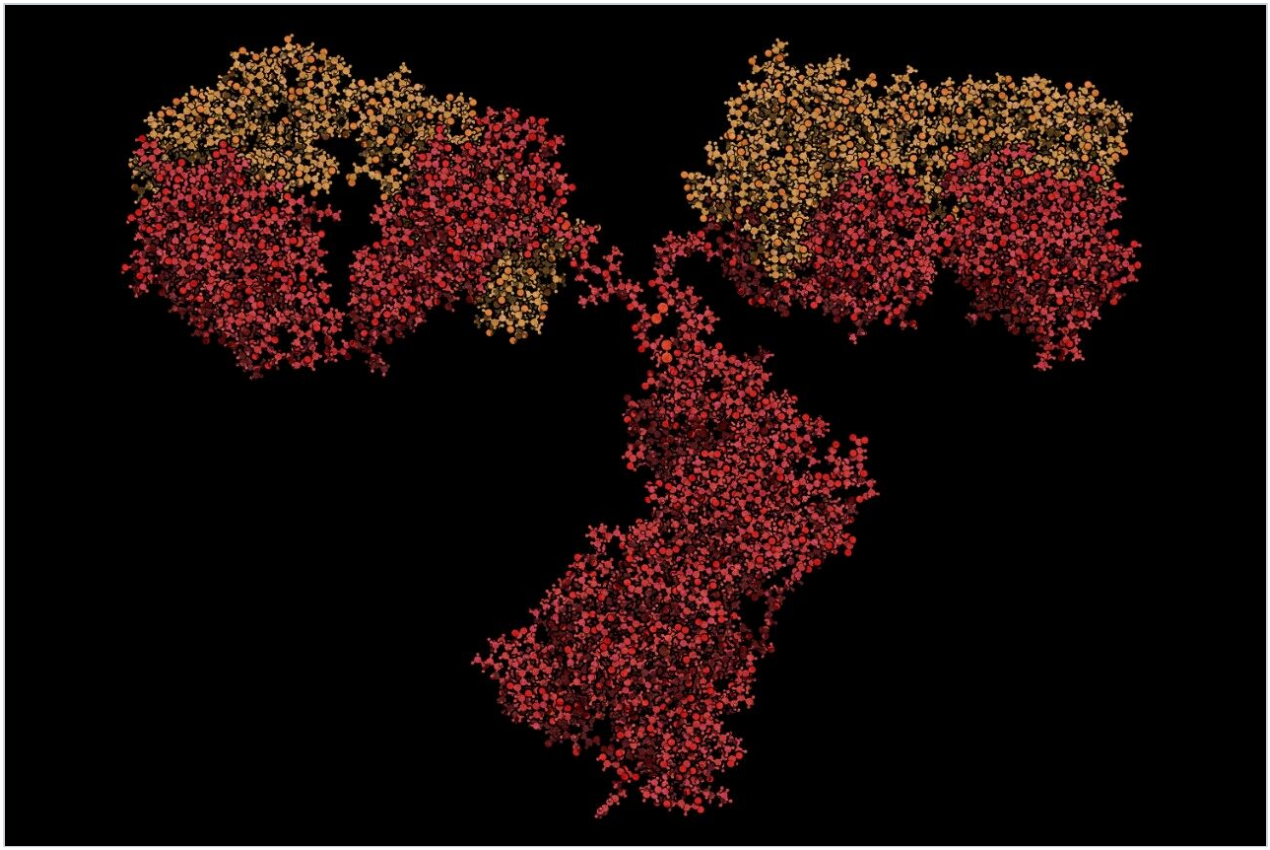
Waters™

Nota applicativa

An Integrated LC-MS Platform for Multi-Attribute Monitoring of CQA's of mAbs at the Subunit Level

Nilini Ranbaduge, Henry Shion, Ying Qing Yu, Weibin Chen

Waters Corporation



Abstract

This application note demonstrates the use of LC-MS platform under compliant-ready software (UNIFI Scientific Information System) for Fc-core fucosylation and aglycosylation confirmation and monitoring their relative levels. Details on sample preparation, LC-MS setup and data processing are discussed in this application note.

Benefits

- Streamlined workflows that can be used for subunit level MAM in Waters UNIFI Scientific Information System
- Consistent, easy-to-use data processing method with MaxEnt 1 deconvolution suitable for subunit level CQA monitoring with minimum modifications to default parameters
- Automated relative abundance measurements and reporting for CQAs

Introduction

Many protein modifications of monoclonal antibody (mAb) products, in addition to their influence on structural heterogeneity, have profound impact on function, efficacy, pharmacokinetic properties, and safety. Such modifications are known as critical quality attributes (CQAs) of the mAb. Quality-by-Design (QbD) principles supported by the US Food and Drug Administration (FDA) mandates a thorough understanding of these CQAs in the early stages of manufacturing. Implementation of QbD requires deployment of monitoring methods to assess the levels of these critical quality attributes at pre and post-development in addition to the QC environment.

Several biopharmaceutical companies have developed mass spectrometry-based methods that confirm and relatively quantify multiple CQAs of mAbs. This approach is commonly referred to as multi-attribute monitoring method (MAM). While some methods use a peptide mapping approach[1-2] most recently, mAb subunit level methods have made its way from biopharmaceutical characterization[3] to process development and quality control[4] as a viable approach that benefit from simple sample preparation and reduced data complexity. Subunit level analysis of mAb modifications has been successfully deployed for process development and optimization as well as QC release testing, and was initially developed on a QTof mass

spectrometer platform for rapid LC-MS analysis and monitoring CQAs such as glycosylation and methionine oxidation.[5-6]

Fucosylation of N-glycan core GlcNAcs affects the potency of the mAb through antibody-dependent cell-mediated cytotoxicity (ADCC),[7-8] and is an important CQA to monitor during process development, optimization, product manufacturing, and quality control. Here, we demonstrate the use of a similar LC-MS platform under compliant-ready software (UNIFI Scientific Information System) for Fc-core fucosylation and aglycosylation (Figure 1) confirmation and monitoring their relative levels. Details on sample preparation, LC-MS setup and data processing are discussed in this application note. In addition, we also performed instrument-to-instrument and day-to-day repeatability assessment to demonstrate that this method has the potential to be validated according to ICH guideline Q2R1.

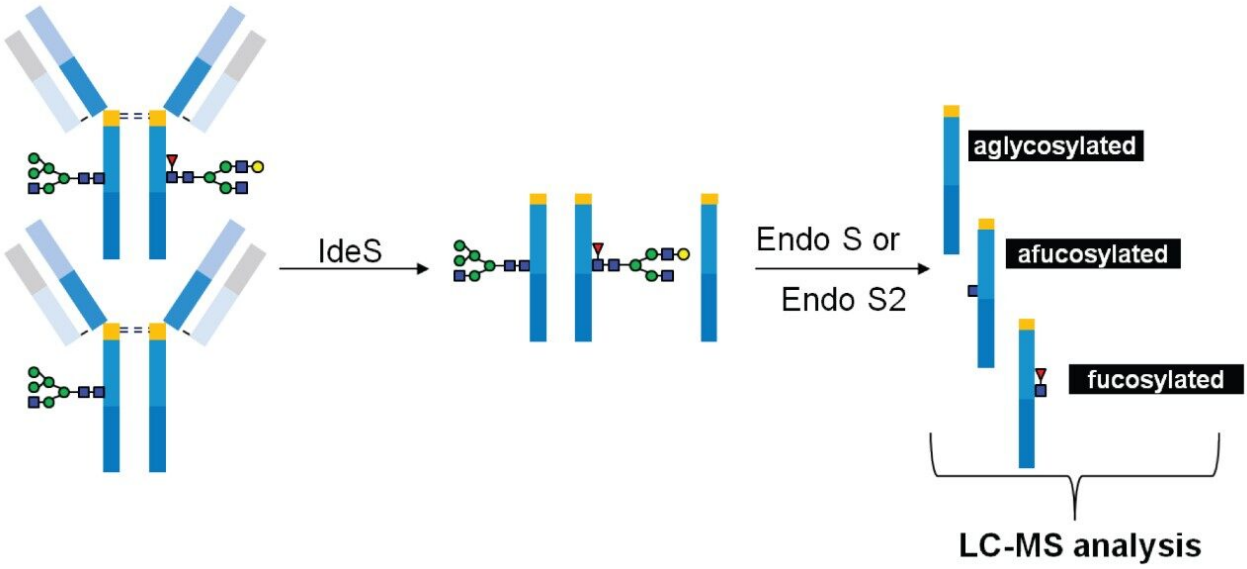


Figure 1. The digestion of trastuzumab with IdeS cleaves the mAb in the hinge region generating scFc and F(ab) fragments. Further digestion with Endoglycosidase cleaves the glycan structure between the two core GlcNAc's at the reducing end of the N-glycans.

Experimental

Sample information

Trastuzumab (samples from two different batches)

Reagents, solvents, and sample preparation

Subunit sample preparation

An aliquot of formulated trastuzumab (20 mg/mL) was diluted in 25 mM ammonium acetate buffer and digested with FabRICATOR/IdeS (Genovis, Cambridge, MA, USA) and Endoglycosidase (GlycNATOR, Genovis, Cambridge, MA, USA) enzymes (1:1 enzyme to mAb ratio by weight) at 37 °C for 1.5 h. The final concentration of the solution was adjusted using 3% acetonitrile, 0.1% formic acid to 0.01 mg/mL for injection.

The trastuzumab samples were treated with both Endo S (IgGZERO)/IdeS and Endo S2/IdeS separately to determine the level of high mannose structures in the sample using the change in Fc-afucose levels.

LC conditions

Column type:	BEH C_4 (P/N 186004495)
Column temperature:	80 °C
Sample temperature:	6 °C
Wavelength:	280 nm

LC gradient

Time (min)	Flow rate (mL/min)	0.1% FA in H_2O (%)	0.1% FA in ACN (%)
Initial	0.40	95.0	5.0
1.00	0.40	95.0	5.0
1.10	0.40	74.0	26.0
3.00	0.40	70.0	30.0

Time (min)	Flow rate (mL/min)	0.1% FA in H_2O (%)	0.1% FA in ACN (%)
3.10	0.40	5.0	95.0
4.00	0.40	5.0	95.0
4.10	0.40	95.0	5.0
8.00	0.40	95.0	5.0

Vion IMS QTof MS settings

MS system:	Vion IMS QTof
Mass range:	m/z 500–4000 Da
Data acquisition:	ESI positive sensitivity mode
Capillary voltage:	2.75 kV
Cone voltage:	70 V
Source offset:	80 V
Source temperature:	125 °C
Desolvation temp.:	600 °C
Desolvation gas low:	600 L/h
Lock mass:	Glu-Fibrinopeptide B (P/N 700004729) at 320 fmol/µL in 50/50 H_2O/ACN, 0.1% FA

Informatics for data acquisition and processing

UNIFI Scientific Information System 1.8.2

Vion IMS QTof with Driver pack 2.0

The UNIFI intact protein workflow method was used in core fucosylation analysis of trastuzumab subunits. The total ion chromatogram peaks for selected retention times were integrated and the full charge distribution of the summed spectrum was used in MaxEnt1 deconvolution (Figure 2). Custom field formulas for protein modification quantification in UNIFI were used for fucosylation% and aglycosylation% determination. Relative abundance of each modification was calculated as a percentile of total MS response for afucose, fucose, and aglycosylated scFc.

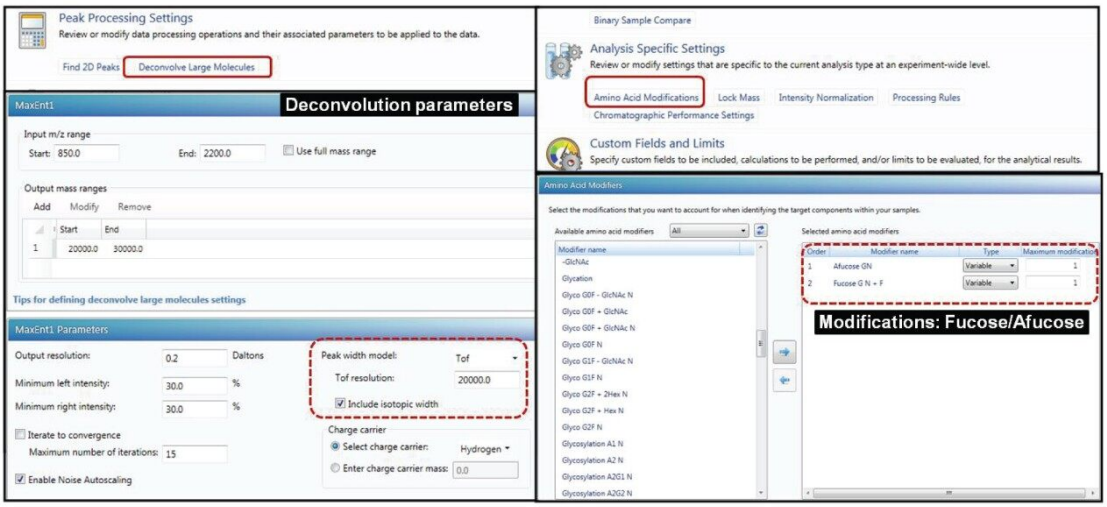


Figure 2. Subunit data was processed in UNIFI 1.8.2 using MaxEnt 1 deconvolution.

Results and Discussion

UPLC separation and MS analysis

The MS response-based core Fc-fucosylation assessment of the subunit components provides a simple, direct analysis method compared to peptide mapping and released glycan analyses. Enzymatic digestion of trastuzumab with Endo S2 cleaves the β, 1-4 glycosidic bond between the two core N-acetylglucosamines (GlcNAcs) leaving scFc with one GlcNAc ± an α, 1-6-fucose attached. This simplifies the glycosylated scFc

into two basic components: afucosylated scFc (+1 GlcNAc) and fucosylated scFc (+1 GlcNAc +1 Fucose) in addition to the aglycosylated scFc form. These scFc subunit components eluted in one peak during UPLC separation (Figure 3). BEH C_4 Column was used with a 3.0 min LC-gradient for the LC-MS analysis. The scFc peak eluted at 2.6 minutes.

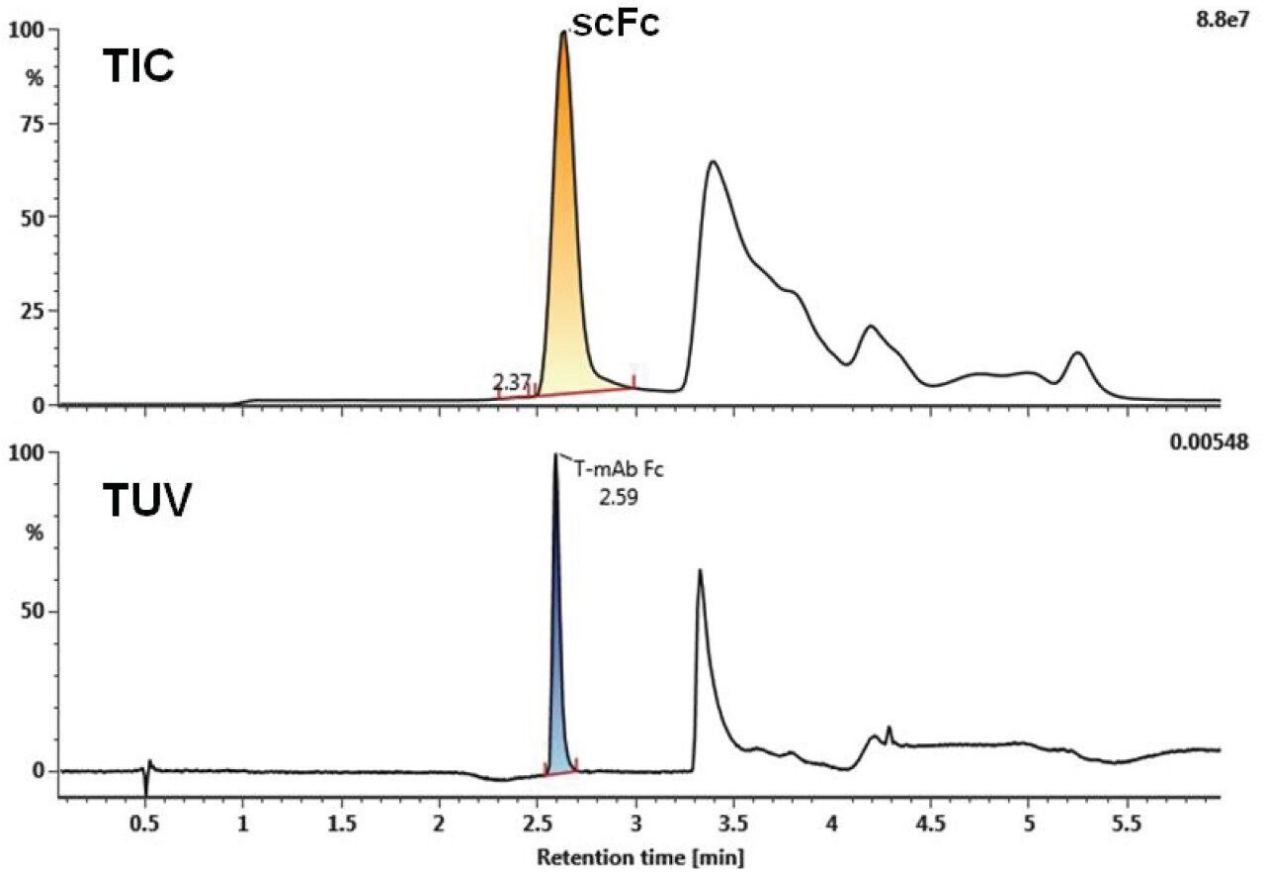


Figure 3. Trastuzumab subunit treated with Endo S2 was analyzed by reversed-phase chromatography coupled to a Vion IMS QTof System. The 3.0 min gradient method was used to resolve the scFc subunits from other components for the MS analysis.

For the LC-MS analysis, 0.1 µg (on-column) of digested trastuzumab from two different batches (Trastuzumab-1 and Trastuzumab-2) was used and MS spectra for both samples were processed using UNIFI processing parameters given in Figure 2. Figure 4 shows the deconvoluted mass spectrum for Trastuzumab-1 containing aglycosylated, afucosylated and fucosylated scFc subunits with a mass accuracy of less than 5 ppm. The relative abundance of each modification was determined based on the total MS response for these peaks. The UNIFI software platform has the ability to calculate and present the data in a user selected format such as a bar graph that can be easily incorporated into the final report (Figure 4). As shown in the bar

graphs in Figure 4 the two samples contain equal amounts of aglycosylated scFc form, while Trastuzumab-2 has the highest MS response for afucosylation.

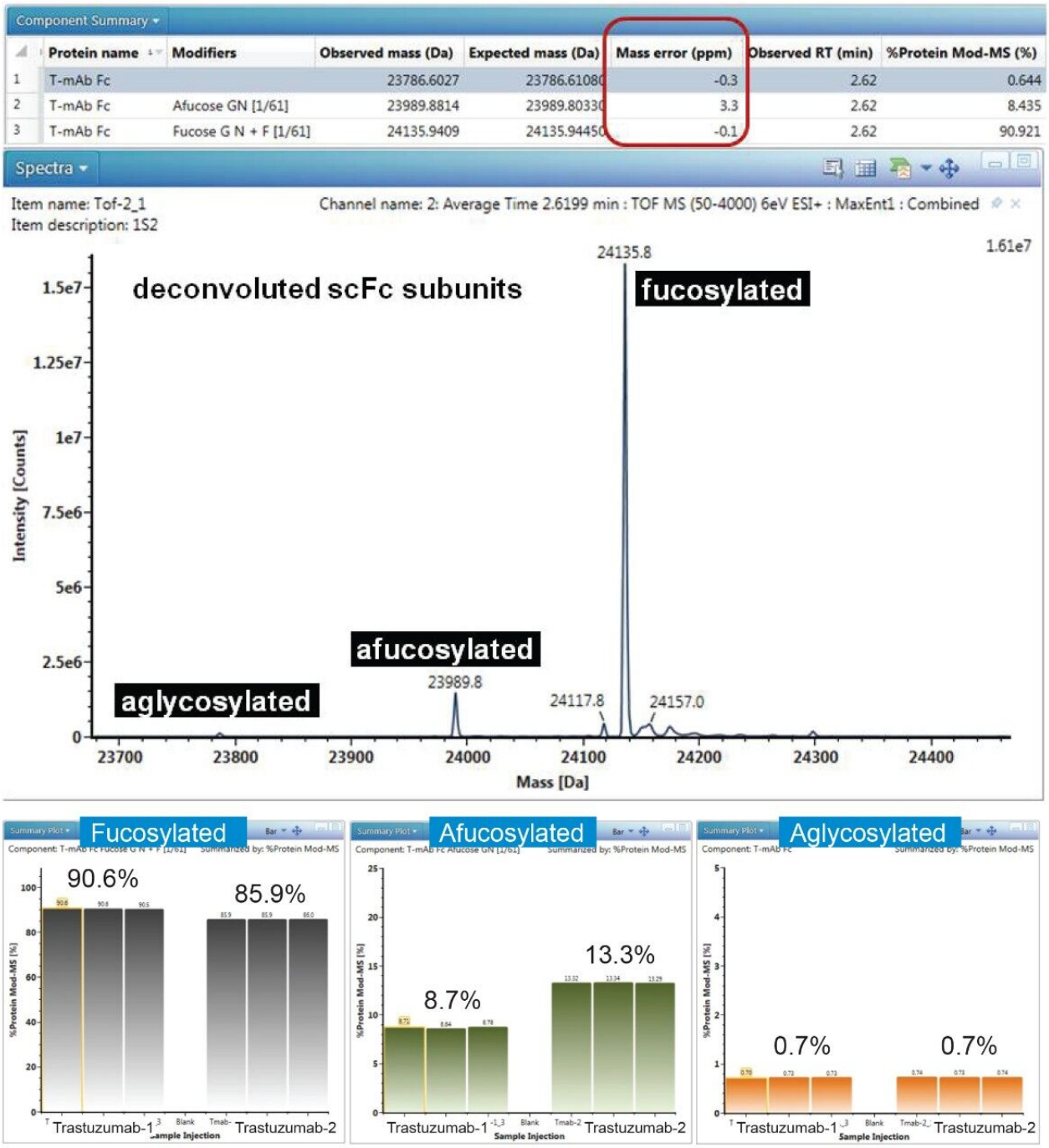


Figure 4. The component summary for one injection representing the expected and observed masses and the respective delta mass difference (ppm) for fucosylated, afucosylated, and aglycosylated scFc subunits. We observed less than 5 ppm mass accuracy for the identified components. The bar graphs at the bottom present the summary of %relative abundance measured for the respective modifications in both Trastuzumab-1 and Trastuzumab-2 samples.

Analytical assays implemented in regulated cGMP environment require validation for repeatability to establish its stability as described in ICH guidelines. Pharmaceutical companies interested in incorporating mass spectrometry into their CQA monitoring/validation methods for mass confirmation of protein modifications have initiated evaluating the MS system-to-system reproducibility. To demonstrate the above criterion, we measured the RSD% for scFc subunits analyzed on two QTof MS systems to determine our MS system's suitability. Figure 5 shows the RSD% for fucose%, afucose%, and aglycosylated scFC% at 0.12%, 0.56%, and 5.51% respectively. Both Vion systems (QTof-1 and QTof-2) produced consistent results (Figure 5) for the most abundant and the least abundant protein modification% levels. The %RSD calclated for six replicates of trastuzumab completed on both instruments reported less than 10% RSD.

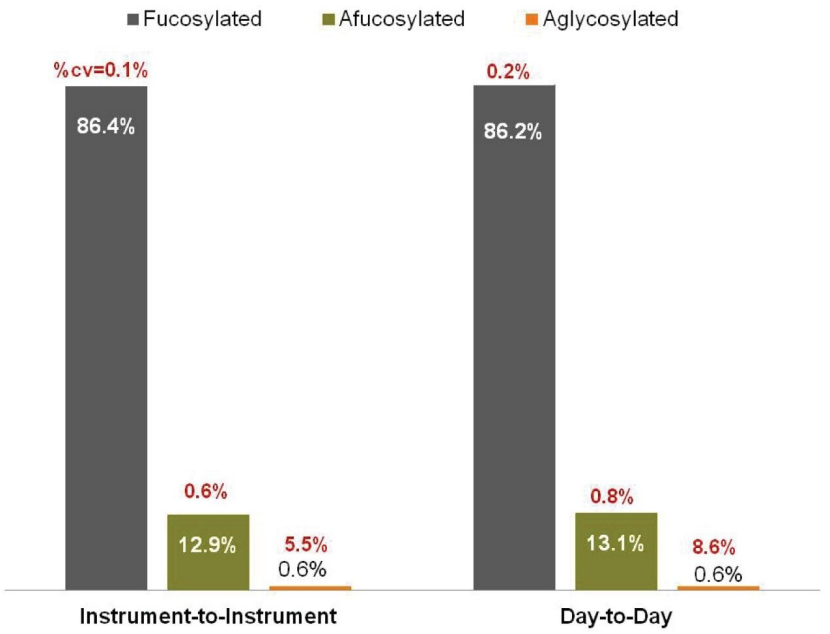


Figure 5. The bar-graph presents the relative abundance levels measured for fucosylated, afucosylated and aglycosylate scFc in Trastuzumab-2 sample. This reports the Instrument-to-Instrument and Day-to-Day measurement robustness in terms of the mean relative abundance for each protein modification and the coefficient of variance/CV% (in red). The reported CV% is less than 10% for all selected modifications.

Similarly, we investigated the repeatability of the assay over time, and replicated the assay over a period of four weeks using one of the QTof MS systems. The RSD% for samples analyzed four weeks apart in triplicate injections also reported less than 10% RSD for all scFc subunit modifications indicating a high robustness of

instrumentation and repeatability of the assay.

Measure relative abundance of high mannose and hybrid glycan structures

Endo S cleaves the β, 1-4 bond between core GlcNAc's of N-glycan structures except that of high mannoses and hybrid glycans whereas Endo S2 also removes the high mannoses and hybrid glycans. The difference in afucose levels when digested with Endo S2 compared to Endo S provides the content of these structures in the mAb sample. If the UNIFI workflow analysis contains these two types of samples in one sample list, the difference can be viewed under maximum and minimum afucose of the "result summary" (Figure 6). The maximum shows the total afucosylation level in Endo S2 digested sample and the minimum in Endo S digested sample. The total amount of high mannose and hybrid glycan structures can be automatically calculated using the given maximum and minimum afucosylation levels by UNIFI custom calculations.

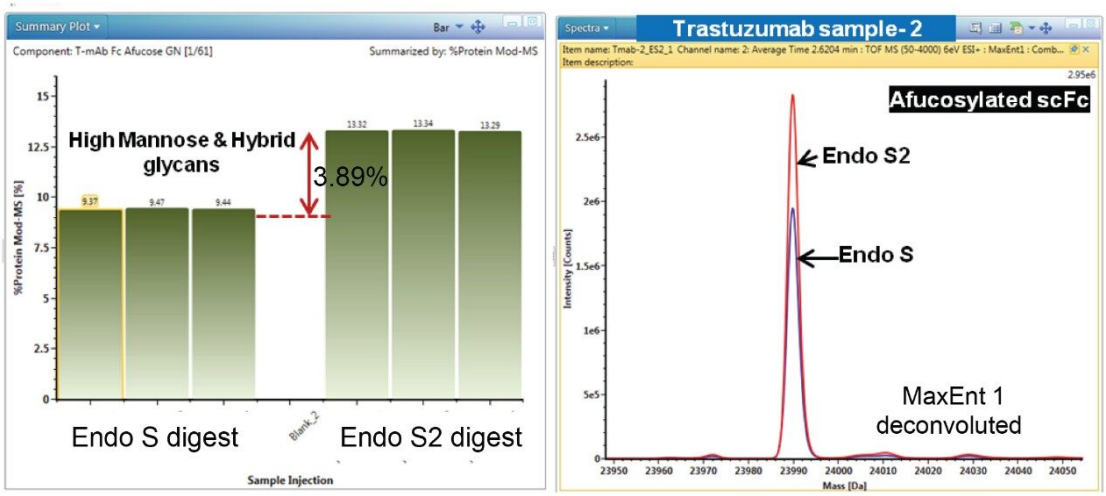


Figure 6. The total amount of high mannose and hybrid glycans can be conveniently calculated based on the change in afucose% level in Endo S2 compared to Endo S digested samples using UNIFI customizable calculations function.

Automated data reporting capability

As previously demonstrated, automated reporting capabilities in UNIFI provide the option to generate customizable reporting formats according to user requirements.[9] The UNIFI report shown here includes the summary tables and plots of the processed data that can be easily adopted into a late stage mAb characterization or QC protocol (Figure 7).

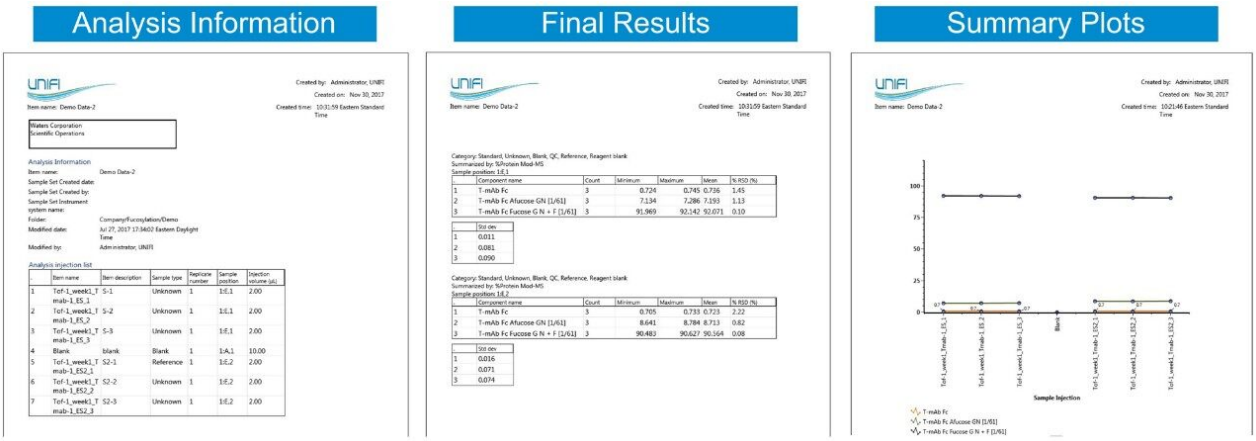


Figure 7. The customizable UNIFI reporting formats provide a user friendly compliant system for reporting the analysis results including the custom calculated measurements. The formats can be organized in accordance with the protocols established in each organization.

Conclusion

MAM workflow: Versatile built-in workflows that can be used for protein characterization and subunit level MAM deployment.

- Simplified sample preparation method and fast LC-MS run time: has the potential to enable near real-time monitoring of the processes.
- Ease-of-use: the fully automated UNIFI workflow with data acquisition and processing capability can be easily deployed to monitor CQAs of mAbs (e.g. fucosylation levels) with minimal user intervention.
- Quantification capability: UNIFI allows user defined formulas to be used in calculations. These calculations can be used to calculate not only the core fucosylation levels of N-glycans but also the monitoring of other attributes levels such as high mannoses, hybrid glycan structures, oxidation, etc.
- Robustness of the method and instrumentation: the UNIFI software and Vion IMS QTof MS system demonstrated that less than 10% RSD was delivered for samples analyzed on different instruments and different times. The reproducible measurement acquired from the current study highlights the applicability of the platform in late stage development or QC environments.

References

1. Xu, W.; Jimenez, R. B.; Mowery, R.; Luo, H.; Cao, M.; Agarwal, N.; Ramos, I.; Wang, X.; Wang, J. A Quadrupole Dalton-Based Multi-Attribute Method for Product Characterization, Process Development, and Quality Control of Therapeutic Proteins. *mAbs* 2017, 9 (7), 1186–1196.

2. Bomans, K.; Haberger, M.; Bonnington, L.; Wagner, K.; Kailich, T. Multi-Attribute Monitoring of Antibody Modifications by Semi-Automated Liquid Chromatography Mass Spectrometry Peptide Mapping. *American Pharmaceutical Review* 2016.

3. Upton, R.; Bell, L.; Guy, C.; Caldwell, P.; Estdale, S.; Barran, P. E.; Firth, D. Orthogonal Assessment of Biotherapeutic Glycosylation: A Case Study Correlating N-Glycan Core Afucosylation of Herceptin with Mechanism of Action. *Analytical Chemistry* 2016, 88 (20), 10259–10265.

4. Haberger, M.; Bonnington, L.; Bomans, K.; Maier, M.; Eltner, S.; Dembowski, M.; Reush, D.; Bulau, P. Application of Electrospray Ionization Mass Spectrometry in a Quality Control Laboratory. *American Pharmaceutical Review* 2016.

5. Sokolowska, I.; Mo, J.; Dong, J.; Lewis, M. J.; Hu, P. Subunit Mass Analysis for Monitoring Antibody Oxidation. *mAbs* 2017, 9 (3), 498–505.

6. Dong, J.; Migliore, N.; Mehrman, S. J.; Cunningham, J.; Lewis, M. J.; Hu, P. High-Throughput, Automated Protein A Purification Platform with Multiattribute LC–MS Analysis for Advanced Cell Culture Process Monitoring. *Analytical Chemistry* 2016, 88 (17), 8673–8679.

7. Chung, S.; Quarmby, V.; Gao, X.; Ying, Y.; Lin, L.; Reed, C.; Fong, C.; Lau, W.; Qiu, Z. J.; Shen, A.; Vanderlaan, M.; Song, A. Quantitative Evaluation of Fucose Reducing Effects in a Humanized Antibody on Fcγ Receptor Binding and Antibody-Dependent Cell-Mediated Cytotoxicity Activities. *mAbs* 2012, 4 (3), 326–340.

8. Shields, R. L.; Lai, J.; Keck, R.; O'Connell, L. Y.; Hong, K.; Meng, Y. G.; Weikert, S. H. A.; Presta, L. G. Lack of Fucose on Human IgG1 N-Linked Oligosaccharide Improves Binding to Human FcγRIII and Antibody-dependent Cellular Toxicity. *Journal of Biological Chemistry* 2002, 277 (30), 26733-26740.

9. Waters Corporation, Biopharmaceutical Platform Solution with UNIFI - Key Applications. Waters Application Notebook. 720004887EN. 2017.

Featured Products

ACQUITY UPLC H-Class PLUS Bio System <https://www.waters.com/10166246>

Vion IMS QTof Ion Mobility Quadrupole Time-of-flight Mass Spectrometry <https://www.waters.com/134845751>

ACQUITY UPLC Tunable UV Detector <https://www.waters.com/514228>

UNIFI Scientific Information System <https://www.waters.com/134801648>

720006166, December 2017