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Nota de aplicación

Establishment of a Robust mAb Subunit Product Quality Attribute Monitoring Method Suitable for Development, Process Monitoring, and QC Release

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Abstract

This application note presents a subunit MAM method as a complement to peptide MAM approaches in biopharmaceutical development. Typical peptide mapping MAM methods provide a more targeted capability for attribute-based analysis but are challenged by laborious sample preparation, longer run times (lower throughput), and more complex data analysis. A subunit-based MAM approach addresses each of these challenges, while providing an opportunity to monitor a wide range of biotherapeutic product attributes.

Benefits

- Faster, simpler sample preparation with fewer method-induced artifacts
- Shorter LC-MS data acquisition and processing times
- Additional information gained compared to an intact mAb analysis
- Compliance-ready waters_connect Informatics for streamlined automated data analysis

Introduction

Biopharmaceutical developers of both innovators and biosimilars face increased competitive pressures to be faster to the market, lower their costs, and keep their reputations for quality high. It is important to characterize and monitor critical quality attributes (CQAs) throughout the drug development process, and increasingly extend these assays for process monitoring and lot release. Therefore, the analytical methods used for CQA monitoring must be as robust, sensitive, and fast as possible.

LC-MS peptide mapping multi-attribute method (MAM) gained popularity due to the wealth of information that it provides compared to traditional optical-only detection methods. However, many labs using this approach find that sample preparation can introduce method-induced artifacts and suffer from irreproducibility. In addition, the data acquisition is usually lengthy, limiting throughput, resulting in data sets that can be quite complex. In recent years, biotherapeutic developers turned to mAb subunit MAM analysis to produce critical information more quickly and robustly. For example, Dong ***et. al*** established a method for automated purification, subunit digestion, and LC-MS analysis of mAbs for cell culture process monitoring.[1] Through this workflow, they were able to monitor glycosylation profiles and nonezymatic lysine glycation in near real time and make adjustments to the cell culture process. A similar method was used by Sokolowska et al, in which Fc subunit methionine oxidation was found to be a critical product attribute and was monitored following photo and chemical stress studies.[2] This GMP compliant Xevo QTof LC-MS method has been validated for use in QC for commercial product release and stability studies.[3]

In this study, we demonstrate the implementation of a subunit MAM method on two additional Tof-based MS systems (BioAccord and Vion) to monitor antibody glycosylation, glycation, oxidation, and sequence variants. This method demonstrates that subunit based analysis is a core capability of Tof based LC-MS platforms and when deployed on a compliant-ready informatics platform, such as UNIFI/waters_connect, can be utilized to support mAb development, in manufacturing, and quality functions within a biopharmaceutical organization.

Experimental

Sample Description

50 µg antibody sample was incubated with 50 units of Fabricator (IdeS) enzyme (Genovis) in digestion buffer (25 mM NaCl, 25 mM Tris, 1 mM EDTA, pH 8.0), at a final concentration of 1 mg/mL, for 1 hour at 37 °C. DTT (dithiothreitol) was then added to a final mAb concentration of 5 mM for a partial reduction of inter-chain disulfides by incubation for 30 min at 37 °C. For the deglycosylated samples, 50 µg antibody sample was incubated with PNGaseF* at 37 °C, at final mAb concentration of 1 mg/mL, prior to the IdeS digestion and reduction steps.

*For 50 µg sample, 4 µL PNGaseF from the *Rapi*Fluor-MS Kit was used and scaled up as needed.[4] All samples were diluted to 0.1 mg/mL with 0.1% formic acid in water prior to analysis.

Method Conditions

LC Conditions

LC system:	ACQUITY UPLC I-Class
Detection:	ACQUITY UPLC TUV
Vials:	QuanRecovery with MaxPeak HPS 12 x 32 mm Screw Neck Vial, 300 µL (P/N: 186009186)
Column(s):	Waters ACQUITY BEH C_4 300 Å, 1.7 µm, 2.1 x 50 mm (P/N:186004495)
Column temp.:	80 °C
Sample temp.:	6 °C
Injection:	0.5 µg IdeS-digested mAb (5 µL injection of 0.1 mg/mL sample)
Flow rate:	0.25 mL/min

LC Conditions

Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile

Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.25	80	20	6
10.0	0.25	60	40	6
10.3	0.25	20	80	6
11.3	0.25	20	80	6
11.6	0.25	80	20	6
15.0	0.25	80	20	6

MS Conditions

MS System	ACQUITY RDa	Vion IMS QTof
Ionization mode	Positive	Positive
Acquisition range	400–7000 *m/z* (High Mass)	500–4000 *m/z*
Capillary voltage	1.5 kV	2.75 kV
Cone voltage	50 V	70 V
Desolvation temp.	550 °C	600 °C
Source temp.	N/A	125 °C

Data Management

Data was acquired, processed, and reports generated using the UNIFI v1.9.4 Intact Protein Analysis workflow.

Results and Discussion

Multi-attribute methods show great promise to provide direct and selective product attribute analysis for biopharmaceutical drug candidates. Here we demonstrate a fast and efficient subunit MAM method, using IdeS enzyme to cleave the mAb at the hinge region followed by reduction of inter-chain disulfide bonds to generate free light chain (LC), Fd, and Fc subunits (see Figure 1) of ~25 kD mass. The free subunits were subjected to reverse phase (RP) chromatography in a 15-minute LC method and the resulting resolved peaks were analyzed via mass spectrometry for qualitative and relative quantitative analysis.

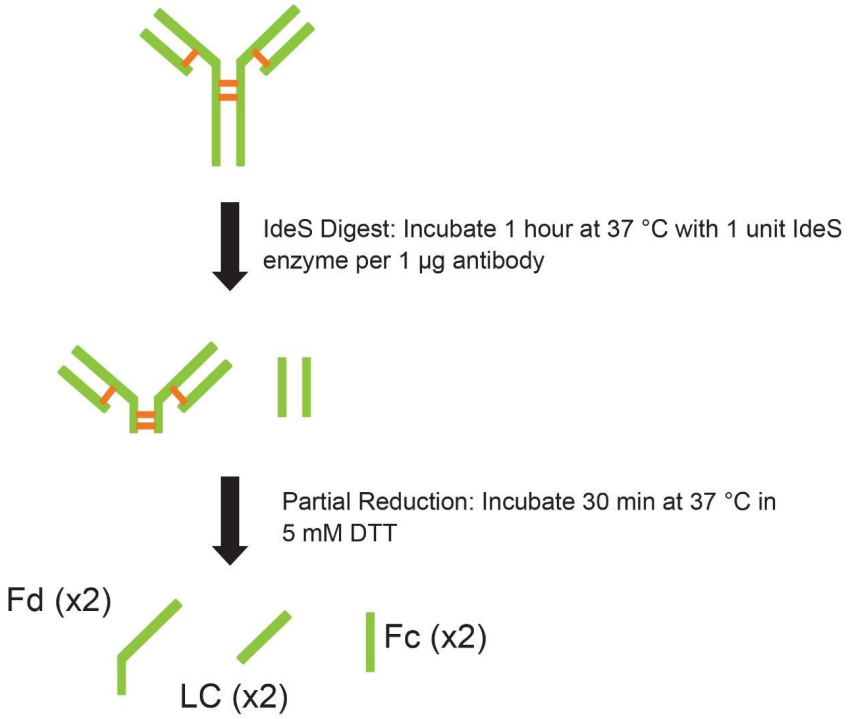

Figure 1. Sample preparation for mAb Subunit MAM, including IdeS digestion and partial reduction.

MAM methods destined for process monitoring, QC release testing, and other studies conducted within regulated labs must be robust and reproducible.[5] To assess the robustness of subunit attribute based analysis on the BioAccord System, triplicate injections of an IdeS-digested trastuzumab sample were analyzed on two

systems with the same set of three columns evaluated on each system. Acquired mass spectra for each of the three subunit peaks (Fd, Fc, and LC) were automatically deconvoluted using MaxEnt1 and the resulting masses were matched to trastuzumab species during automated UNIFI data processing (using a 10 ppm mass accuracy threshold). Relative percentages of glycosylated and glycated species were calculated via integrated MS response of the deconvoluted mass peak. LC and Fd glycation were measured at 1.6% and 1.2%, respectively, with less than 8% RSD evident over all injections (n=18). Fc N-glycosylated species ranged from 0.1 to 40% abundance (as shown in Figure 2) and all Fc glycosylation species over 0.5% relative abundance had an abundance variation less than 5% RSD across all injections. This assessment is well within typical expectations for assays supporting CQA monitoring during bioprocessing or product release. After demonstrating the primary robustness of this methodology, it was applied to three case studies similar to analyses typically performed to support developability, clone selection, and formulations.

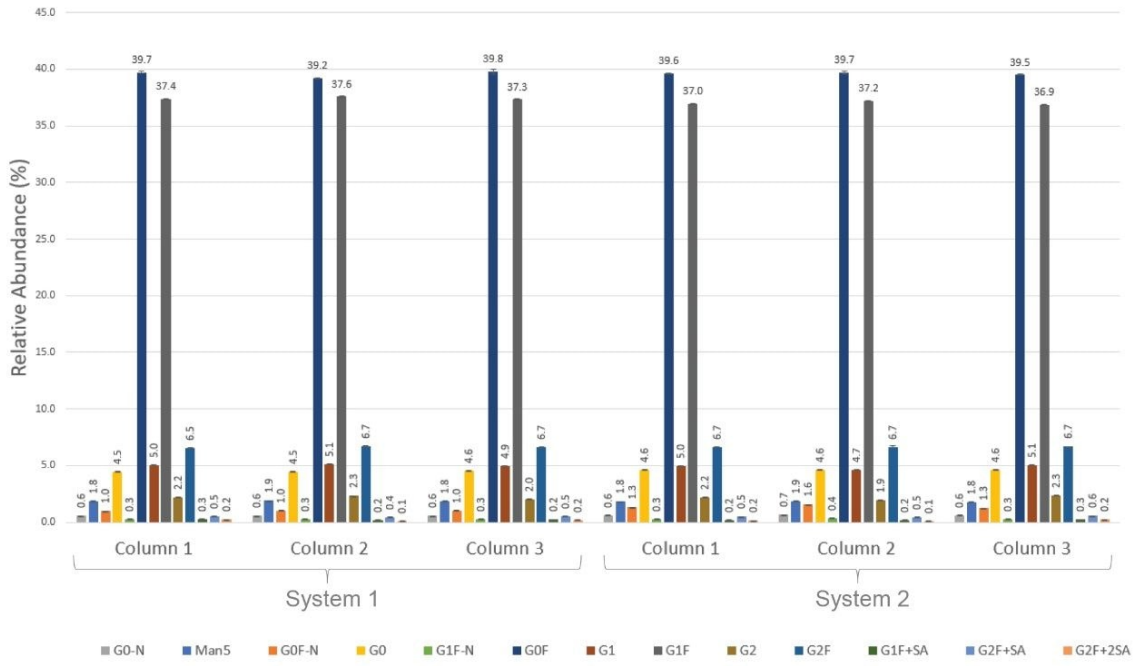

Figure 2. Trastuzumab Fc N-glycosylation species analyzed via subunit MAM method on two BioAccord systems, three columns each, triplicate injections. For all species >0.5% relative abundance, %RSD is <5%.

The first study relates most closely to questions of developability that might be asked during clone screening and

those questions typical for cell culture process monitoring. We applied this subunit method to glycoprofiling of cetuximab, an antibody with an N-glycosylation site in the Fd region of the heavy chain, in addition to the typical IgG1 Fc N-glycosylation sites on the heavy chains (Figure 3). Traditionally, N-glycans are profiled by released glycan assays such as 2AB labelling using HILIC-FLR analytical system or HILIC-FLR-MS using MS enhancing tag, such as *Rapi*Fluor-MS.[4] However, if a released glycan assay is used in the case of cetuximab, one would obtain the global picture of all N-glycans present but not specific to which site they occupy. This information is important because Fd and Fc glycosylation have differing effects on antigen recognition, immunogenicity, and serum half-life.[6] The IdeS-digested cetuximab sample was analyzed in triplicate and the results for Fc and Fd are displayed in Figure 4. The observed Fd N-glycosylated species (right) are more complex branched structures than the typical Fc N-glycosylation profiles (left). The calculated %RSD for relative abundance of all Fc and Fd glycosylated species was less than 3% in these analyses. These observations are consistent with previously published findings, in which an IdeS subunit digest of cetuximab was separated, fraction collected, and a released N-glycan assay was performed for isolated Fc and Fd separately.[7] With the use of a subunit MAM method, fractionation of the Fd and Fc is not necessary to localize the glycoforms associated with each domain. An additional advantage of using this subunit MAM method is that the Fc C-terminal lysine variant and other CQAs can be monitored simultaneously within the same analysis.

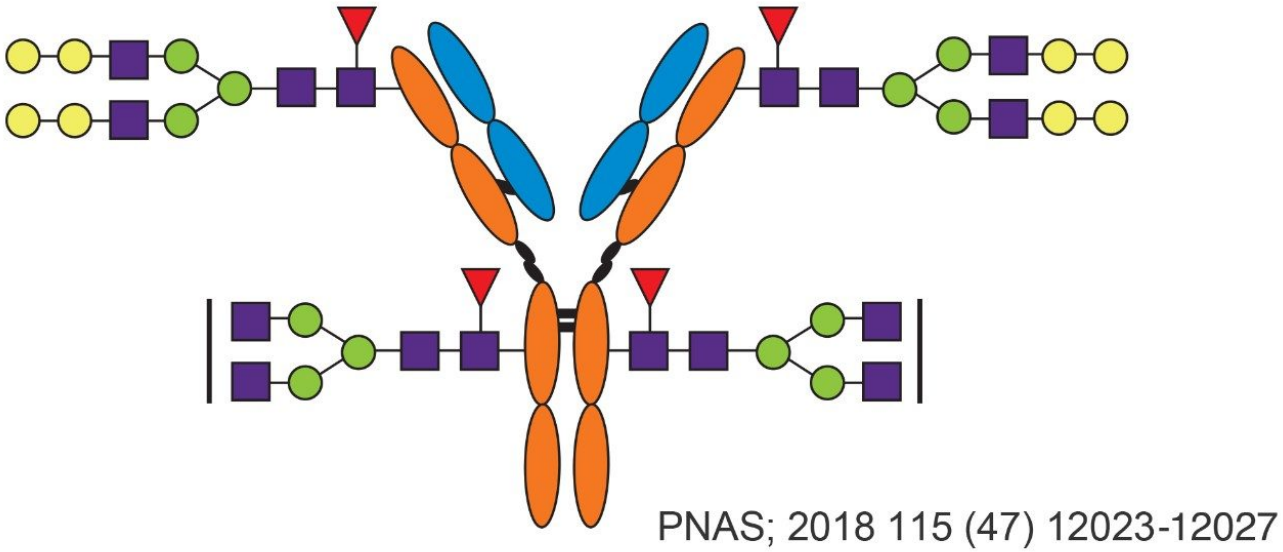

Figure 3. Depiction of cetuximab showing the N-glycosylation sites in the Fd in addition to the typical sites in Fc.

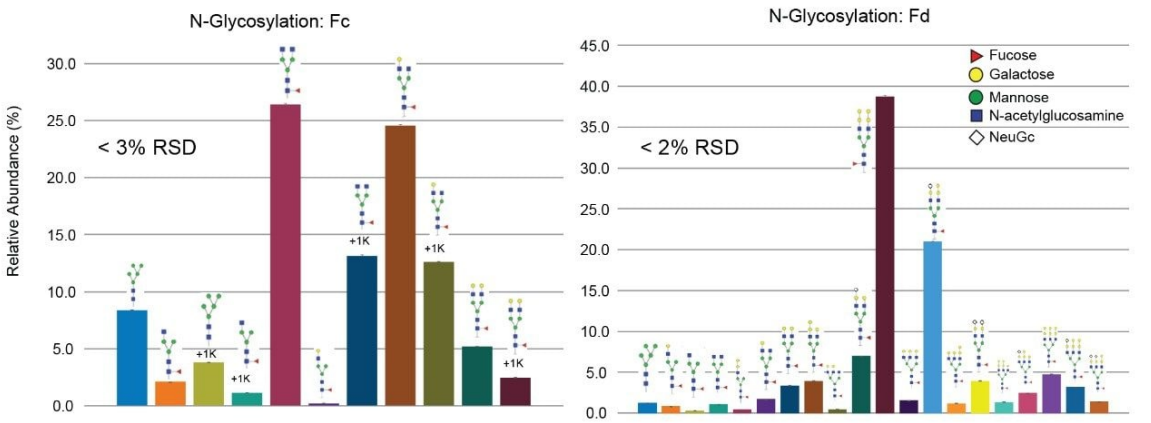

Figure 4. Cetuximab Fc and Fd N-glycosylation species.

The second case study demonstrates how a subunit MAM approach could be applied to clone selection or process development to monitor product sequence variants. These can occur during production as a result of misincorporation of amino acids due to sequence mutation or suboptimal cell culture conditions.[8] To mimic a typical analysis, we used a sample of trastuzumab containing 3 known point mutations – one in the light chain (V104L), resulting in +14 Da mass shift and two in the Fc (E359D and M361L), resulting in a combined -32 Da mass shift. This sample was spiked into originator trastuzumab at levels between 0.5%–50% and analyzed for accuracy in quantitation and linearity. For this study, the samples were deglycosylated prior to IdeS digestion to simplify data analysis of the spiked Fc sequence variants on a BioAccord System. Figure 5A shows a representative component plot with masses for LC, Fd, and Fc subunits conformed within the 10 ppm tolerance and expected lower-level sequence variants for LC and Fc detected with the same criteria. Relative percentages agreed with the expected spiked value and results were linear (R^2 = 0.9994) over the 1–50% range (Figure 5B).

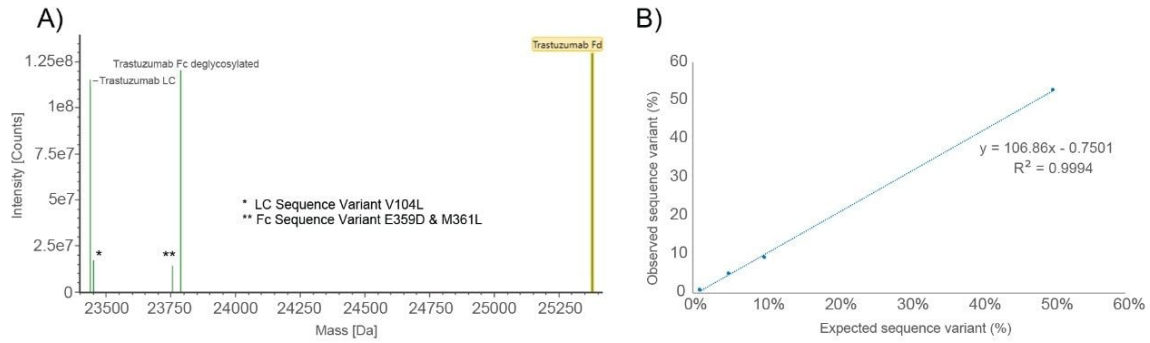

Figure 5. Trastuzumab sequence variant analysis.
(A) Component plot for 10% sequence variant spiked sample with automatically labelled LC, Fd, Fc, and the two sequence variant species. LC sequence variant is labelled with (*) and Fc sequence variant species is labelled with (**). (B) % observed sequence variant vs % expected sequence variants, linear from 1–50%.

The final example is a forced oxidation experiment that mimics efforts common to formulations and product stability studies. Oxidation that occurs during storage of a final drug product can affect its efficacy and therefore impact its shelf life. A control sample of the NIST Reference mAb was stressed with 0.003% or 0.01% hydrogen peroxide (H_2O_2) for 24 hours at room temperature prior to IdeS digestion, reduction, and subunit MAM analysis on a Vion IMS System. The LC and Fd subunits proved resistant to the stress conditions but a significant increase in oxidation was observed for the Fc subunit (Figure 6). The 0.003% H_2O_2 treatment level converted almost all of the Fc species to an oxidized (single and double oxidation) form with a further shift towards doubly oxidized species for the 0.01% H_2O_2 stressed sample.

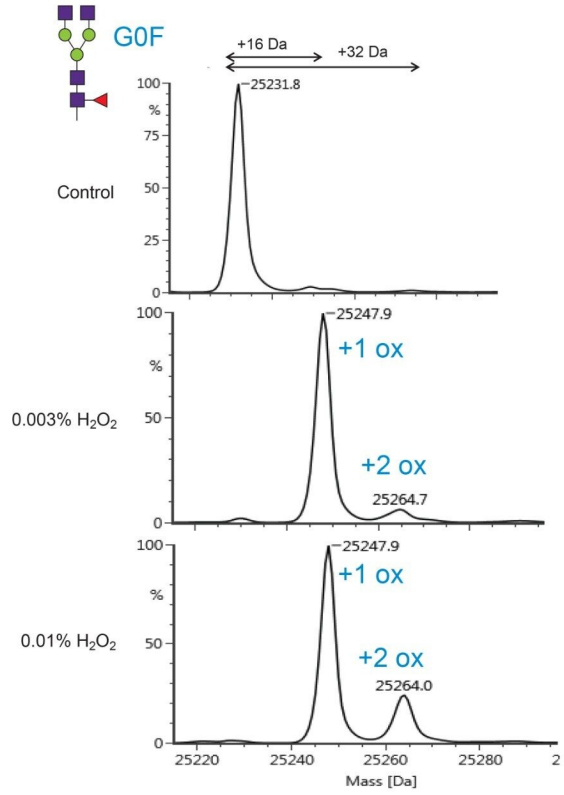

Figure 6. NIST mAb forced oxidation study, Fc species. MaxEnt1 deconvolution (zoom of G0F species), showing +1, +2 oxidation following incubation in 0.003% and 0.01% hydrogen peroxide (H_2O_2).

While subunit MAM methods offer a potentially faster and more efficient way to analyze for mAb CQAs, there are two practical limitations to consider. First, the monitored CQAs must be mass resolved by protein mass spectrometry. For example, isobaric and near-isobaric species such as isomerization and deamidation will not be resolved using this method and resolution of smaller modifications, such as Met oxidations (+16 Da), are more easily quantified on an 25kD IdeS subunit than a 50kD reduced heavy chain. Second, subunit MAM can only localize the modifications to the LC, Fd, or Fc subunit and the presence of multiple instances of a modification on a given subunit may confound direct interpretation of the results. For these situations, site-specific peptide mapping approaches may be required.

Conclusion

In this work we demonstrated subunit based monitoring of mAb quality attributes across multiple TOF platforms. Subunit MAM based analysis is more amenable to higher sample throughput and creates less complex data than peptide based MAM methods. These benefits come with potential limitations on the selectivity for specific attributes residing within the same subunit and an inability to monitor deamidation and isomerization based attributes. However, common CQAs such as glycosylation profile, glycation, oxidation, and product sequence variants can be monitored using this simpler approach. The BioAccord and Vion systems, operating under the compliance-ready waters_connect/UNIFI informatics platform, show excellent reproducibility and repeatability for this type of analysis consistent with previous extensive studies with the Xevo QTof platform.

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720007129, January 2021