LC-MS Analysis of Monoclonal Antibody Glycoforms using a Novel FcR Receptor Affinity Stationary Phase Paired with High Resolution Mass Spectrometry

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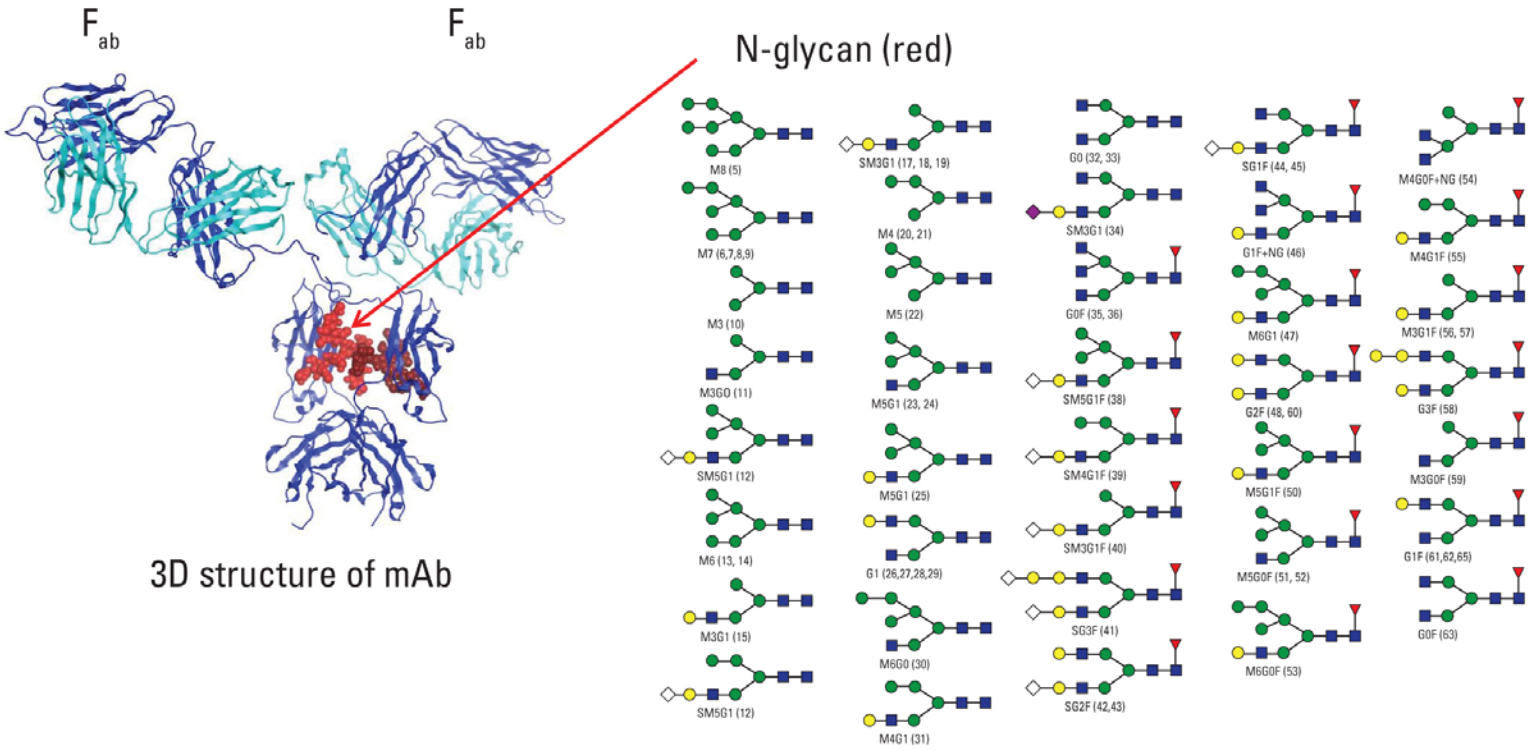
Introduction

- Glycosylated proteins used as biopharmaceuticals, including monoclonal antibodies (mAbs), are among the most promising candidates in the drug development pipeline.
- Glycosylation is a critical factor in drug product solubility, kinetics, stability, efficacy, and immunogenicity.
- Understanding the inherent heterogeneity of N-glycan moieties of the F_c region of mAbs (Figure 1) is an important part of cell line development and ultimately in the manufacture and quality control of these active pharmaceutical ingredients.

Introduction (cont)

Figure 1: Three Dimensional Structure of a Typical 150 kD Monoclonal Antibody



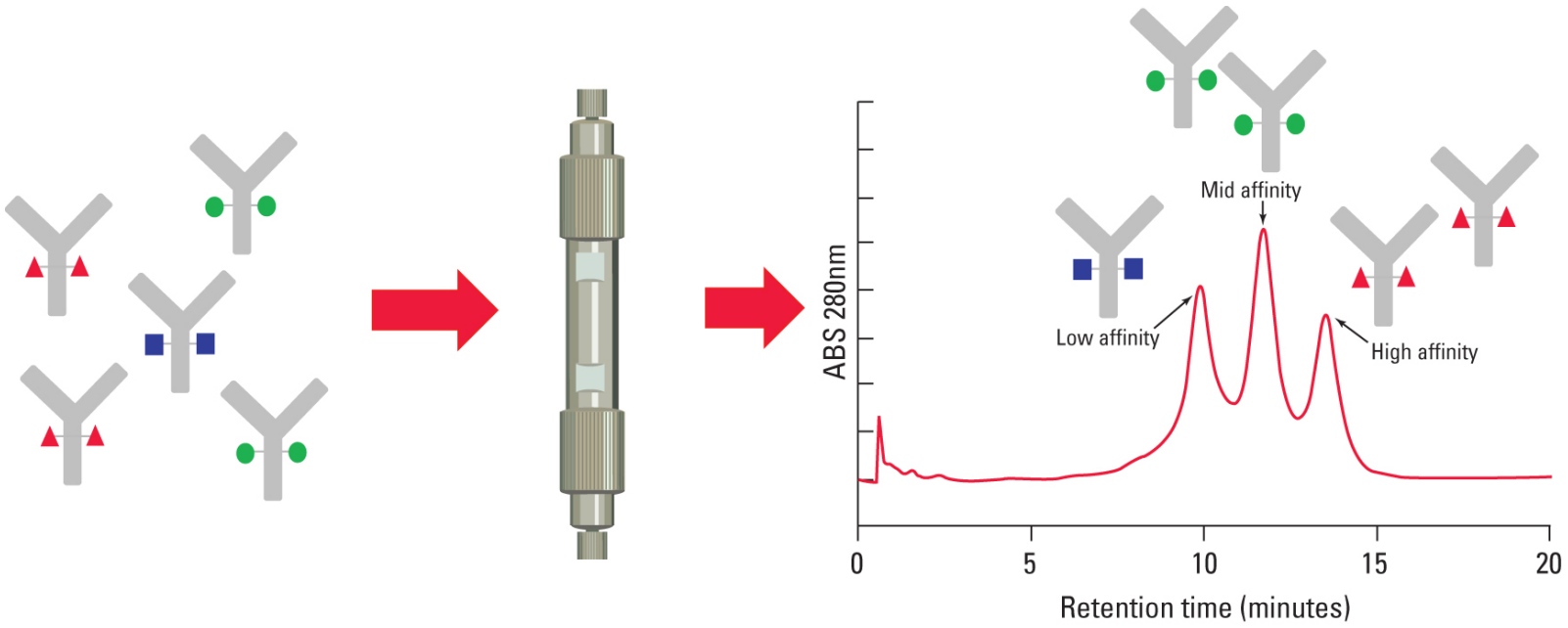
- mAbs consist of two variable regions, F_{ab}, and one conserved region, F_C, with a linkage site for N-glycans.
- The N-glycan moiety of the F_C is heterogeneous and plays a critical role in drug efficacy and safety.[1,2]

Introduction (cont)

- High performance liquid chromatography (HPLC) paired with high resolution mass spectrometry (MS) can be utilized to analyze glycosylation of both intact and digested protein molecules.
- The purpose of this work is to evaluate a new affinity stationary phase based on a novel FcR protein ligand (Figure 2) using offline MS detection to obtain spectral characterization of intact glycosylation for a typical IgG_1.
- This type of top down approach has utility to confirm results of subunit or released N-glycan analysis and as a characterization tool in early drug development.

Introduction (cont)

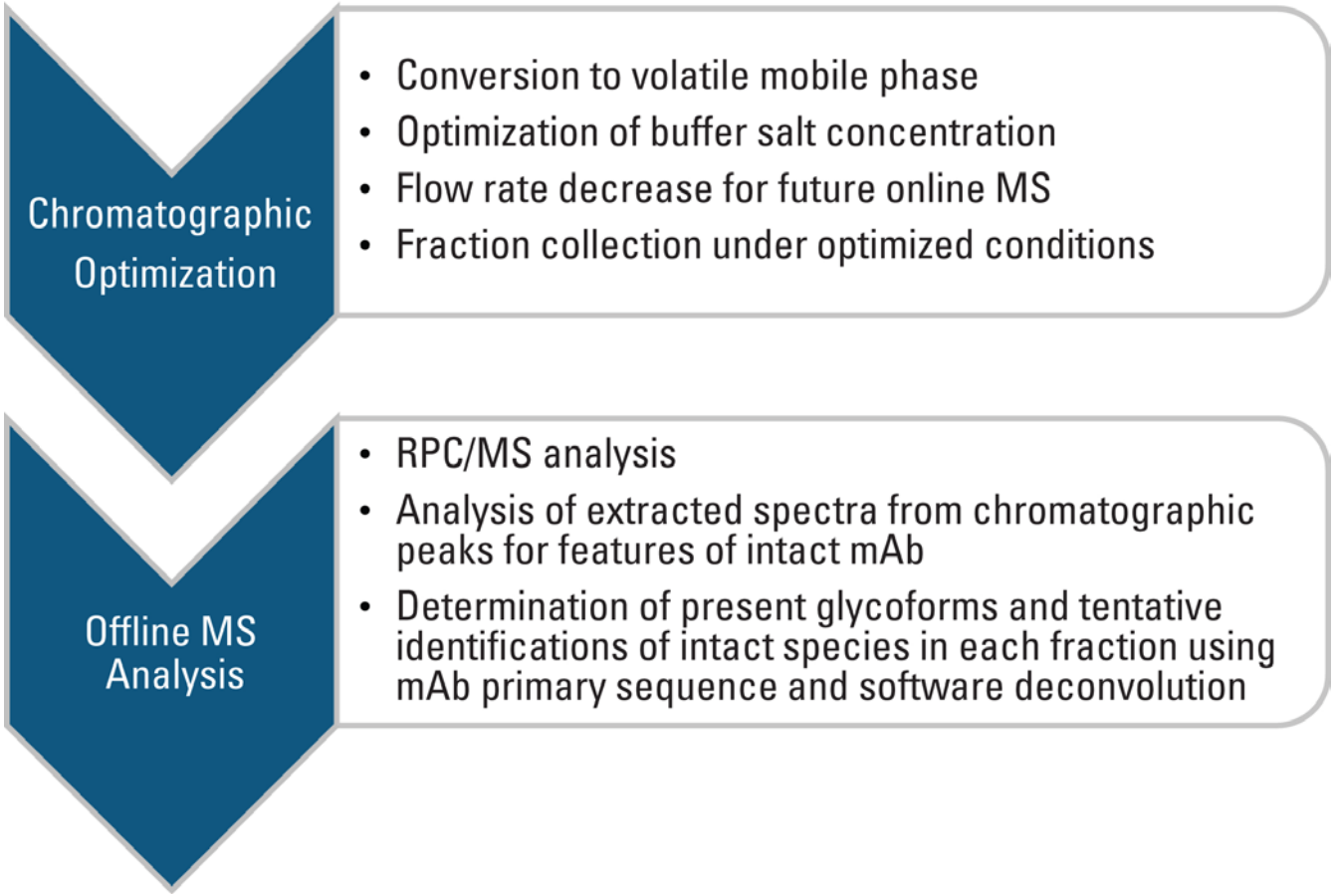
Figure 2: Schematic of the Separation Mechanism for the Novel FcR Affinity Stationary Phase



A heterogenous mAb is injected on the column and separated based on the affinity for the FcR ligand.

Materials and Methods

Experimental Design



Chromatographic Optimization for MS Compatibility

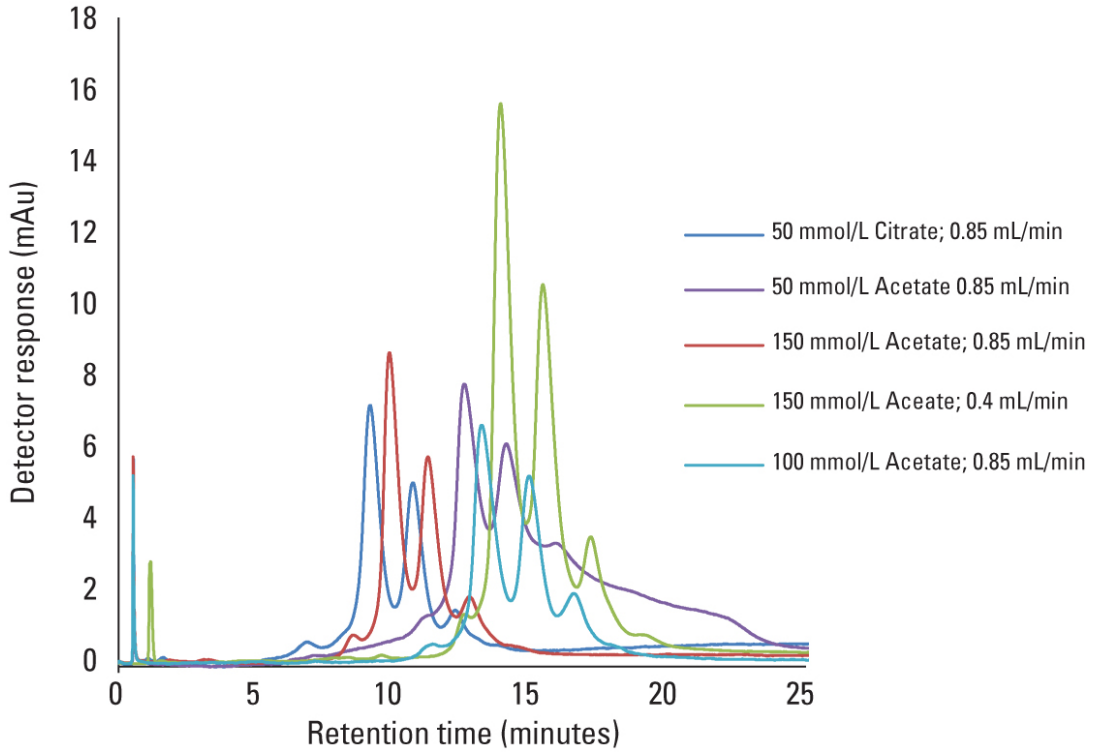
Instrument: Agilent 1200
Column: novel FcR receptor column, 4.6 mm ID ×7.5 mm
Mobile phase: see table
Flow rate: 0.85 mL/min, gradient 0 to 100% B in 20 min
Temperature: 15º C
Injection vol.: 5 uL
Sample: Ipilimumab API, 5.0 mg/mL

Table 1: Mobile Phase Conditions for Chromatographic Optimization Experiments

Mobile Phase A	Mobile Phase B	Flow Rate mL/min
50 mmol/L sodium citrate pH 6.5	50 mmol/L sodium citrate pH 4.5	0.85
50 mmol/L ammonium acetate pH 6.5	50 mmol/L ammonium acetate pH 4.5	0.85
100 mmol/L ammonium acetate pH 6.5	100 mmol/L ammonium acetate pH 4.5	0.85
150 mmol/L ammonium acetate pH 6.5	150 mmol/L ammonium acetate pH 4.5	0.85
150 mmol/L ammonium acetate pH 6.5	150 mmol/L ammonium acetate pH 4.5	0.4

Chromatographic Optimization for MS Compatibility: Results

Figure 3: Chromatographic Overlay of Different Mobile Phase Conditions Used During the Conversion and Optimization to MS Compatible Conditions



150 mmol at 0.4 mL/min (green trace) was chosen for offline fraction collection.

Chromatographic Optimization for MS Compatibility: Conclusions

- Initial experiments showed that a conversion to ammonium acetate in equivalent concentration to sodium citrate did not yield adequate resolution.
- Resolution using ammonium acetate was improved by increasing the buffer salt concentration, with the optimum between 100-150 mmol/L.
- Decreasing the flow rate did not impact resolution; however, there was an increase in signal, suggestive of greater recovery at lower flow rate.
- Though not evaluated experimentally, keeping the temperature at 15º C proved beneficial for column longevity.

Offline MS Analysis

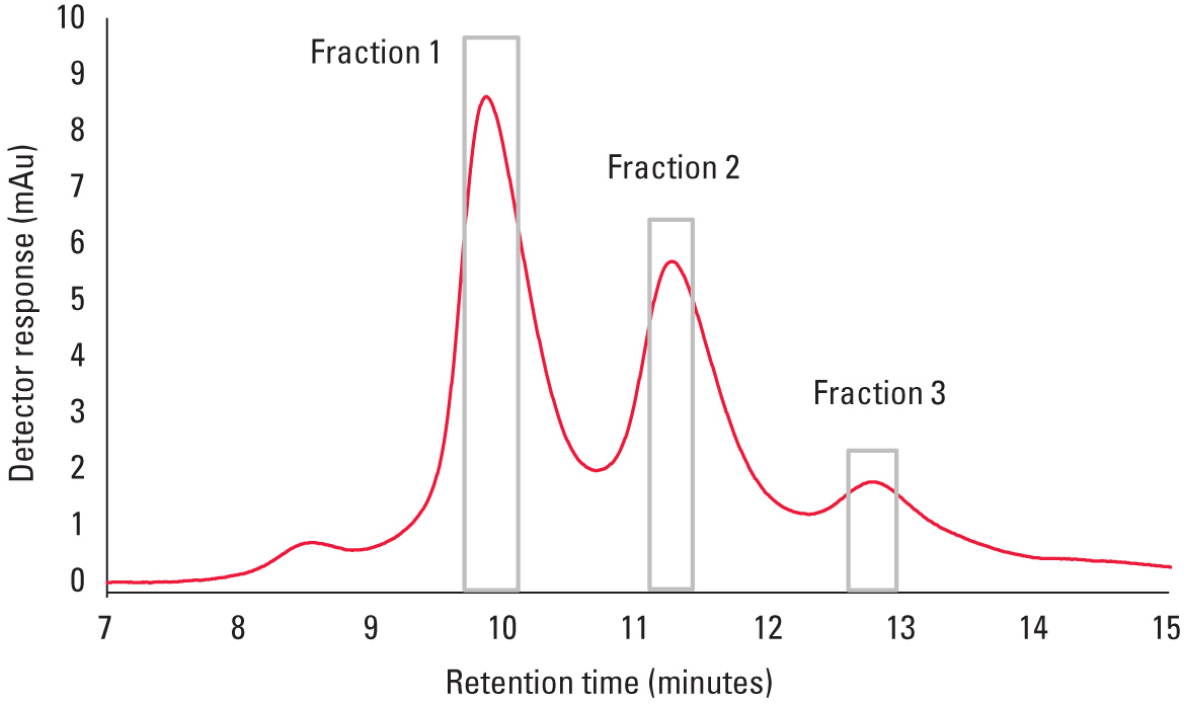
Fraction Collection

Instrument:	Agilent 1200
Column:	novel FcR receptor column, 4.6 mm ID × 7.5 mm
Mobile phase:	A: 150 mmol/L ammonium acetate, pH 6.5 B: 150 mmol/L ammonium acetate, pH 4.5
Flow rate:	0.85 mL/min, gradient 0 to 100% B in 20 min
Temperature:	15º C
Injection vol.:	5 uL
Sample:	Ipilimumab API, 5.0 mg/mL

- Fractions of each of the three major peaks for Ipilimuma were collected.
- Fractions were collected manually from the outlet of the UV detector.
- Collection attempted to minimize peak overlap and collect the fractions from the high intensity portion of the eluting peak.
- Fractions of several runs were pooled for future offline analysis.

Offline MS Analysis

Figure 4: HPLC Analysis of Ipilimumab Using a Novel FcR Receptor Affinity Column



The sample chromatogram from a fraction collection experiment shown above indicates the approximate cuts taken for each fraction and the representative separation in ammonium acetate buffer.

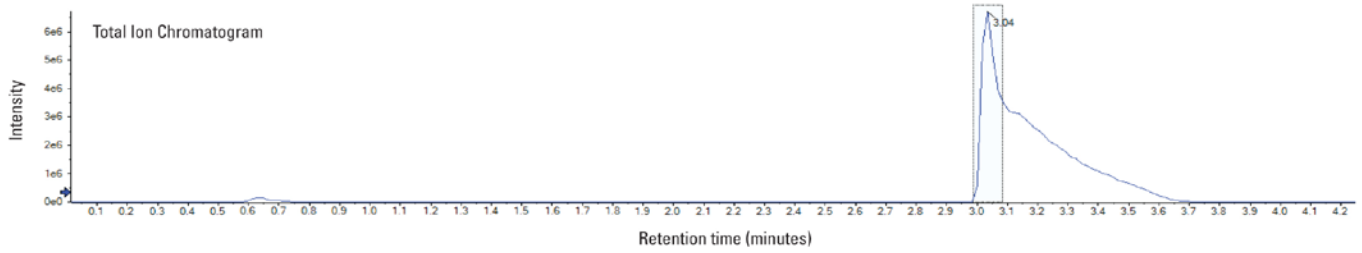
Offline MS Analysis

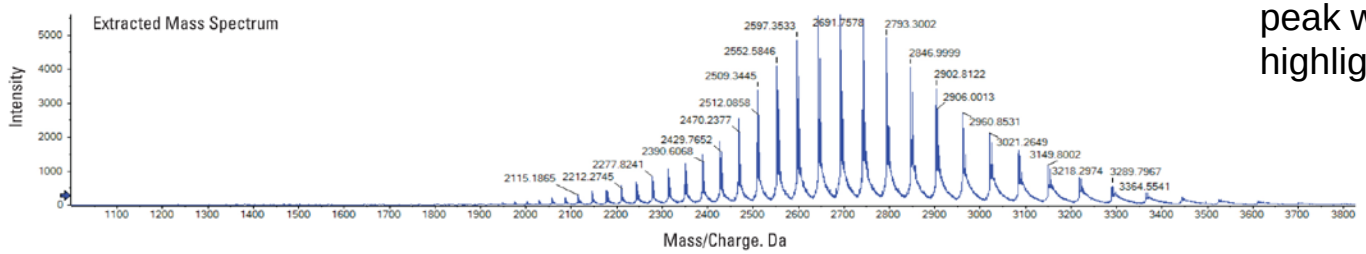
Reversed Phase LC-MS

Instrument:	Sciex M3 MicroLC
Column:	C4 stationary phase, 3.0 µm, 1.0 mm ID × 5 cm
Mobile phase:	A: water with 0.1% formic acid B: water with 0.1% formic acid
Flow rate:	0.1 mL/min, gradient 20% B to 80% B in 6 min
Temperature:	60º C
Detection:	Sciex TripleTOF® 6600, ESI+, 1000-5000 m/z, 1.0 s accumulation time
Injection vol.:	1 uL
Sample:	Ipilimumab standard, 500 µg/mL peak 1 fraction, 25 µg/mL peak 2 fraction, 15 µg/mL peak 3 fraction, 2.5 ug/mL

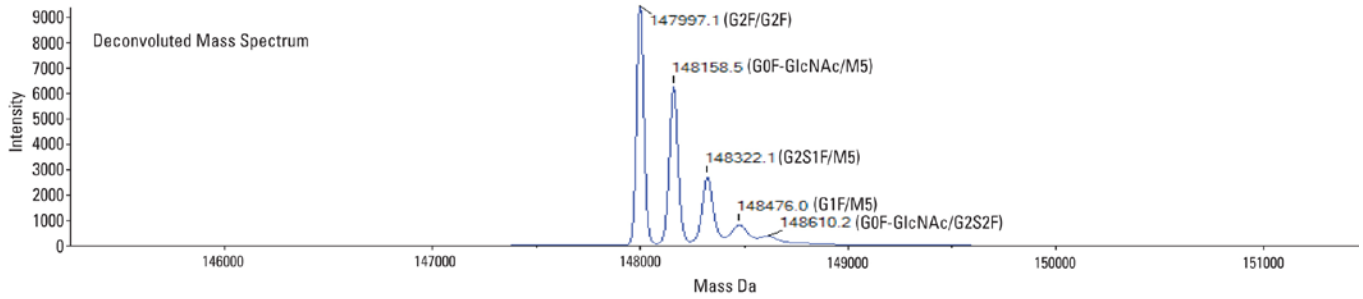
Offline MS Analysis: Results

Figure 5: RPC/MS Analysis of Ipilimumab



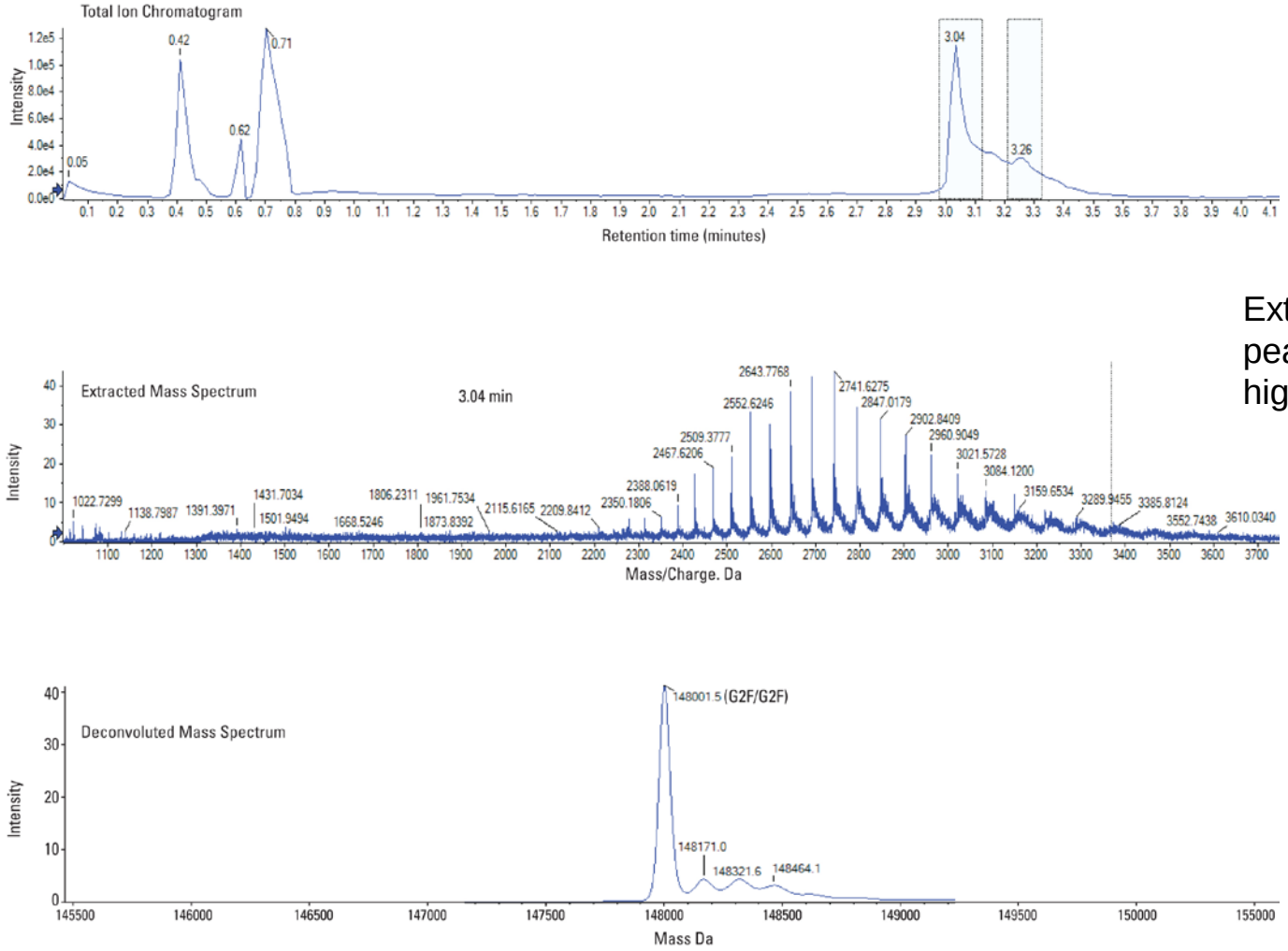


Extracted spectra of the mAb peak was taken from the highlighted region at 3.04 min.



Offline MS Analysis: Results

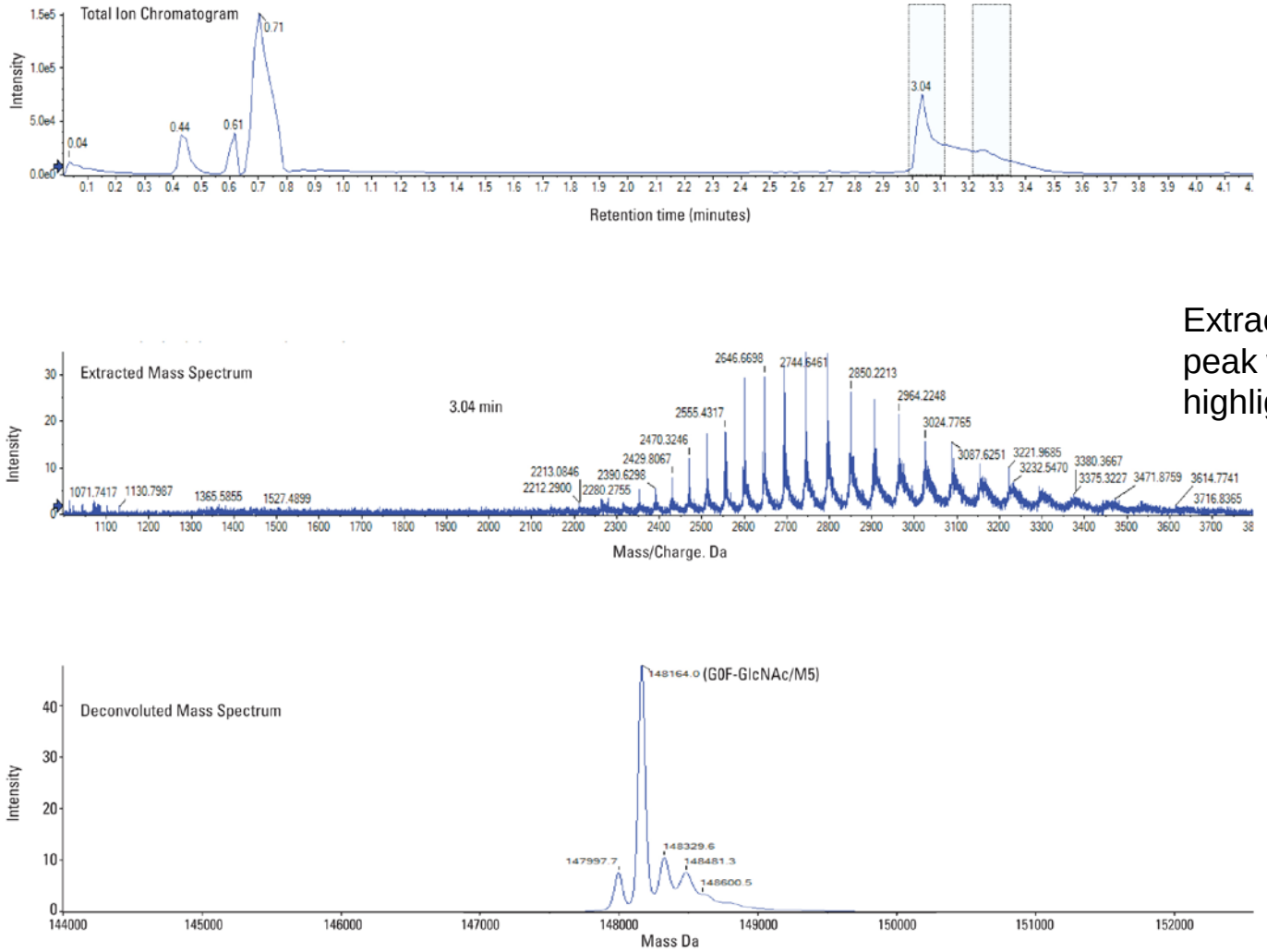
Figure 6: RPC/MS Analysis of Fraction 1 from FcR Affinity Column Separation



Extracted spectra of the mAb peak was taken from the highlighted region at 3.04 min.

Offline MS Analysis: Results

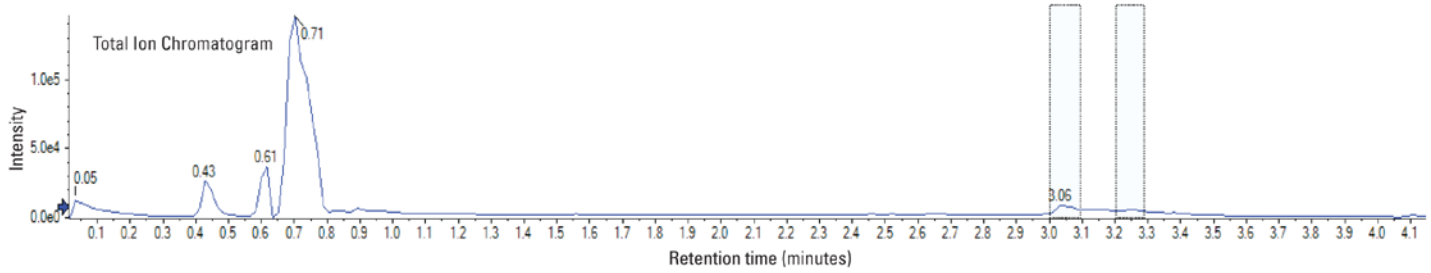
Figure 7: RPC/MS Analysis of Fraction 2 from FcR Affinity Column Separation

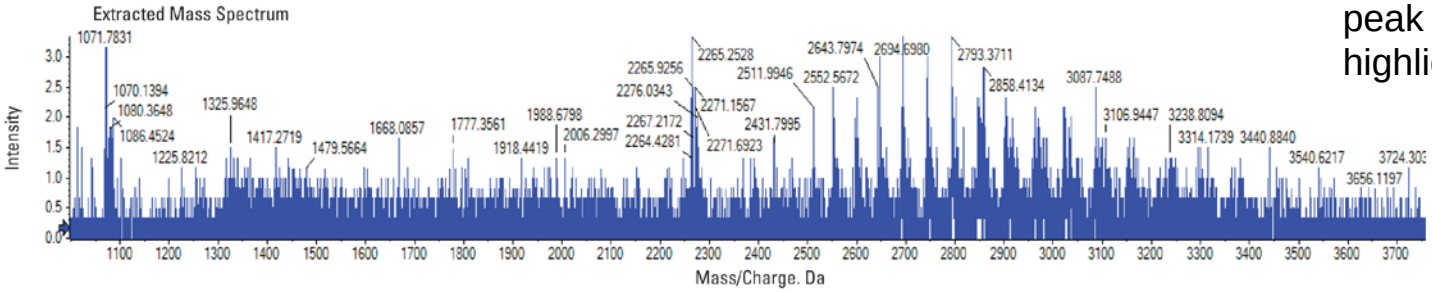


Extracted spectra of the mAb peak was taken from the highlighted region at 3.04 min.

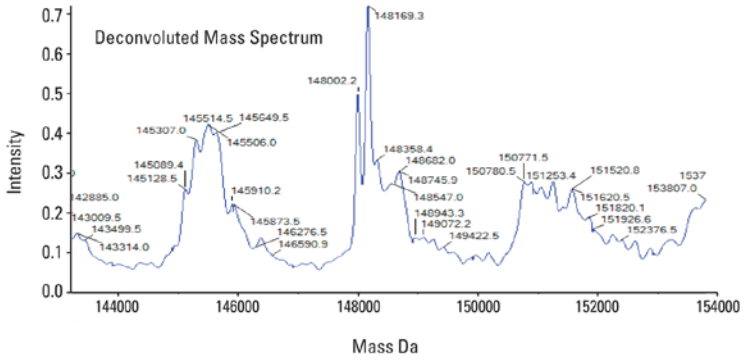
Offline MS Analysis: Results

Figure 8: Total Ion Chromatogram and Extracted Spectra for Peak Fraction 3 from FcR Affinity Column Separation



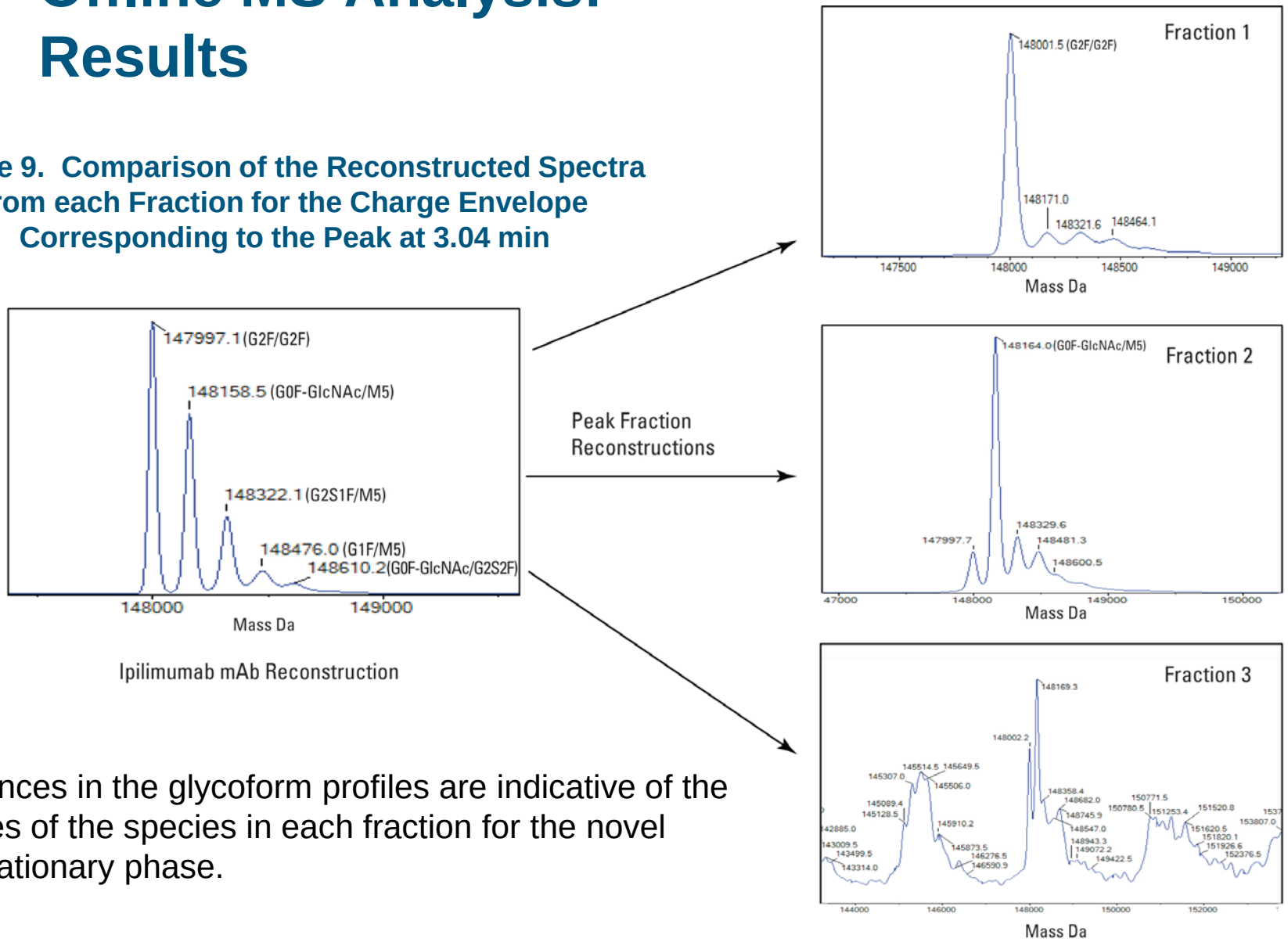


Extracted spectra of the mAb peak was taken from the highlighted region at 3.04 min.



Offline MS Analysis: Results

Figure 9. Comparison of the Reconstructed Spectra from each Fraction for the Charge Envelope Corresponding to the Peak at 3.04 min



Differences in the glycoform profiles are indicative of the affinities of the species in each fraction for the novel FcR stationary phase.

Offline MS Analysis: Conclusions

- The reconstructed spectra for each fraction show distinctly different masses, suggestive of different glycoforms.
- The presence of difference glycoforms in each fraction demonstrates the efficacy of the original separation and provides a method for characterization of these species using MS data that may have otherwise gone undetected.
- The peak at 3.04 min was identified as the mAb, but other peaks in the chromatogram suggest the analyte may not have been stable in the elution buffer from the first separation; spectral analysis of these other degradation products may elucidate more features of the mAb glycobiology.

Offline MS Analysis: Conclusions (cont.)

- Original chromatographic conditions were adapted to a volatile buffer suitable for MS analysis. The resulting fractions made for fast offline MS characterization and the conditions have potential for an online, native style analysis.
- Deconvolution software was used to identify the major species in each reconstructed error with a mass accuracy of +/- 2 amu. This tandem approach for analyzing mAb glycoforms was capable of elucidating intact structural information of these molecules.

Future Work

- Subunit N-glycan analysis of Ipilimumab for comparison to existing offline separation data to aid in glycoform identification
- Further optimization of the LC method for MS compatibility; study pH, flow rate and temperature in using higher amounts of volatile salt
- Optimize MS conditions for potential online analysis

References

1. Shinkawa T *et al., J.Biol. Chem.* **278**, 3466-3473 (2003).
2. Pablo Umana *et al*., *Nature Biotech*. **17** FEB, 176-180 (1999).