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Applikationsbericht

Similis Bio - Streamlined mAb Subunit LC-MS Workflow for Multiple Attribute Monitoring of Biosimilar mAb Candidates During Bioprocessing and Development

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Abstract

This application note summarizes a collaboration between Similis Bio and Waters Corporation™ to demonstrate the feasibility of an automated Protein A purification and LC-MS mAb subunit analysis of bioreactor samples to screen biosimilar monoclonal antibody (mAb) candidates in comparison to the innovator to support process development. The method was demonstrated to have sufficient sensitivity and precision to quantitatively assess N-linked oligosaccharides at levels known to impact pharmacokinetics and effector function activity, in addition to other post-translational modifications (PTMs) such as C-terminal lysine variants and other subunit-specific (LC & Fd') modifications. Subunit mAb LC-MS monitoring methods such as this can be utilized to support mAb bioprocessing, development, and quality control within biopharmaceutical and biomanufacturing organizations.

Benefits

- Automated ProA purification of cell culture samples with subsequent FabRICATOR™ subunit digestion using

the Andrew+™ Pipetting Robot

- Simplified LC-MS acquisition using BioAccord™ LC-MS System, a benchtop TOF MS designed for ease of operation for analysts of all levels of expertise
- Utilization of the waters_connect™ Intact Mass App for streamlined automated data acquisition and analysis with compliance-ready features to ensure data integrity
- Localization of Fab vs Fc modifications is possible using this mAb subunit LC-MS method
- Demonstrated orthogonality between mAb subunit LC-MS workflow and released N-glycan assay results

Introduction

The development of biosimilars has gained significant momentum in recent years due to their potential to provide more affordable and effective treatments for a range of diseases. However, the development of biosimilars is a complex and challenging process that requires extensive characterization and comparison to the reference product. Clone selection, early in the development lifecycle, has an outsize impact on the ability to match the quality target product profile for a given product. N-linked oligosaccharide profiles and common PTMs, such as C-terminal lysines, sidechain oxidation and glycation, may vary between clones as a result of expression dynamics and cellular stress responses. Therefore, the evaluation of these product quality attributes during clone selection can reduce subsequent process development costs by selecting a clone with a profile that closely matches the innovator. Additionally, design-of-experiment (DoE) studies to evaluate cell culture feed media and supplementation generate many samples that require testing across numerous analytical methods to guide process development. This creates significant analytical testing burdens and necessitates rapid turnaround times to enable iterative development workflows.

A rapid, automated at-line mAb subunit attribute monitoring workflow (Figure 1) was used to comparatively screen mAb biosimilar product quality attributes during clone selection and upstream process development. Biosimilar samples taken from Sartorius Ambr250™ High Throughput bioreactors were subjected to an automated Protein A (ProA) affinity capture and subunit enzymatic digestion with Genovis FabRICATOR using the Andrew+ Pipetting Robot. The purified and FabRICATOR-digested samples were then analyzed via intact mass analysis of the liberated subunits using the BioAccord LC-MS System. The Intact Mass App within waters_connect Informatics Platform was used to automatically acquire, process, and visualize data as each

sample completed acquisition.

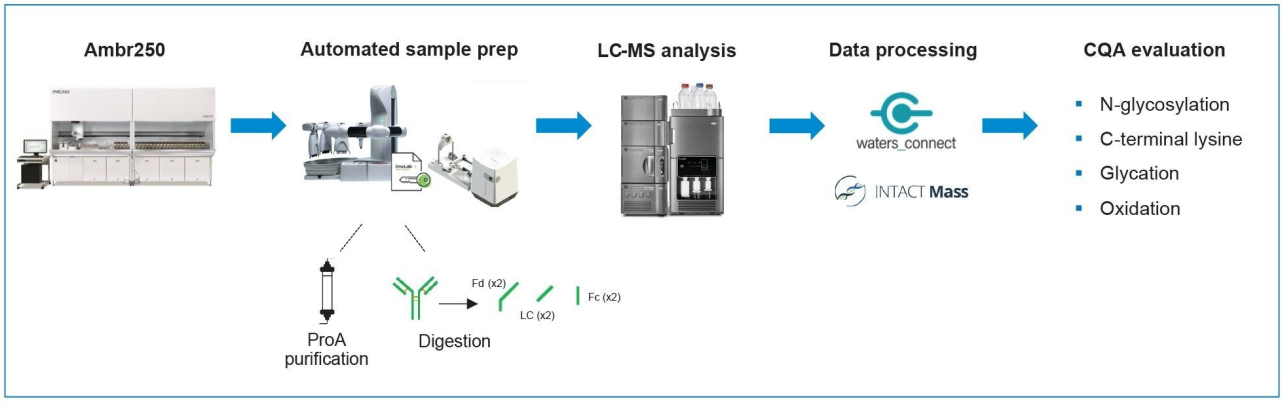

Figure 1. mAb Subunit MAM workflow, with automated sample preparation using Andrew+ Robotics Platform for ProA purification & FabRICATOR digestion, followed by LC-MS data acquisition and processing on the BioAccord LC-MS System with waters_connect informatics.

This higher-throughput method provided N-linked glycosylation profile, unprocessed C-terminal lysine abundance, glycation, and oxidation levels comparable to orthogonal analytical techniques for each quality attribute, while also providing significant benefits in terms of automation, scalability, and throughput. Method sensitivity for high mannose and afucosylated glycans are of particular interest to decision makers, as these species can have a significant impact on mAb pharmacokinetics (pK) and effector function activity at a low relative abundance. This mAb subunit LC-MS workflow is successful in meeting data sensitivity, precision, and reproducibility requirements for early biosimilar development. A similar mAb subunit LC-MS monitoring workflow has been recently published by Genovis, demonstrating that the BioAccord System is an ideal platform for routine and efficient product quality attribute monitoring at the mAb subunit level.[1]

Experimental

Sample Description (Automated Purification)

Cell culture samples were purified with an Andrew+ Robotics Platform (equipped with the Extraction+ module)

workflow that was published recently.[2] Briefly, using a 0.2 µm filter plate, each well was supplied with ProA resin, washed, and the sample was loaded. Each sample was first washed, then eluted into a collection plate containing neutralization buffer (Fig. 2A). Two clones were used to produce distinct cell culture samples for the same product, called "Biosimilar" and "Biosimilar -Negative Control" below.

Sample Description (Subunit Digestion)

Using Andrew+ automation (including the Heater/Shaker+ module), 20 µL of the resulting ProA-purified sample (and innovator sample (commercial drug product)) at ~1 mg/mL, were transferred to a PCR plate for a 1-step subunit digestion with partial subunit reduction. 10 µL of a prepared master mix (Fig. 2B) (2 units/µL FabRICATOR enzyme, 50 mM Tris, 10 mM DTT, pH 7.5) was added to each sample well, and the plate was incubated at 37 °C for 30 minutes (Fig. 2C). Samples were diluted 5x (to 0.1 mg/mL) with 0.1% formic acid in water for LC-MS analysis (Fig. 2D).

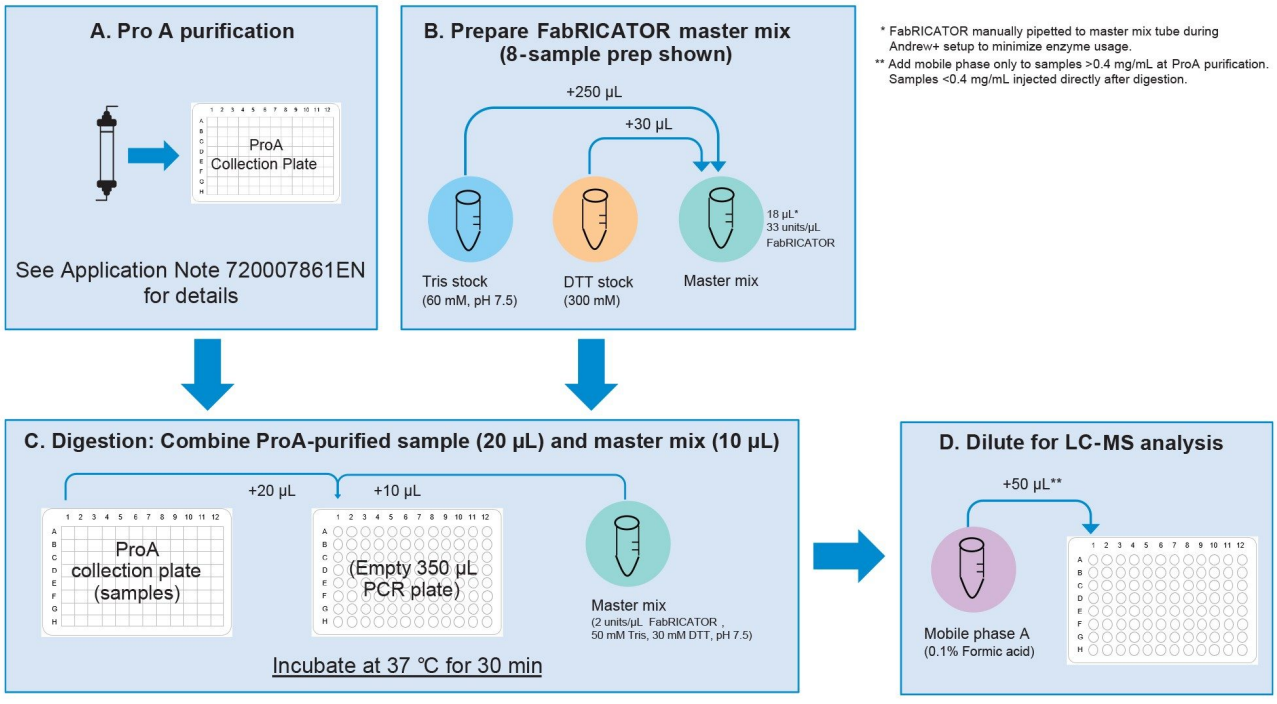

Figure 2. Subunit sample preparation using Andrew+ Pipetting Robot.

Protein A & FabRICATOR Sample Preparation

Robotics system:	Andrew+ Pipetting Robot with Extraction+ and Heater/Shaker+ Modules
Software:	OneLab (Andrew Alliance / Waters)
Filter plate:	Pall AcroPrep™ Advance 96-well Filter Plates- 350 µL, 0.2 µm Supor™ membrane (p/n: 8019)
Collection plate:	QuanRecovery™ 700 µL 96-well plate (p/n: 186009184)
Subunit digestion plate:	Eppendorf twin.tec™ PCR Plate 96 LoBind™ (***e.g.*** p/n: 0030129555)
Protein A resin:	Cytiva MabSelect™ (p/n: 17519901), slurries ~25% (1:1, PBS:50% resin). For 50% resin, centrifuge at 1000 x g for 3 mins and replace supernatant with volume of 400 mM NaCl, 20% ethanol equal to resin volume.
PBS:	Phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4, pH 7.4)
Neutralization buffer (NB):	1M Tris HCl, pH 7.5
Elution buffer (EB):	100 mM Glycine, pH 3.0
Orbital shaker:	Eppendorf Thermomixer® C (8 °C)
FabRICATOR enzyme:	Genovis (p/n: A0-FR1-020)
Dithiothreitol (DTT):	Pierce No-Weigh DTT (p/n: A39255)

LC Conditions

LC system:	ACQUITY™ Premier UPLC System
Detection:	ACQUITY UPLC TUV (280 nm)
Column(s):	BioResolve™ RP mAb Polyphenyl, 2.7 µm, 2.1 x 100 mm (p/n: 186008945)
Column tempertaure:	60 °C
Sample temperature:	6 °C
Injection:	0.5 µg FabRICATOR-digested mAb (5 µL injection of 0.1 mg/mL sample)
Flow rate:	0.3 mL/min
Mobile phase A:	0.1% Formic Acid in Water
Mobile phase B:	0.1% Formic Acid in Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.3	80	25	6
10.0	0.3	60	40	6
10.3	0.3	20	80	6
11.3	0.3	20	80	6
11.6	0.3	80	25	6
15.0	0.3	80	25	6

MS Conditions

MS system:	ACQUITY RDa™
Ionization mode:	ESI Positive
Acquisition range:	400–7000 *m/z* (High Mass)
Capillary voltage:	1.5 kV
Cone voltage:	50 V
Desolvation temperature:	550 °C

Data Management

Data was acquired and processed through the Intact Mass App (v 1.4.0.0) in waters_connect Informatics Platform (v 3.1).

Results and Discussion

mAb subunit LC-MS attribute monitoring workflows offer a compromise of shortened 15-minute sample analysis times while still providing a means monitoring vital localized product quality attributes of innovator and biosimilar mAbs.[3–7] Here we demonstrate an automated ProA purification and FabRICATOR subunit digestion of mAbs sampled at-line from Ambr250 bioreactors and analyzed via LC-MS. Using the Acquire and Process function within the Intact Mass App (Figure 3), the user can set up an analysis sequence, monitor real-time data readouts, and view deconvoluted/processed data as each injection completes data acquisition. The ability to view processed data while the instrument is running subsequent samples enables quicker decision-making which benefits the development process.

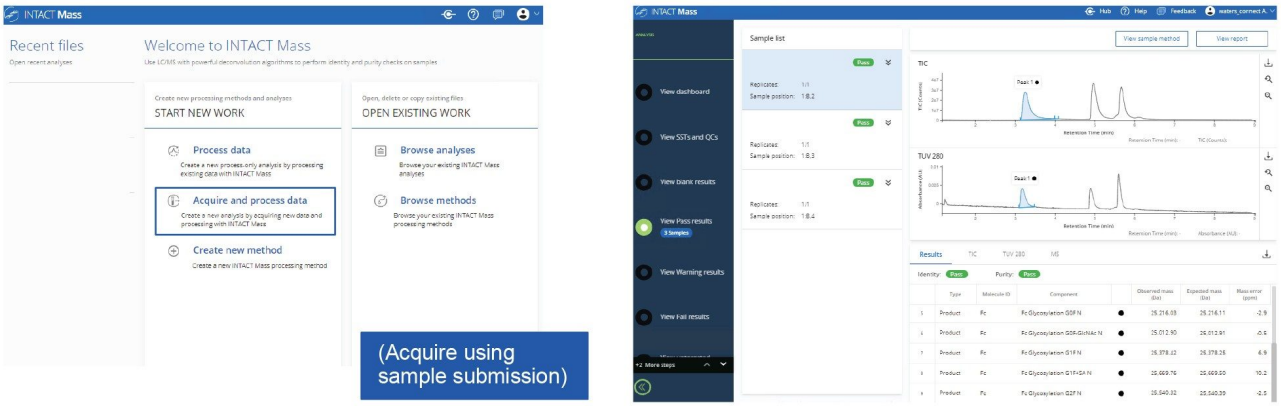

Figure 3. Integrated acquisition and data processing using the Intact Mass App within the waters_connect Informatics Platform. Data was acquired through Sample Submission App and automatically processed by Intact Mass App after each injection was acquired.

The primary aim of this study was to determine if the sensitivity and precision of the mAb subunit LC-MS attribute monitoring approach for Man5 and afucosylated N-glycosylation levels of biosimilar mAb samples reduces the need for other orthogonal assays. FabRICATOR digestion with reduction of inter-chain disulfide bonds produces three protein chains to monitor via RPLC-MS (Fc, LC, & Fd'), as shown (Figure 3 chromatogram), all having masses between 23–25 kDa. The N-glycosylation site is located on the Fc subunit. The MaxEnt1-deconvoluted spectra for the Innovator (Figure 4A, top), Biosimilar (Figure 4A middle), and a Biosimilar-Negative Control (Figure 4A bottom), the latter known to contain high levels of Man5. The Innovator and Biosimilar share relatively consistent N-glycoprofiles but differ significantly in the level of unprocessed C-terminal lysine present in the Biosimilar (0% and 15%, respectively in Figure 4B). The Biosimilar-Negative Control, as expected, is observed with roughly 10% Man5 species, while the Biosimilar sample contains lower levels similar to the Innovator (Figure 4B).

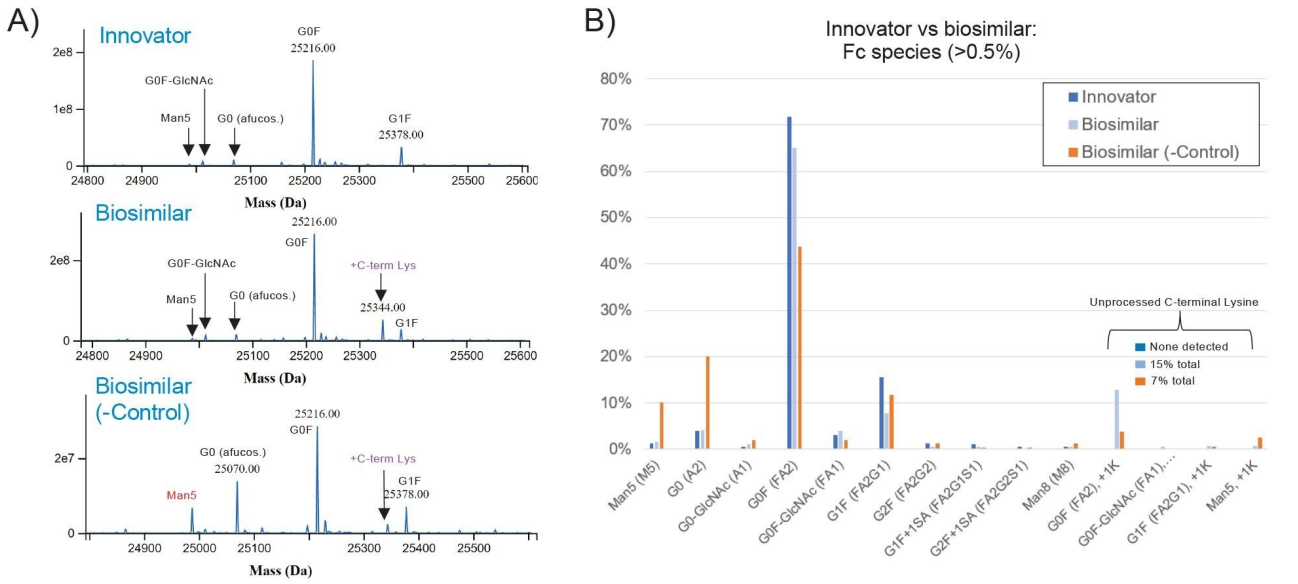

Figure 4. A) Deconvoluted spectra for Fc species generated by the Intact Mass App, comparing innovator, ProA purified biosimilar, and negative control biosimilar sample (containing high Man5 level); B) Relative quantitation of Fc species, including N-glycoforms and unprocessed C-terminal lysine.

The Similis Bio Standard Operating Procedure (SOP) for monitoring glycan levels has been a released N-glycan assay, where glycans that are enzymatically released from the mAbs are labelled with a fluorescent tag (RapiFluor-MS) and analyzed via HILIC-FLR-MS.[8] This method is very sensitive and robust, but it provides only a very targeted set of information limited to the N-glycans present in the sample. If a mAb subunit LC-MS method could produce comparable results to those from the released glycan assay, plus provide additional quality attribute information about the mAb, it could replace the current workflow and potentially additional assays. The LC-MS results for the top five abundant N-glycoforms* (Fig 5A) were compared to the released glycan assay results performed in-house at Similis Bio. The difference in relative percentage (Δ%) for each sample and species is plotted in Figure 5B. All values for relative percentage of these N-glycan species detected in LC-MS subunit analysis were within 2% of the reported value from released glycan. This demonstrates acceptable comparability of results between the two methods.

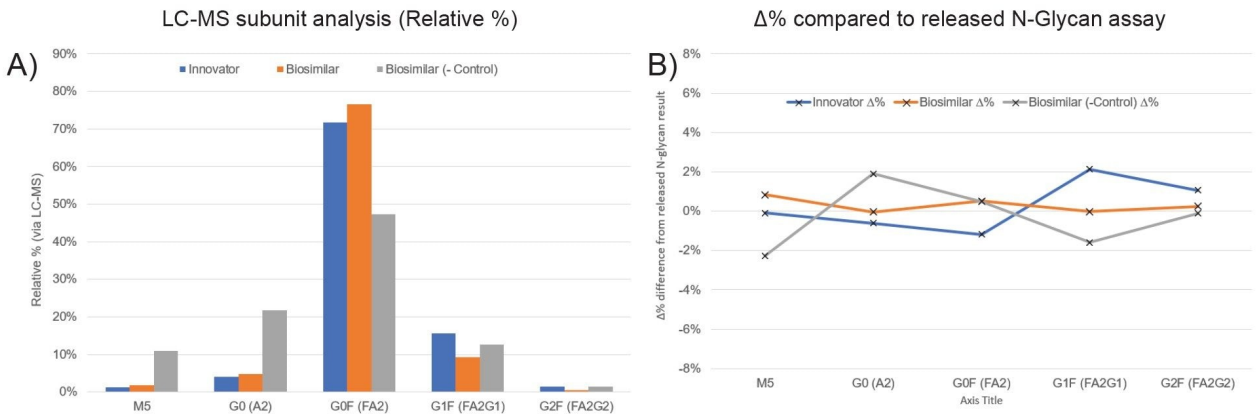

Figure 5. Comparison of Fc Subunit and released RFMS N-glycan results (Top 5 abundant species). Panel A: Relative % detected via LC-MS subunit method (adjusted for C-terminal lysine variants not observable in released N-glycan assay). Panel B: Difference (Δ%) between LC-MS & the released N-glycan assay results.

In addition to the N-glycoprofile, the mAb subunit LC-MS monitoring method provided insights into other quality attributes such as the unprocessed C-terminal lysine, Fd' & LC glycation, and oxidation. Similar efforts have been shown in subunit assays for process development and subsequently validated for mAb QC implementation.[9, 10]

These attributes would normally be assessed via orthogonal methods such as peptide mapping (Optical or LCMS) or using indirect optical LC and CE methods at the peptide, intact, or subunit level. C-terminal lysine, for instance, could be investigated by comparing the charge variant profiles for an untreated sample vs the same sample treated with carboxypeptidase B (CPB) enzyme (for removal of the remaining positively charged C-terminal lysine). In the charge profile, the unprocessed C-terminal lysine species usually appear as basic variants, which then decrease with CPB digestion. Logically, the difference in basic species between the samples should give a rough estimation of unprocessed C-terminal lysine present in the sample. This approach is generally very useful for mAbs with simple charge profiles, but as the complexity increases, the more ambiguous the analysis becomes. The demonstrated mAb subunit LC-MS method simplifies the analysis of this attribute.

Additionally, this mAb subunit LC-MS monitoring method can provide a view of potential modifications on the Fd' and LC chains, which contain the parts of the sequence responsible for binding, and therefore the activity, of the mAb drug product. These potential modifications include, but are not limited to, glycation and oxidation and/or unconverted N-terminal pyroglutamic acid (non-pyroQ). (Note, the oxidation and non-pyroQ modifications are not readily distinguished at subunit level by LC-MS.) In this case study, low levels of Fd' and LC

glycation (<2%) were detected for both the Innovator and Biosimilar samples. In the case of oxidation/non-pyroQ, there was no observable level for the LC in either Innovator or Biosimilar, but the Fd' species contained 10–15%. To distinguish the Fd' oxidation and/or non-pyroQ species, further investigation would be required.

To test the robustness of the mAb subunit LC-MS monitoring method, the same Innovator (drug product) sample was prepared at two different sites (manual vs automated FabRICATOR digestion), and the samples were run on a BioAccord System located at each of the labs. The results for all Fd', LC, & Fc species of >0.5% relative abundance (Fc species shown in Fig 6) were consistent (within 1.5%). The mAb subunit LC-MS method, performed with manual or automated sample preparation, is found to be robust and reliable across multiple laboratories.

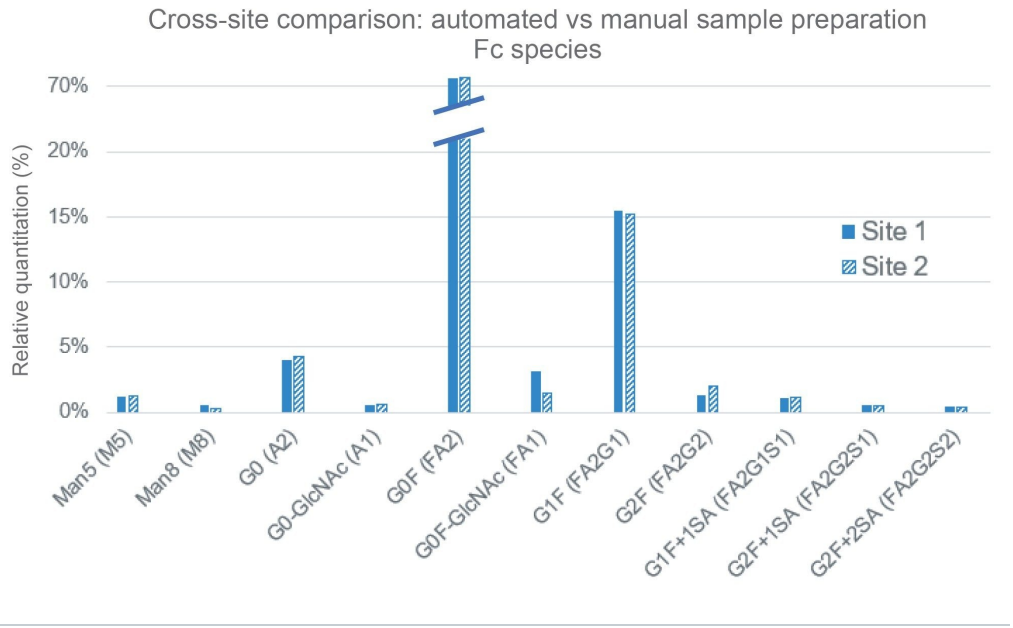

Figure 6. Comparable Fc N-glycosylation results generated in a cross-site comparison (Site 1=automated sample preparation, Site 2=manual sample preparation).

**LC-MS subunit N-glycoform relative percentages were adjusted for the comparison to the released glycan assay results by combining values with and without the terminal lysine residue. This was to exclude the response generated from C-terminal lysine-containing species, which would not be accounted for by released glycan assay.*

Conclusion

The collaborative effort between Similis Bio and Waters Corporation has demonstrated the utility of a workflow for monitoring of select product quality attributes of mAbs using subunit LC-MS analysis of an innovator and of biosimilar drug candidates sampled directly from a bioreactor. The at-line workflow consists of automated ProA purification and FabRICATOR digestion using the Andrew+ Robotics Platform, followed by LC-MS analysis using a BioAccord System. The Intact Mass App allows for seamless data acquisition, monitoring, and processing- all within the compliance-ready waters_connect Informatics Platform. The results generated using this mAb subunit LC-MS workflow are consistent with released glycan results for both Innovator and Biosimilar samples. The same workflow also provided insights into other quality attributes including the unprocessed C-terminal lysine, glycation, and oxidation, reducing the time and effort needed to acquire this information through orthogonal methods.

References

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