were performed with the Heating Shaking Dry bath. Magnetic beads, included in the GlycanAssure HyPerformance kit, were prepared according to the manual during the labeling and utilized for final sample clean-up. The labeled glycans were bound to the beads and washed of excess dye in three steps to ensure purification of the sample before the final elution with elution buffer. Then, 15 µL of the supernatant, containing samples solvated in elution buffer, was removed and added to 45 µL acetonitrile. This provided a sufficiently non-polar solution that was suitable for HILIC chromatographic starting conditions allowing effective loading retention and subsequent gradient separation. The labeled glycan samples were aliquoted and stored in sample vials at 4 °C in the Vanquish autosampler prior to analysis. Additionally, the dextran ladder was labeled with the APTS reagent mixture and cleaned up with magnetic beads according to the manual (MAN0016959).

Instrumentation

The separation was achieved in HILIC mode with the Accucore 150 Amide HILIC LC column. The column was operated by the Vanquish Flex Binary UHPLC system (Table 1 and Table 2). Detection was performed using the Thermo Scientific™ Vanquish™ Fluorescence Detector F with a 2 µL biocompatible micro flow cell.

Vanquish Flex UHPLC system consisting of:

- Flex System Base (P/N VF-S01-A)
- Binary Pump (P/N VF-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A) with 25 µL sample loop
- Fluorescence Detector F (P/N VF-D50-A) with 2 µL micro flow cell biocompatible (P/N 6079.4330)
- Static Mixer for 200 µL mixing volume (P/N 6044.5110)

Separation conditions

The separation conditions are listed in Tables 1 and 2.

Table 1. Chromatographic conditions.

Column	Accucore Amide HILIC column, 2.6 µm, 150 A, 2.1 × 150 mm
Mobile Phase	A: Acetonitrile B: 100 mM Ammonium formate, pH 4.4 Buffers filtered through 0.2 µm filter membrane before use
Gradient	As per GlycanAssure HyPerformance APTS kit manual and described in Table 2.
Flow Rate	0.45 mL/min
Temperature	50 °C still air
Injection Volume	2.5 µL of labeled glycan infliximab and NIST mAb sample
FLD Detection	Excitation: 455 nm Emission: 500 nm Filter wheel: 435 nm Sensitivity: 7 Power mode: High Data collection: 10 Hz

Table 2. Chromatographic gradient conditions.

Time (min)	*A*	*B*
0	68%	32%
45	55%	45%
45.5	40%	60%
47	40%	60%
47.5	68%	32%
50	68%	32%
65	68%	32%

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System, version 7.2.5, was used for data acquisition and data analysis.

Results and discussion

Using the GlycanAssure HyPerformance APTS kit, the total sample preparation was achieved in less than 1.5 hours. The sample preparation included the release, labeling with APTS, and sample clean-up of four samples of originator, four samples of biosimilar, and four samples of NIST mAb standard; moreover, a sample of dextran ladder was included in the procedure.

All the UHPLC runs were included in the same injection sequence with the NIST mAb standards, required for the SST, and the dextran ladder was run before the analytical samples. Figure 1 shows an example chromatogram of the labeled glycans of the NIST mAb standard. In total 38 peaks were observed for the NIST mAb, with 29 peaks having relative area larger than 0.85%. The injection of the dextran ladder produced the typical chromatographic profile expected in HILIC mode (Figure 2). To assign the correct number to the dextran ladder peaks, the dextran ladder chromatogram was compared to the chromatograms of APTS-labeled linear glucose tetraose and triose. A dedicated Chromeleon 7.2 processing method and report template (included in Thermo Scientific™ AppsLab Scientific Library of Analytical Applications) was utilized to fit the dextran ladder glucose unit (GU) values to a 5th order polynomial model, as described in a previous Technical Note.[6] The report has several curve fitting formulas, and in this case the fifth order polynomial fit was used. This GU calibration curve was then used to assign GU values to the NIST mAb standard sample peaks. The suitability of the workflow was assessed solely on the 29 peaks with the largest relative area. Table 3 shows retention and relative area for the selected peaks. The results of Table 3 matched very well the glycan profile observed in multiple laboratories previously (data not shown), indicating the suitability of the full workflow. As the analysis of NIST mAb serves as suitability assessment, major discrepancies of retention time and relative area compared to the values reported in Table 3 may indicate failure of one or more steps of the workflow that will need troubleshooting.

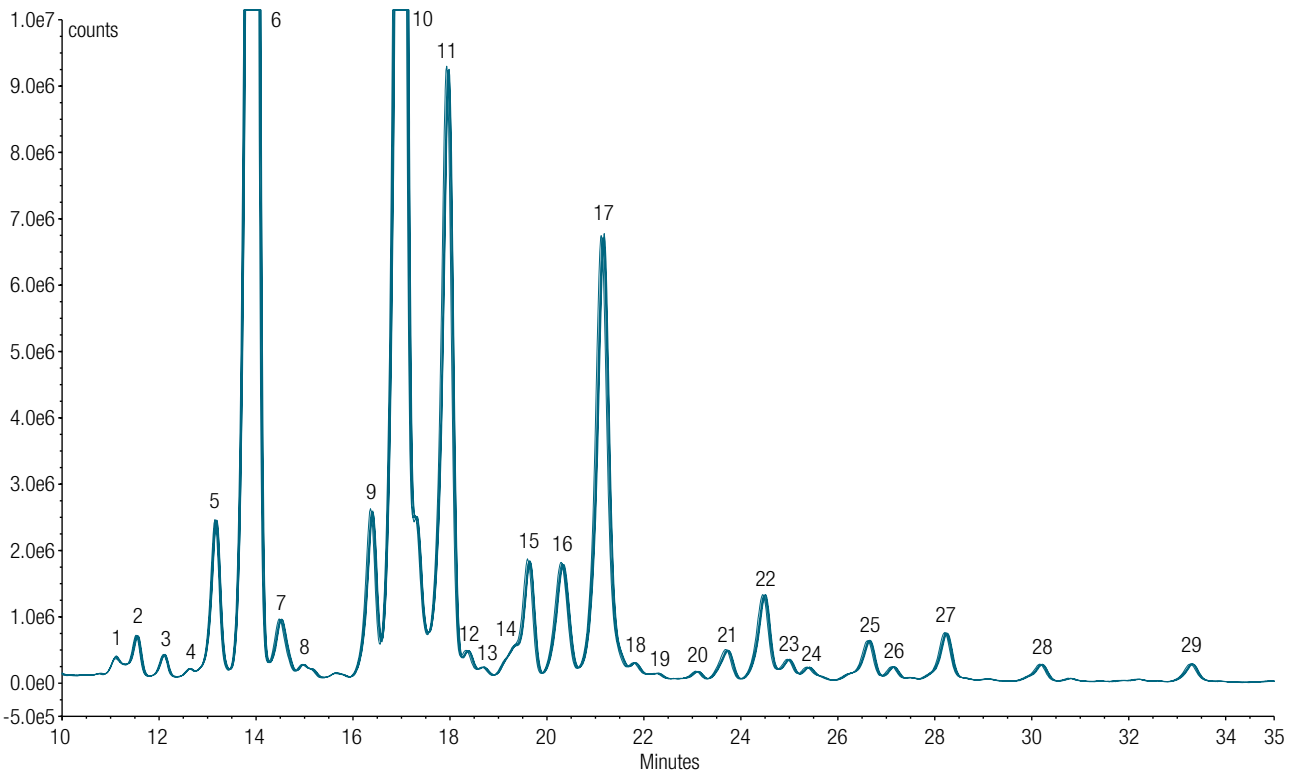

Figure 1. FLD chromatogram overlay of four subsequent (2.5 µL) injections of NIST mAb released glycans, highlighting the excellent retention time and peak area precision. Of the 38 peaks that were detected, 29 peaks with relative area > 0.85% are labeled.

Table 3. Table of retention time and peak area standard deviations of NIST mAb APTS-labeled glycans of Figure 1.

Peak	Average Retention Time (min)	Average Glucose Unit Value (GU) ± Standard Deviation	Average Relative Peak Area (%)	Relative Standard Deviation of Peak Area (n=4) (%)
1	11.110	4.057 ± 0.003	0.253	3.549
2	11.550	4.224 ± 0.006	0.490	2.836
3	12.110	4.433 ± 0.005	0.240	1.409
4	12.637	4.577 ± 0.081	0.093	3.537
5	13.177	4.819 ± 0.006	2.083	0.576
6	13.947	4.947 ± 0.123	36.835	0.214
7	14.513	5.281 ± 0.007	0.960	0.385
8	14.971	5.434 ± 0.006	0.235	2.669
9	16.392	5.894 ± 0.006	2.298	0.196
10	17.002	6.081 ± 0.007	29.353	0.269
11	17.961	6.384 ± 0.006	9.275	0.243
12	18.378	6.507 ± 0.006	0.363	2.794
13	18.708	6.606 ± 0.006	0.140	1.979
14	19.140	6.639 ± 0.053	0.258	32.368
15	19.634	6.875 ± 0.031	1.955	4.578
16	20.322	7.104 ± 0.006	2.103	0.574
17	21.157	7.359 ± 0.008	7.808	0.321
18	21.811	7.560 ± 0.008	0.238	1.855
19	22.264	7.627 ± 0.065	0.093	5.444
20	23.099	7.962 ± 0.007	0.103	4.670
21	23.725	8.111 ± 0.080	0.485	1.498
22	24.484	8.405 ± 0.006	1.430	0.537
23	24.986	8.569 ± 0.007	0.335	1.734
24	25.396	8.705 ± 0.008	0.230	2.118
25	26.646	8.914 ± 0.201	0.720	0.559
26	27.149	9.207 ± 0.077	0.160	1.219
27	28.229	9.574 ± 0.154	0.828	0.564
28	30.200	10.362 ± 0.007	0.313	0.370
29	33.298	11.466 ± 0.009	0.320	0.561

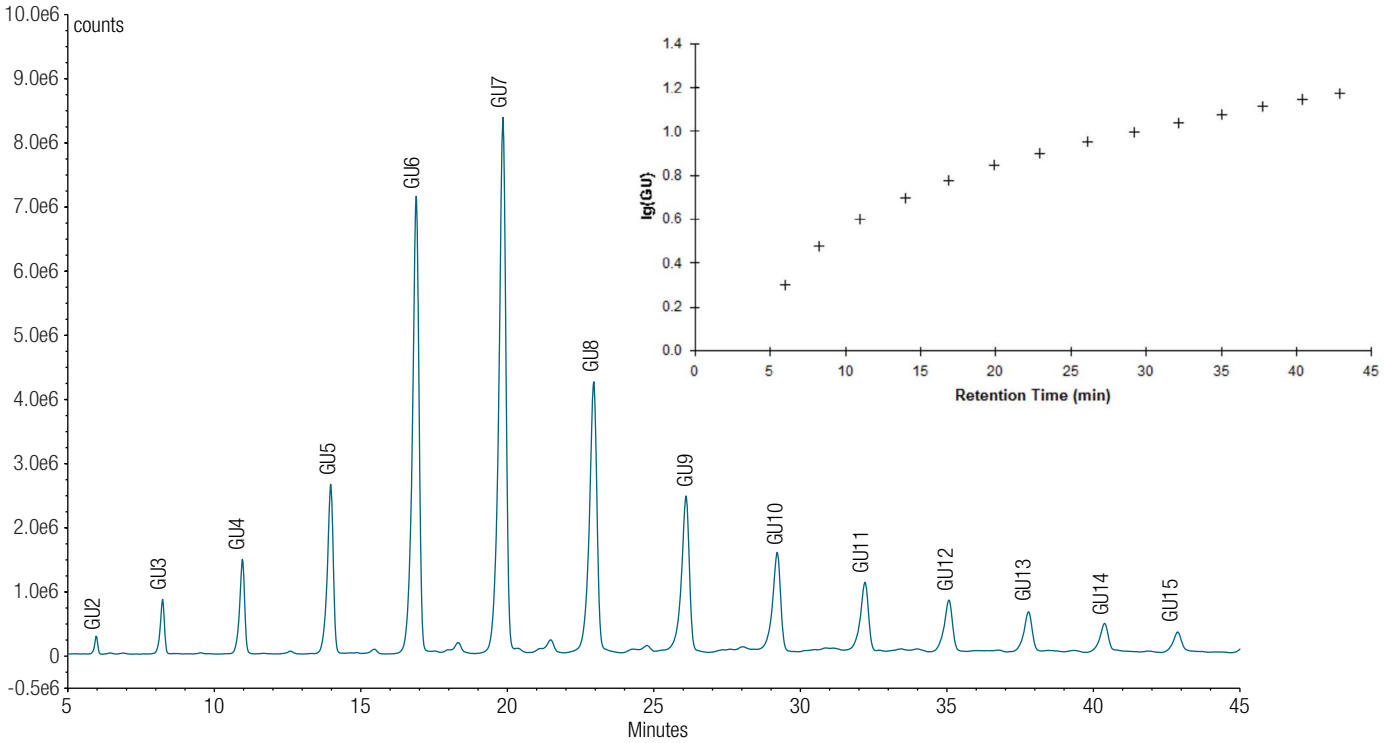

Figure 2. FLD chromatogram, showing the dextran ladder for peaks GU2–GU15 and the associated calibration curve that was used to assign GU values to the unknown sample peaks.

The same sample preparation kit, UHPLC system, and column were used to process and analyze the infliximab samples (originator and biosimilar); the same chromatography conditions as shown in Figure 1 were also used. The originator and biosimilar demonstrated 37 well-resolved *N*-glycan peaks at ≥0.01% relative peak area. Figures 3a and 3b show the chromatogram of the originator and biosimilar, respectively. Data for the qualitative and quantitative comparison of the two glycan samples were limited to the most intense peaks; for this purpose, only peaks with relative area larger than 1.5% were compared. Data collected are shown in Table 4 and Table 5. Relative peak area repeatability (n=4) was excellent for the 22 peaks, with the relative area RSD below 5.5% (Tables 4 and 5). The same dextran ladder run used for the SST (labeled with the GlycanAssure HyPerformance APTS kit, Figure 2) was used to assign GU values to the unknown sample peaks.

When comparing the originator drug (Remicade) and the biosimilar drug (Inflectra), it is clear that the two drug products have very similar glycoprofiles. The GU values indicate that the same peaks are present in both samples, with slightly differing relative abundances, in keeping with the EMA Assessment Report.[7] Peaks 4, 5, 15, and 17 make up a higher relative area in Remicade compared to Inflectra, and conversely, peaks 8, 9, 13, 18, and 20 make up a higher relative area in Inflectra compared to Remicade (Figure 4). These small differences are a result of the two completely independent manufacturing processes by which the two drug products are produced. It is important that neither drug product deviate significantly over time. Therefore, the monitoring of CQAs during the manufacturing process and upon batch-release is essential.

The suitability of the workflow is proven for the analysis of *N*-glycans found in many mAbs. However, for more complex glycoproteins (e.g. Enbrel® - Etanercept)—which contain many sites of glycosylation including *N*- and *O*-linked glycans—this workflow would only be capable of elucidating the profile of the *N*-linked glycans. In these situations, it is worth considering subsequent *O*-glycan analysis or considering a protein sub-unit middle-down mass spectrometry approach.

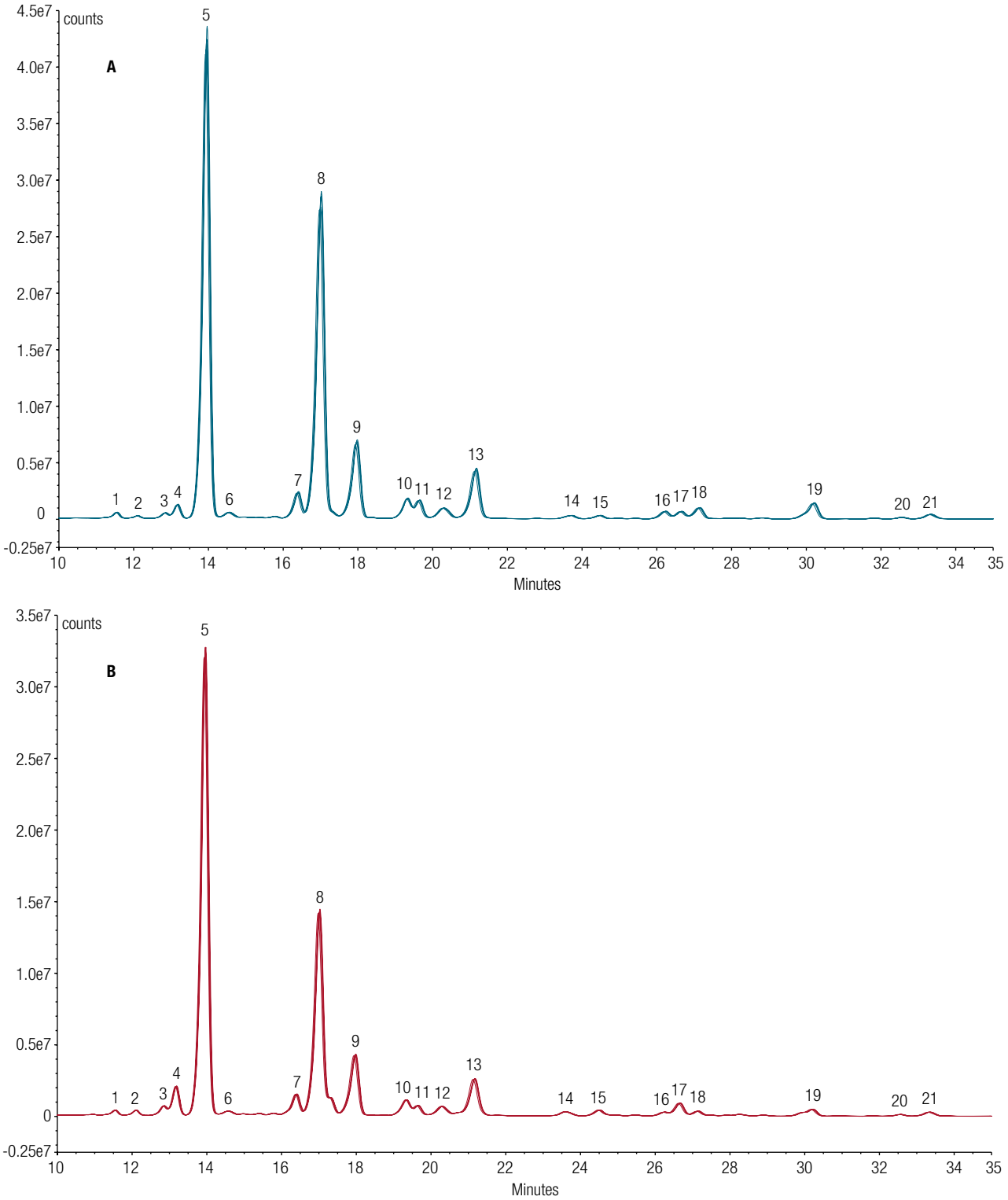

Figure 3. (A) FLD chromatogram overlay of four subsequent (2.5 µL) injections of Inflectra (blue) and (B) injections of Remicade (red) released glycans (all injections 2.5 µL), highlighting the excellent retention time and peak area precision and the difference between the two drug products. Of the 37 peaks detected, 21 peaks with relative area > 1.5% are labeled.

Table 4. Table of retention time and peak area standard deviations of Inflectra APTS-labeled *N*-glycans.

Peak	Average Retention Time (min)	Average Glucose Unit Value (GU) ± Standard Deviation	Average Relative Peak Area (%)	Relative Standard Deviation of Peak Area (%)
1	11.547	4.223 ± 0.005	0.488	3.242
2	12.101	4.430 ± 0.006	0.200	1.840
3	12.842	4.699 ± 0.006	0.460	3.309
4	13.171	4.817 ± 0.005	1.055	2.831
5	13.946	5.073 ± 0.011	40.593	2.777
6	14.545	5.239 ± 0.084	0.510	2.652
7	16.389	5.893 ± 0.006	2.308	3.082
8	17.003	6.087 ± 0.006	30.318	2.774
9	17.960	6.383 ± 0.006	7.480	2.953
10	19.325	6.801 ± 0.004	2.020	2.799
11	19.638	6.847 ± 0.044	1.563	2.565
12	20.295	7.096 ± 0.004	1.340	2.766
13	21.151	7.357 ± 0.007	5.135	2.819
14	23.707	8.155 ± 0.008	0.420	2.059
15	24.483	8.405 ± 0.008	0.348	2.916
16	26.219	8.978 ± 0.008	0.840	4.317
17	26.643	9.050 ± 0.066	0.788	2.957
18	27.133	9.205 ± 0.076	1.195	2.197
19	-	-	-	-
20	30.194	10.360 ± 0.009	2.035	3.173
21	32.559	11.203 ± 0.007	0.173	3.059
22	33.322	11.406 ± 0.114	0.570	3.762

Table 5. Table of retention time and peak area standard deviations of Remicade APTS-labeled *N*-glycans.

Peak	Average Retention Time (min)	Average Glucose Unit Value (GU) ± Standard Deviation	Average Relative Peak Area (%)	Relative Standard Deviation of Peak Area (%)
1	11.554	4.225 ± 0.007	0.488	2.055
2	12.109	4.432 ± 0.005	0.475	1.529
3	12.844	4.703 ± 0.005	0.918	2.811
4	13.180	4.820 ± 0.006	2.720	0.979
5	13.952	5.090 ± 0.006	46.460	1.370
6	14.571	5.300 ± 0.007	0.423	1.007
7	16.396	5.895 ± 0.005	2.253	0.956
8	17.009	6.084 ± 0.008	24.183	1.245
9	17.965	6.385 ± 0.006	7.018	0.804
10	19.338	6.761 ± 0.072	1.955	0.986
11	19.646	6.851 ± 0.043	0.975	2.523
12	20.292	7.095 ± 0.006	1.338	1.496
13	21.158	7.359 ± 0.007	4.910	1.258
14	23.587	8.117 ± 0.007	0.628	0.623
15	24.503	8.411 ± 0.009	0.708	1.784
16	26.243	8.860 ± 0.117	0.428	3.036
17	26.649	9.057 ± 0.060	1.583	1.298
18	27.140	9.208 ± 0.077	0.473	1.737
19	29.901	9.902 ± 0.004	0.318	7.230
20	30.205	10.364 ± 0.009	0.940	0.284
21	32.561	11.204 ± 0.009	0.220	2.260
22	33.332	11.408 ± 0.118	0.595	2.057

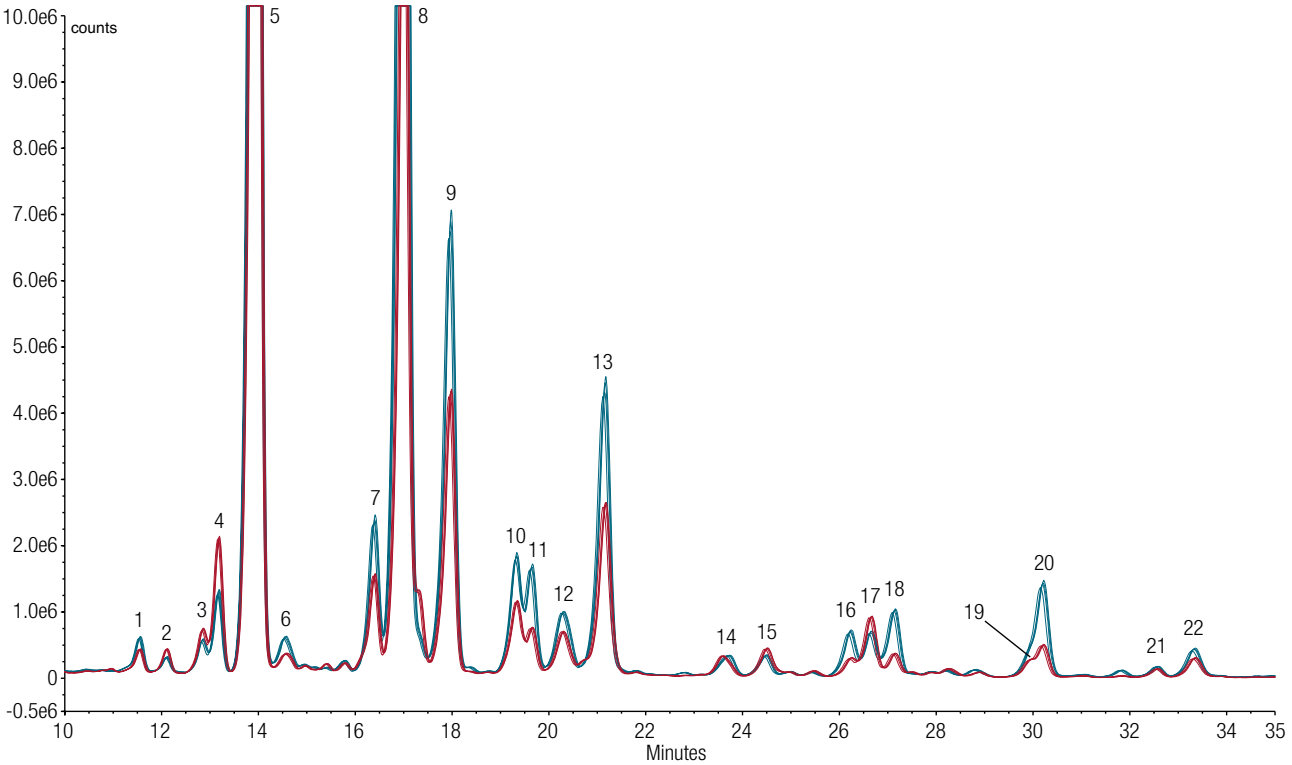

Figure 4. FLD chromatogram overlay of four subsequent (2.5 µL) injections of Inflectra (blue) against four subsequent (2.5 µL) injections of Remicade (red) released glycans, highlighting the excellent retention time and peak area precision and the difference between the two drug products. Of the 37 peaks detected, 22 peaks with relative area > 1.5% are labeled.

Conclusion

- The Vanquish Flex UHPLC system, combined with FLD detection and in conjunction with the GlycanAssure HyPerformance glycan labeling kit, provides a simple and robust platform method to characterize and monitor *N*-glycosylation of mAbs.
- The Accucore 150 Amide HILIC column facilitates reproducible and reliable GU assignment when using an oligosaccharide ladder, by providing stable retention time and peak area precision when operated by the Vanquish Flex UHPLC system.
- The GlycanAssure HyPerformance APTS kit greatly simplifies the *N*-glycan mapping workflow and saves significant time through sample preparation with fewer steps, providing a way to keep current with the demands of the modern laboratory and regulatory bodies.
- The inclusion of a SST based on GU assignment and relative abundance assessment of *N*-glycans released from the NIST mAb standard, greatly increases the confidence of the workflow.

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