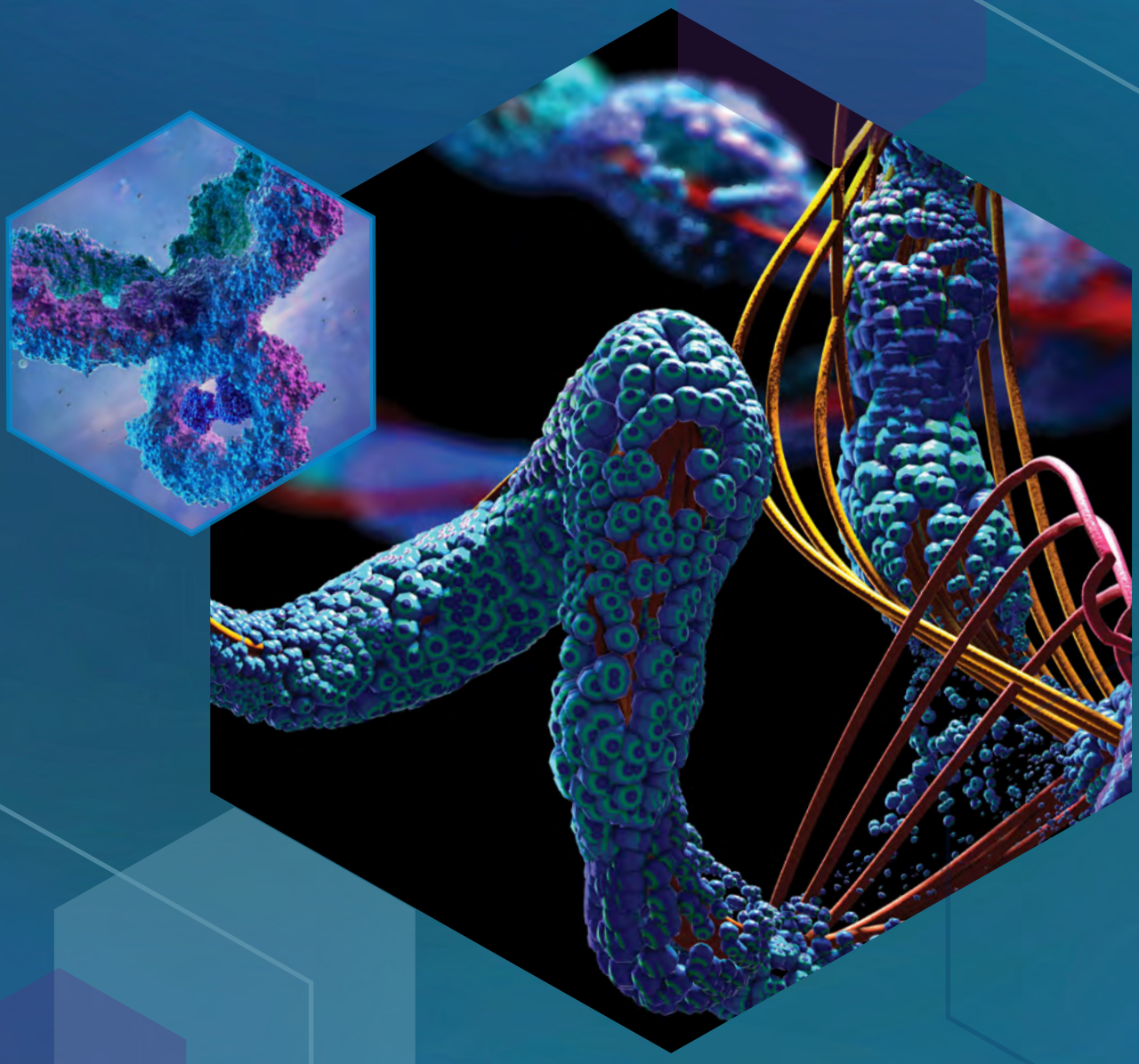
[APPLICATION NOTEBOOK]

Multi-Attribute Methods for Biopharmaceutical Analysis

Application Notes



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Introduction

The adoption of LC-MS-based multi-attribute method (MAM) analysis for routine monitoring of biotherapeutic variation has progressed greatly over the last five years. The ability to directly assess the molecular attributes that contribute to efficacy, safety, stability, and process robustness is enabling analysts and their organizations to obtain more data without the ambiguity of traditional chromatographic and electrophoretic assays that measure product variation indirectly. In many cases, this data is generated with greater sensitivity and dynamic range than these legacy assays, furthering the discussion whether they complement or eventually replace these traditional analyses in development, manufacturing, and quality organizations.

This notebook compiles several recent application notes generated by the Biopharmaceutical Scientific Operations Team at Waters. They illustrate key attribute monitoring workflows that have been developed to improve and streamline MAM analysis of biotherapeutic proteins.

- In the first section, we demonstrate the power of integrating high performance analytics (UPLC and Tof based high-resolution mass detection) with the fit for purpose compliant-ready waters_connect™ informatics platform to streamline Peptide MAM analysis. Analysts can obtain product Identification, targeted attribute quantification, and detection of potential new product variants/impurities (new peak detection) within a single experiment. The ability to deploy these capabilities into organizations with limited or no previous LC-MS experience, by simplifying and automating instrument operations and data processing workflows, has greatly expanded the utility of this approach without forcing changes to organizational structures.
- Peptide MAM experiments can also be carried out with nominal mass detection using the ACQUITY™ QDa Mass Detector under control of Empower™ CDS informatics. The second section highlights the capability to acquire product ID and targeted attribute analysis results on a platform already familiar to most pharmaceutical organizations.
- Lastly, the third section demonstrates the benefits of MAM for mAb data acquired at the subunit-level. The simplicity of subunit sample generation and capabilities for quick LC-MS analysis cycles make subunit level MAM analysis attractive for higher throughput MAM assay development, and for more rapid deployment of MAM assays into regulated lab environments.

Along with highlighting some additional MAM resources and examples from the peer-reviewed literature, we hope his body of work informs the reader on what can be accomplished using attribute-based LC-MS analysis and encourages further discussions with your colleagues and the biopharmaceutical team at Waters.

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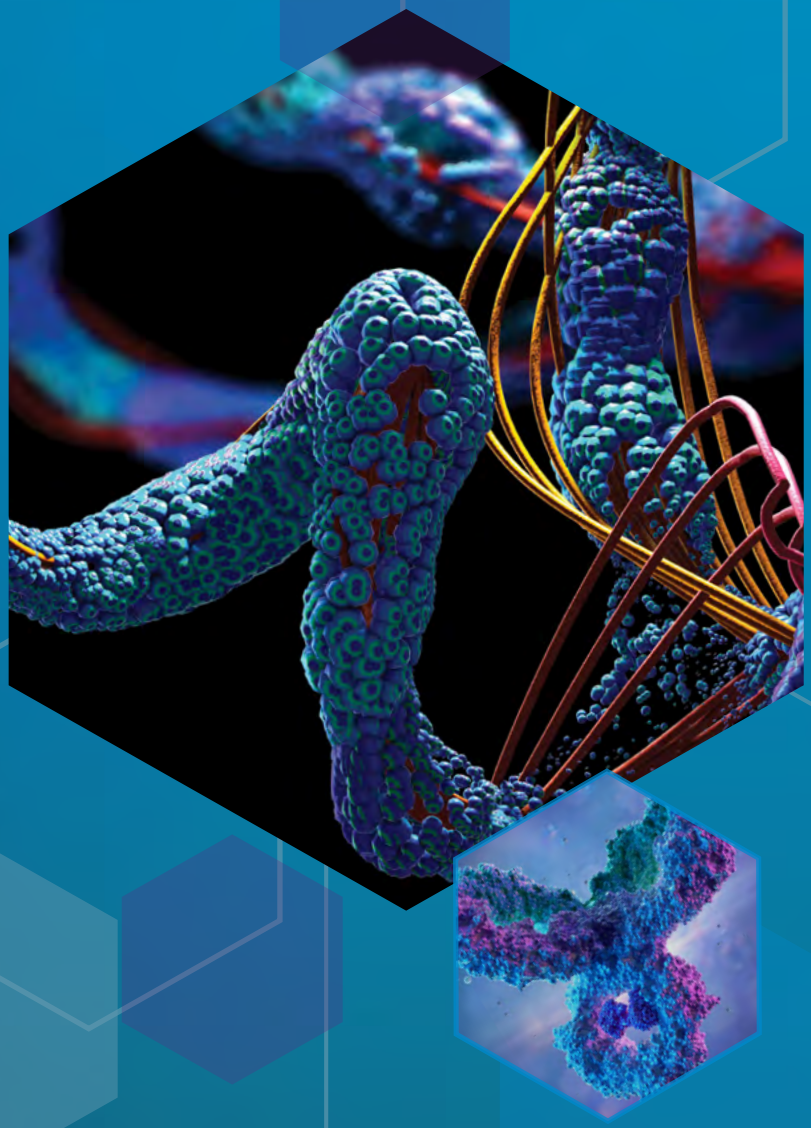

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Application Note

A Streamlined Compliant Ready Workflow for Peptide-Based Multi-Attribute Method (MAM)

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Waters Corporation

Abstract

Here we demonstrate an end-to-end analytical workflow developed for peptide-based multi-attribute method (MAM). The waters_connect informatics platform provided a compliant-ready environment for automated data acquisition, processing, and reporting of product quality attributes (PQA) for a monoclonal antibody (mAb) standard. The peptide MAM workflow application coordinates a seamless transition between waters_connect apps to track and quantify product quality attributes arising from protein bioproduction and degradation. In this study, these include modifications such as oxidation, deamidation, succinimide modification, glycosylation, C- and N- terminal modifications, and isomerization. Expanding the assay to support purity assessment, the Peptide MAM App also provided new peak detection (NPD) capabilities against a designated reference sample.

Benefits

- End-to-end compliant-ready peptide MAM workflow for product quality attribute analysis
- Small footprint, SmartMS-enabled system for routine and reliable generation of high quality data by non-MS experts

- Streamlined transfer of attributes from peptide mapping analysis to MAM analysis within a common compliant-ready informatics platform
- Targeted peptide-based attribute tracking and relative quantification
- Robust algorithms for new peak detection of potential impurities providing high sensitivity with low false-positive detection rate

Introduction

Monoclonal antibodies remain one of the most important classes of biotherapeutics in development. They are subjected to various co- and post-translational modifications during manufacturing and storage.[1] Some of the product attributes are critical to potency, efficacy, and safety of the molecule – these are termed critical quality attributes (CQAs). Control and monitoring of PQAs and CQAs throughout the product life cycle ensures high quality mAbs that meet regulatory expectations and target product profile requirements. Additionally, a quality by design (QbD) approach that focuses on establishing the relationship of various manufacturing processes to achieve this target profile benefits directly from the high information content of MAM based analysis.[2,3] Establishing QbD design space throughout biotherapeutic manufacturing involves implementation of analytical methods that can efficiently monitor these quality attributes across larger sample sets.

Multi-attribute method (MAM) is an LC-MS methodology established for monitoring multiple PQAs and CQAs with the additional capability for detecting unknown impurities within a single analysis.[4] Unlike conventional chromatographic methods such as ion exchange chromatography (IEX) and hydrophilic interaction chromatography (HILIC) that depends on optical detection, peptide MAM is built on reversed-phase chromatography coupled with mass spectrometry (RPLC-MS). Utilizing the power of mass spectrometry and advanced informatics tools, MAM can monitor multiple PQAs with greater throughput, sensitivity, and dynamic range than these single attribute optical based detection assays.

A peptide MAM analysis is designed to target and quantify multiple product quality attributes by analysis of an enzymatic digest of a biotherapeutic protein. This is accomplished by quantifying digested peptides and their modified counterparts, typically focusing on those with established impact on product safety and efficacy or

those that indicate process stability. Peptide attribute monitoring is often combined with a purity assessment step commonly referred to as new peak detection (NPD). During NPD data processing, the detected peaks within an experimental sample are compared to those from a reference control sample to identify new peaks or those with an altered abundance relative to user defined thresholds. Currently, many biopharmaceutical companies are working on evaluating MAM across multiple vendor platforms. Reports of these efforts emphasize the importance of consistency in attribute quantitation and the need for minimized NPD false positive rates to reduce user interventions and automate analysis workflows.

Here, we describe a streamlined peptide MAM workflow on the Waters BioAccord LC-MS System operated under the compliant-ready waters_connect informatics platform. The BioAccord System is comprised of an ACQUITY UPLC I-Class PLUS and an ACQUITY RDa TOF MS System designed to support routine LC-MS analysis across biopharmaceutical development, manufacturing, and QC organizations.

Experimental

Sample Preparation

Reference sample: a 10 mg/mL NISTmAb (RM 8671) sample was initially diluted to 1 mg/mL in denaturing buffer (6 mol/L guanidine HCl, 250 mmol/L Tris, pH 7.8). The sample was incubated in dithiothreitol (DTT, 5 mmol/L) for 30 min under room temperature followed by alkylation with iodoacetamide (IAM, 10 mmol/L) for 20 min in the dark at room temperature. The reduced alkylated sample was desalted using a NAP-5 column (GE Healthcare), followed by digestion with recombinant porcine trypsin at 1:20 enzyme to substrate ratio, for a period of 4 h at 37 °C.

Stressed mAb sample: NISTmAb (RM 8671) at 10 mg/mL was buffer exchanged into 50 mmol/L Tris base at pH 8.0 using BioRad 10K MWCO spin columns, transferred to tightly capped 0.5 mL Eppendorf LoBind tubes, and incubated at 40 °C for 8 days. On day 8, samples were removed from the incubator and digested in the same manner as the Reference Sample.

Spiked samples: The spiked samples were derived from control and stressed NISTmAb digest by spiking in Pierce Peptide Retention Time Calibration (PRCS) Standard. Each spiked sample contained PRCS at a concentration of 0.5 pmol (Pierce standards) per 3 µg of mAb digest.

System suitability standard: MassPREP Peptide Mixture (p/n: 186002337 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186002337-massprep-peptide-mixture.html >)

Instrumentation

1. The BioAccord LC-MS System

- ACQUITY RDa Detector
- ACQUITY UPLC I-Class PLUS
- ACQUITY TUV Detector
- Column Heater (CH-A) module

Target match tolerance:	10
Fragmentation identification, fragment match tolerance:	10 ppm
Minimum primary fragment ions for confirmation:	3
Analysis specific settings, amino acid modifiers:	Fixed: Carbamidomethyl Variable: Oxidation (M, W) Lysine C-TERM Deamidation (N, Q) N and O-Glycosylation Pyroglutamic acid Q N-TERM
Digest settings, selected digest reagent:	Trypsin
Missed cleavages:	2
Minimum sequence length:	4

Table 1. Peptide mapping (characterization) workflow processing parameters.

LC Conditions

Detection:	TUV, 10 mm analytical FC, λ=214 nm
Vials:	QuanRecovery with MaxPeak HPS (p/n 186006937)
Column(s):	(ACQUITY UPLC Peptide CSH C_{18} Column, 2.1 × 100 mm, 1.7 µm (p/n 186005297)
Column temp.:	60 °C
Sample temp.:	6 °C
Injection volume:	2.0–10.0 µL
Flow rate:	0.20 mL/min
Mobile phase A:	H_2O, 0.1% formic acid
Mobile phase B:	Acetonitrile, 0.1% formic acid

Table 2. ACQUITY UPLC I-Class PLUS System conditions/parameters applicable to mAb digest analysis.

Gradient Table

Time	Flow (mL/min)	% A	% B	Curve
Initial	0.200	99	1	6
2.00	0.200	99	1	6
52.00	0.200	65	35	6
57.00	0.200	15	85	6
62.00	0.200	15	85	6
67.00	0.200	99	1	6
80.00	0.200	99	1	6

Table 3. LC gradient for the mAb sample analysis.

MS Conditions

MS system:	ACQUITY RDa Detector
Mode:	Full scan with fragmentation
Mass range:	50–2000 *m/z*
Polarity:	Positive
Scan rate:	2 Hz
Cone voltage:	20 V
Capillary voltage:	1.2 kV
Desolvation temperature:	350 °C
Intelligent data capture:	On

Table 4. ACQUITY RDa Detector settings used for peptide analysis.

Data Management

Data were acquired and processed using the waters_connect informatics platform with the Peptide MAM App (monitoring).

Results and Discussion

Characterization: Defining product quality attributes for monitoring and transferring them to the scientific library.

Before MAM attribute monitoring studies can be conducted, biotherapeutic characterization analysis must be completed to define the range of potential attributes that could be targeted for MAM analysis. The waters_connect informatics platform supports both characterization and monitoring workflows as well as tools for streamlined information transfer between these applications. Peptide map based protein characterization is performed using peptide mapping workflow in the waters_connect UNIFI App. The focus of this workflow is assignment of peptide sequences to peaks, identifying modifications, and populating retention time and charge state information utilized in MAM analysis.

In this study, the peptide mapping data was acquired using Data Independent Acquisition mode (MS with fragmentation mode on the BioAccord) to identify PQAs. Peptide map data could also be acquired in LC-MS^E mode on a QTof system, where the MS scans alternate between low and elevated collisional energy, providing peptide identification using MS1 (low collision energy) channel, while obtaining fragment ions in the MS2 (elevated collision energy) channel for sequence confirmation. The peptide mapping data for all analytical samples were processed using the parameters elucidated in Table 1. A minimum of three confirmatory fragment ions was selected when identifying the peptide attributes. The data revealed many peptide modifications known to be present on the NISTmAb.[5] Some of the typical "hotspot" modifications observed were oxidation of methionine in the DTLMISR peptide and deamidation of asparagine in the VVSVLTVLHQDWLNGK tryptic peptides. This unmodified VVSVLTVLHQDWLNGK sequence also represents the base peak peptide (peak with the highest MS intensity) that is referenced with regards to the new peak detection approach.

The attributes selected for peptide MAM were based on previous knowledge of the NIST reference mAb.[5] Each set of unmodified and modified peptides representing the PQAs were compiled into a common NISTmAb library with waters_connect Scientific Library (Figure 1). Peptide attributes of interest were exported from the peptide mapping analysis by simply selecting "send to -> Scientific Library" options. These scientific libraries can be shared between laboratories and updated by authorized users as they gain additional knowledge of the molecule.

Figure 1. The Scientific library is available for managing attribute peptide information between characterization and monitoring applications. Attribute peptides assigned in peptide mapping experiments can be exported into a library that is available to the Peptide MAM App for building a target list for attribute monitoring.

Peptide MAM Workflow

Although LC-MS data can be collected in an identical fashion to peptide mapping, the peptide MAM workflow differs in the approach processing of that data. In Peptide MAM, the attributes to be monitored are specifically targeted and quantification is based on specific parameters, often optimized for each attribute. This Peptide MAM workflow is coordinated through the Peptide MAM App which links seamlessly with the other waters_connect Apps (Figure 2A) for simple method set-up, acquisition, and processing. The Apps integrated in this workflow are: Acquisition Method Editor, Sample Submission, Peptide MAM processing, and LC-MS Tool Kit (Figure 2B).

Figure 2. The waters_connect hub (A) enabled rapid navigation between Apps. The workflow for peptide MAM (B) comprises several integrated functions: The ACQUISITION method editor App for method generation, The SAMPLE submission App for data acquisition, The Scientific library for storage and management of attribute information, the Peptide MAM Processing App, and LC-MS toolkit App for facilitating any additional manual data review.

The Acquisition Method Editor generates the LC-MS method that is referenced within Sample Submission App sample list to support data acquisition. The Peptide MAM App utilizes this data and a list of peptide attributes for targeted quantification to report back relative abundances for these attributes and generate a list of potential "new peaks" relative to a reference sample. Following automated Peptide MAM data processing, the LC-MS Tool Kit App can assist in manual follow-up investigations. These functions are discussed in greater detail in the sections below.

LC-MS Method Set Up and Data Acquisition

Data acquisition is managed by a single method that encompasses instrument parameters leading to targeted quantification processing, and New Peak Detection. The Acquisition Method Editor App for a peptide MAM analysis would encompass instrument parameters for both the ACQUITY UPLC I-Class PLUS modules, TUV detector, and ACQUITY RDa Detector. A set of generic instrument parameters optimized for mAb-based peptide MAM is provided in Tables 2 through 4. The system can be set to acquire data for later processing, or it can be set to "Acquire and process" within the Peptide MAM App (Figure 2, Left Panel).

The sample queue for data acquisition of a NISTmAb forced degradation experiment is shown in Figure 3. This list includes blanks, system suitability injections, experimental controls (reference mAb samples), and the forced degradation analytical samples. The system suitability injections (sample type: SST) shown were performed before and after the analytical samples, but the order can be modified as required. "Unknown" was selected as the sample type for all reference/control and analytical samples (Figure 2, Right Panel, Column 3). In addition, reference samples for "New peak detection reference" and the "Retention time alignment reference" were selected to facilitate the post-acquisition data processing within the Peptide MAM App. In this example, the control mAb and spiked in stressed samples were respectively selected as new peak detection and retention time alignment references. Typically, the sample with the highest number of peaks provides the best chromatographic alignment reference.

Figure 3. The sample submission functionality is accessed within the Peptide MAM App when creating a streamlined acquisition and processing method for Peptide MAM studies. The central panel contains information on file location, the acquisition system, and sample locations, while the main table to the right contains the sample queue. Here each injection can be designated a role in the overall analysis and reference sample for NPD and peak alignment can be assigned.

Peptide MAM Data Processing

The end goal of Peptide MAM data processing is the targeted relative quantification of attributes and detection of

potential novel sample impurities. Process-Only and Acquire & Process Analyses both rely on a list of peptides used for determining system suitability and targeted quantification of peptide attributes. Processing for new peak detection is fully automated but requires a designated reference sample for comparative evaluation.

System suitability establishes that the key components of the system are within a performance range sufficient to produce quality data. The system suitability test is based on processing data associated with a targeted list of peptides created manually in the processing method. This list can be populated with user defined limits for chromatographic peak width (FWHM) expected retention time, mass accuracy, and MS intensity criteria. The pass/fail status of system suitability injections from these parameters indicates the overall system readiness for peptide MAM analysis.

The direct import of MAM peptide attribute lists from the Scientific Library remains the simplest and most effective mode as it maintains the traceability of data from characterization through monitoring analyses, but these can be also imported in .csv format into the software for data acquired on other Waters or 3rd party LC-MS systems. Each peptide entry within MAM processing method contains a peptide sequence, modification(s), targeted retention time, and selected charge states for targeted peptide monitoring. Typical processing parameters for peptide MAM (Figure 4) enable attribute quantification based on a target LC retention time and accurate mass, following automated retention time alignment (Figure 5A) and codetection of LC-MS peaks. The processed data (Figure 5B) presents the %modification levels determined for three selected attributes: oxidation of DTLMISR peptide, deamidation of VVSVLTVLHQDWLNGK, and the HC glycopeptide containing a Man5 glycan. Data is displayed in a bar graph format for rapid data comparisons. The bar graph for stressed NISTmAb samples showed elevated DTLMISR oxidation and VVSVLTVLHQDWLNGK deamidation levels (Figure 5B, orange-colored bars) surpassing respective warning thresholding levels at 3% and 2%. Man5, a low abundance glycoform, was unaffected at a 0.83% modification level (vs other monitored glycoforms) across all samples. The %RSD for low abundance Man5 glycopeptide quantification was reported at 7.4% despite detection at only ~0.1% of the base peak intensity for the sample.

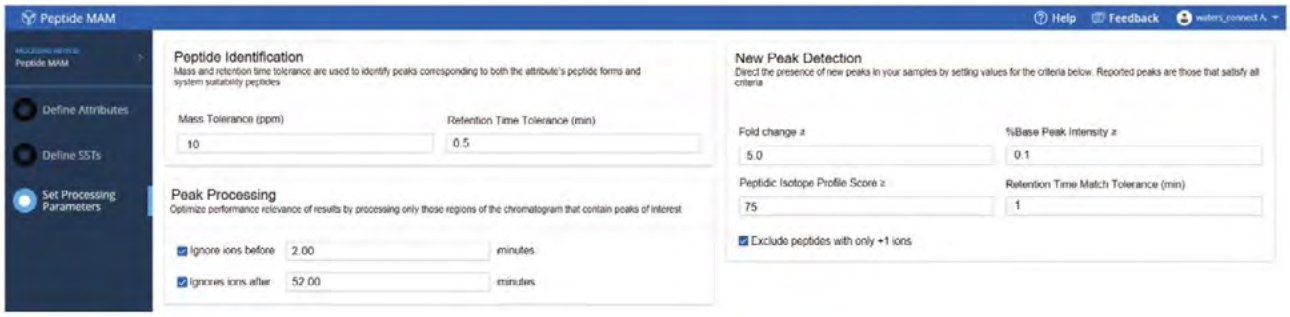

Figure 4. Setting parameters for targeted attribute monitoring and new peak detection. Peptide MAM processing uses fixed mass and retention time tolerances and can be restricted to a user defined retention time range. The new peak detection criteria comprise five potential parameters: minimum fold change, minimum %base peak intensity, an isotopic profile score, retention time match tolerance and the ability to remove +1 ions (chemical noise ions) from the results.

Figure 5. The retention time alignment panels (A) show the pre- and post-alignment of the DTLMISR peptide peaks from the control and alignment reference samples as an overlaid chromatogram. The monitored attribute peptide panel (%Abundance) is displayed for three modified (oxidized, deamidated, and glycosylated) peptides. Predefined pass/fail criteria based on user defined limits determine the color of bars, and the presence of a warning icon above them. The table, below, displays target criteria and results for the DTLMISR Ox attribute from the stressed sample.

Retention Time Alignment

Algorithmic alignment of chromatographic peaks within a defined (0.5 min here) retention time window reduces misassignment of peaks and false positive identifications that produce variability across LC-MS injections. During automated peak processing, retention time alignment (Figure 5A) uses calculated alignment vectors to align chromatograms against a specified alignment reference sample (user defined). The codetection of peaks creates a composite spectrum used for peptide peak tracking and is particularly relevant when aligning data that was acquired on separate days or different systems. In addition, the "upper and lower retention time restriction" limits avoiding processing pre-gradient injection interferences, and post-gradient column wash segments, reducing processing times and simplifying data review.

Consistent Isotope Selection for Peak Integration and Quantification

While users can specify the charge states used for each peptide, the codetection algorithm for peptide component tracking automates selection of individual isotopes used for peptide quantification. This simplifies the MAM processing method setup and minimizes variability in peak processing resulting in lower and narrower %RSD ranges for attribute %modification levels. Isotope selection for the DTLMISR oxidation peak is shown (Figure 6) across all four analytical samples. Both control and stressed mAb samples indicate consistent selection of all detectable isotopes (5 selected) for both charge states (+2 and +1) utilized for DTLMISR peptide quantification.

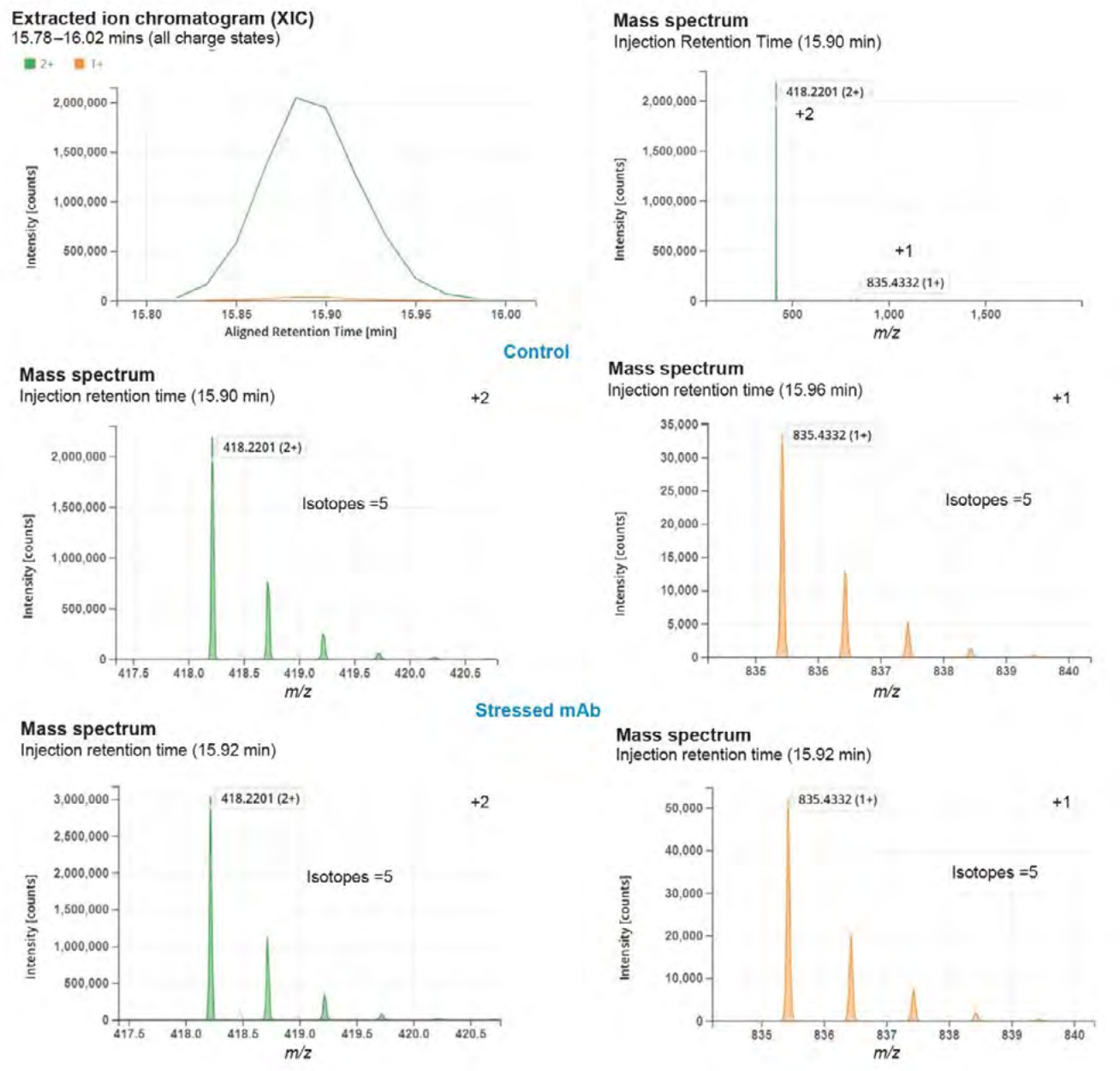

Figure 6. The XIC (top left) and the MS spectrum (top right) for DTLMISR oxidation peptide demonstrate the charged ions and peaks used for quantification of the attribute. DTLMISR has two charge states used for peak area calculation: +2 (the most abundant form) and a +1 minor form ion. The isotope patterns utilized in XIC and peak area calculations for control (middle) and stressed (bottom) mAb digest samples are shown here. For both samples automated peak processing used all five isotopes for each charge state.

New Peak Detection (NPD)

New peak detection is an essential part of peptide MAM when the analysis is utilized as a purity assay. NPD analysis outputs the retention time and neutral mass of potential impurities in a sample that were not detected in the reference sample or those that varied significantly in intensity from a codetected peak in the reference sample. The current challenges associated with NPD, based on industry feedback, includes high rate of false negative/positive peaks, leading to misrepresentation of the sample quality and significant time investments in manual data review. With each new spurious peak requiring careful validation to meet regulatory criteria, the process could delay lot release to investigate these anomalous results. In order to evaluate the NPD functionality, both control and stressed NISTmAb tryptic digests were spiked with 15 heavy labeled standard peptides at 0.5 pmol per 3 µg level of the digest (Figure 7).

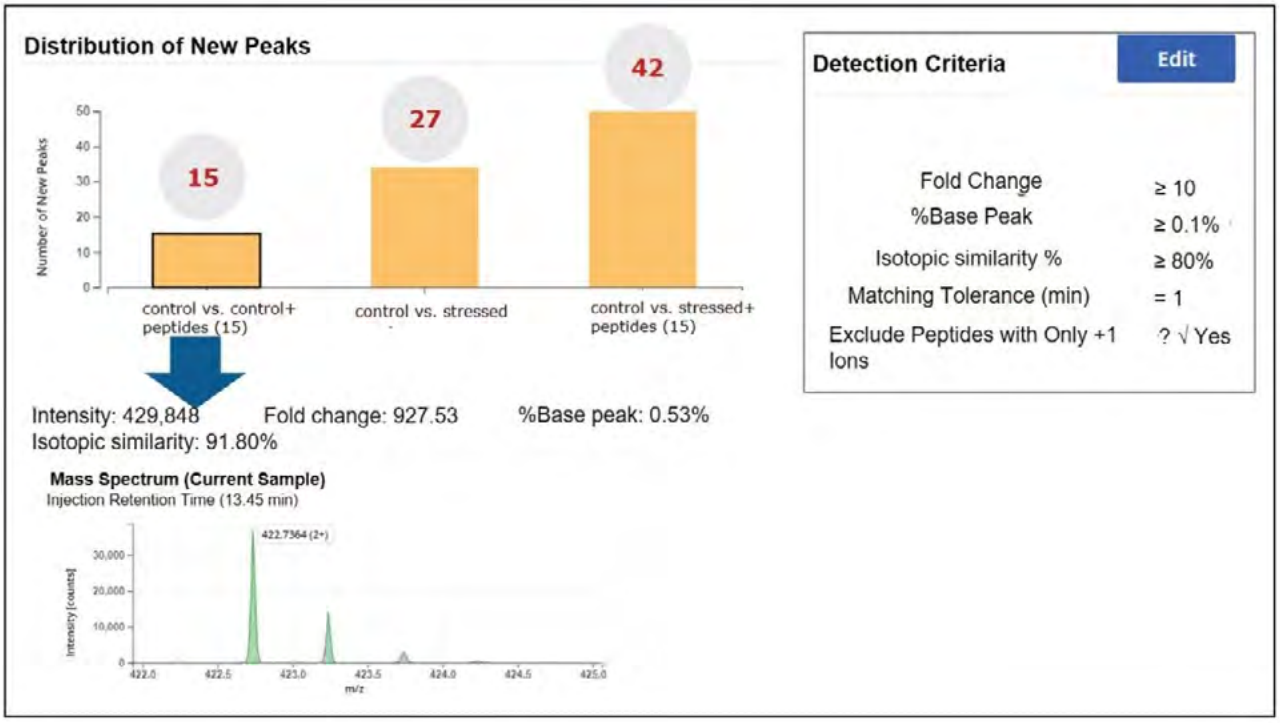

Figure 7. New Peak Detection performed with defined criteria based on fold change, %base peak level, %isotopic similarity and retention time tolerance. Each peak passing the criteria (Right Side) is displayed for review. The peaks can be further examined using MS spectra to manually verify the NPD results.

The NPD filtering criteria can include fold change, %base peak, %isotopic similarity, and retention time tolerance (min). During peak processing the fold change of a new peak is calculated relative to the MS intensity of the codetected peak in the reference/control sample. Any elevation or reduction in peak intensity beyond the thresholds will trigger this criterion for a new peak detection. Since fold-change is calculated relative to a reference sample, the process can standardize impurity level measurements across all analytical samples. In this study, as per industry practice, ≥10 was used as the default fold change.

The %base peak is another criterion used in NPD used to avoid false positive identifications from background ions and chemical noise. This is calculated relative to the most intense peptide's MS response within an LC-MS chromatogram. In the example data for the forced degradation study (Figure 7) the unmodified VVSVLTVLHQDWLNGK peptide was determined as the base peak by the software. The study used a %base peak at a minimum of 0.1% level to set a lower limit for new peak detection.

To further minimize false positive identifications, an isotopic similarity score has been introduced into peptide MAM NPD data processing. The %isotopic similarity is calculated relative to the isotopic distribution of an ion with similar *m/z*. BioAccord data collected with intelligent data capture (IDC) has this score typically set to 75% or higher. The matching tolerance for retention time was maintained at 1 min and excluded all solvent ions and chemical noise by selecting "Exclude peptides with only +1 ions option".

The NPD results (Figure 7) showed identification of 15 new peaks in spiked control sample that corresponded to the neutral mass of spiked heavy labeled peptides. In the stressed NISTmAb sample the number of new peak peaks was 27. These peaks contained modifications such as oxidations and deamidations, typical of previous studies of the NIST mAb. The stressed and spiked mAb sample should result in 42 (15 spike + 27 stressed) new peaks, matching our observations.

If needed, each new peak can be verified using a "review" option, enabling MS spectra to be displayed for the selected new peaks. Authorized users can accept or reject these new peaks prior to finalizing the results.

Reporting

Communicating peptide MAM results effectively, requires the reporting of the processed data, associated quality metrics, and processing conditions. Peptide MAM App utilizes a built-in template for streamlined report generation, where analysts have the flexibility to select chapter-based elements to include in the report. The report provides for a summary of injections, system suitability test criteria and results, attribute quantification, new peak detection, and the LC-MS acquisition method record. An example report (Figure 8) generated for the forced

degradation study is shown with key sections excerpted for presentation. Report results can be exported as a PDF or in .csv formats to enable data reuse in other applications.

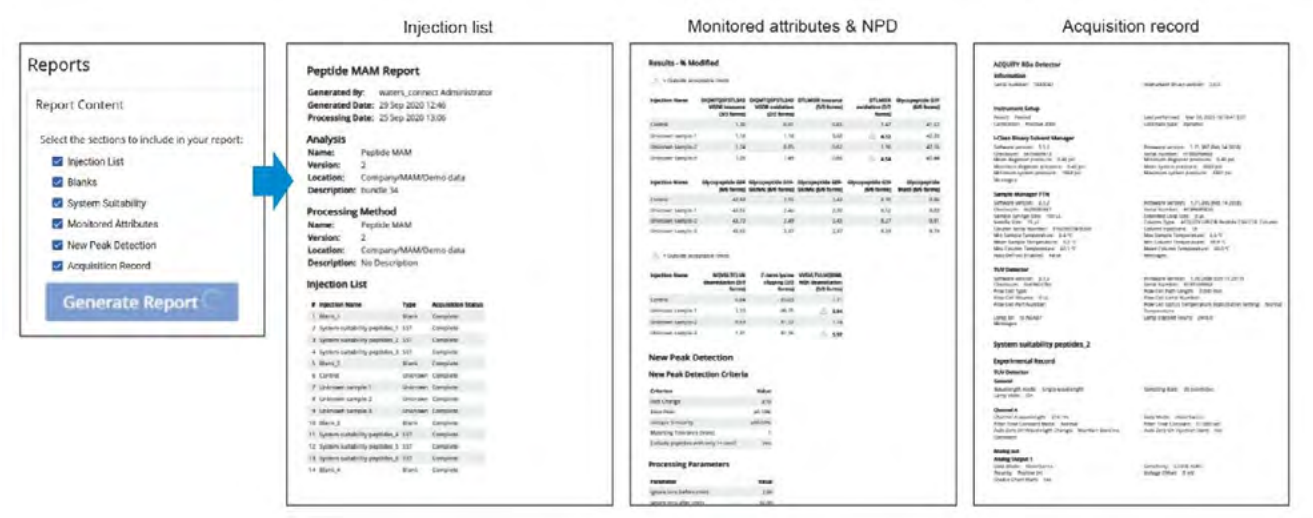

Figure 8. Selectable report template chapters (left panel) are available for configuring peptide MAM data reporting. This report comprises a summary of injections, system suitability test data, peptide attribute tracking data, NPD, and LC-MS method criteria.

Inter-System Reproducibility for Peptide MAM Studies

Data generated from three BioAccord systems (Figure 9) was generated for NIST mAb stress samples using the methodology described above. The data exhibited reproducibility of <10% RSD for the eight monitored attributes. Key to this study was the inclusion of the Man5 glycosylated attribute and VVSVLTVLHQDWLNGK deamidation attribute that represented the widest intensity dynamic range (>1000-fold MS intensity difference from the base peak) on all three BioAccord systems operated under waters_connect.

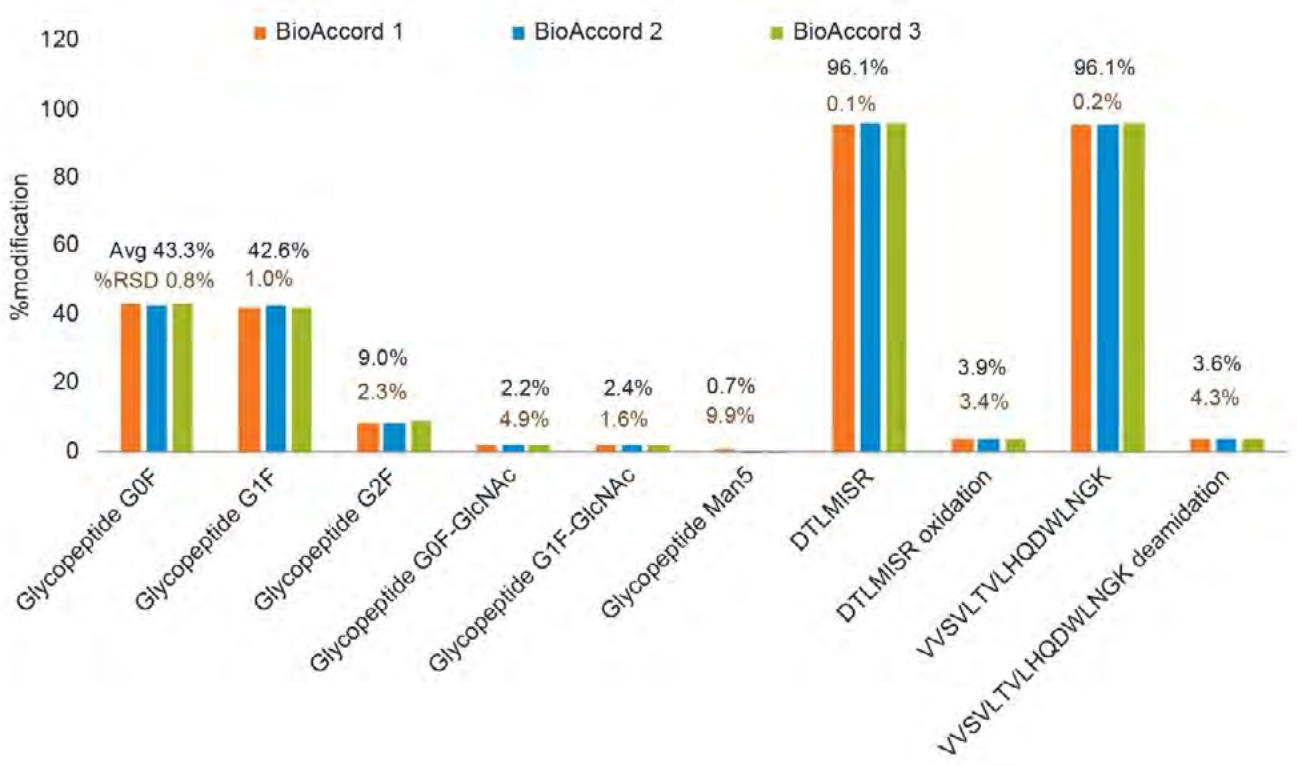

Figure 9. Attribute monitoring data acquired on three BioAccord systems indicate low %RSD variability for attribute-based measurements between replicate experiments on a common instrument and across instruments.

Conclusion

A streamlined Peptide MAM workflow has been demonstrated on the BioAccord LC-MS System under the control of the compliant-ready waters_connect informatics platform. This MAM functionality can support and accelerate biopharmaceutical product development, process development, manufacturing, and QC lot release by enabling direct, sensitive, and selective attribute-based measurements that will complement and potentially replace existing conventional chromatographic and electrophoretic assays.

Specifically, this Peptide MAM workflow will generate clear and superior performance compared to previous efforts in this area: 1) The Peptide MAM workflow that is highly automated and intuitive to use by non-MS experts; 2) Automated RT alignment improves peak component tracking across injections, instruments and

studies providing consistent attribute-based quantification; 3) Advanced algorithms and filtering tools minimize false positives for new peak detection; 4) Scientific Library functionality enables the seamless transition from characterization to monitoring with data traceability; and 5) SmartMS capabilities of the BioAccord System for attribute-based analysis allows experienced and novice MS users to operate a high-resolution MS instrument with confidence. These capabilities should enable routine adoption of peptide LC-MS attribute based biotherapeutic analysis not only within core biopharmaceutical development organizations, but also into the emerging areas of LC-MS process monitoring and QC lot release.

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Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

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

waters_connect HUB <https://www.waters.com/waters/nav.htm?cid=135040165>

Biopharmaceutical Platform Solution with UNIFI <https://www.waters.com/10195515>

BioAccord LC-MS System for Biopharmaceuticals <
https://www.waters.com/waters/nav.htm?cid=135005818>

720007094, December 2020

Waters™

Application Note

The BioAccord System With ACQUITY Premier for Improved Peptide CQA Monitoring

Nilini Ranbaduge, Robert E. Birdsall, Ying Qing Yu, Weibin Chen

Waters Corporation

Abstract

Peptide MAM is an LC-MS based assay for direct biotherapeutic product attribute analysis that is increasingly used in protein biotherapeutic quality assessment. As part of lifecycle management, assays need to remain accurate and consistent to ensure continued drug product quality and safety. In this respect, robust system performance plays a critical role in MAM data quality. Non-specific adsorption of acidic peptides to metal surfaces is a well-known phenomenon affecting LC-MS analyses, causing asymmetric peaks, loss of peptides, and increased variability in detector response for quantitative measurements. This work demonstrates the performance gains of the BioAccord configured with the inert ACQUITY Premier resulting in increased peptide recovery and robust MS response, with more reproducible results for attributes spanning 3-orders of magnitude in dynamic range. The improved performance from MaxPeak High Performance Surfaces (HPS) Technology demonstrates that the BioAccord System with ACQUITY Premier controlled by the compliant-ready waters_connect informatics is optimally suited as a LC-MS platform for MAM-based assays.

Benefits

- Improved acidic peptide recovery from MaxPeak HPS System and column surfaces
- Peptide quality attribute monitoring at low loading levels
- Stable MS signal at high and low measurement levels
- Lowered %RSD levels for monitored attributes

Introduction

LC-MS based multi-attribute method (MAM) assays continue to gain popularity within the biopharmaceutical industry due to their ability to directly measure multiple attributes such as product variation and degradation. These assays complement, and can potentially replace, multiple conventional optical based assays due to their increased specificity and sensitivity in a single analysis.[1] To this end, efforts have been made to develop and validate MAM-based assays using critical quality attributes (CQAs) of the drug product as part of lifecycle management.[1,2] Assays need to be robust, accurate, and consistent to ensure methods can be readily deployed across an organization to support product development, manufacturing, and release activities. It has become clear that everything from sample preparation, chromatographic robustness, detection consistency, and informatic processing can affect overall assay robustness, with abundance (trace-level) of targeted attributes having the greatest challenges for assay performance and reproducibility.

Recently, it was reported that chromatographic performance for peptides containing multiple acidic residues (glutamic/aspartic acid or residues with acidic modifications) can be compromised with generic RPLC-MS methods when performed on conventional stainless-steel LC systems.[3] Peptides bearing electron-rich moieties such as carboxylic acids interact with or adsorb to the metal oxide surfaces in the instrument and column resulting in reduced recovery and increased peak tailing for these metal sensitive analytes. Adsorption phenomena such as these can be particularly challenging for peptide MAM analyses, as suboptimum performance brought on by metal/surface interaction of analytes can reduce the quantitative accuracy and reproducibility of the assay in the assessment of product attributes. Substantial efforts have been made to reduce these metal-peptide interactions in RPLC assays using alternate ion-pairing reagents, passivation, and high ionic strength solvents that may require additional method development efforts that are not always MS-compatible.[4,5] While these mitigation strategies have been shown to improve chromatographic performance, careful consideration must be given during method optimization as introduction of additional method complexity

increases the risk associated with method robustness. The ACQUITY Premier UPLC System is designed to improve the chromatographic performance for acidic compounds without the need for additional method development or extensive optimization efforts. This is made possible through the incorporation of MaxPeak High Performance Surfaces (HPS) Technology in the high surface area components of the LC system and column hardware which introduces a barrier layer to minimize analyte/surface interaction of metal sensitive analytes.[6] The purpose of this study is to demonstrate the impacts of MaxPeak HPS Technology to MAM-based assays through its ability to improve recovery[7] and peak shape of metal sensitive analytes, resulting in improved assay sensitivity and robustness without changing the RPLC-MS method or conditions.

Experimental

Sample Description

mAb Tryptic digestion standard (p/n: 186009126 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009126-mab-tryptic-digestion-standard.html>) was dissolved in 200 µL of 0.1% formic acid to yield a final concentration of 0.2 µg/µL. The injection volume of the sample was 5.0 µL (1.0 µg).

Method Conditions

Data was acquired on both a (1) BioAccord System with ACQUITY UPLC I-Class PLUS (Stainless-Steel) and a (2) BioAccord System with ACQUITY Premier (Inert-MaxPeak HPS) for direct comparison. Column technology consistent with each system architecture were used with a common stationary phase packing.

LC Conditions

Detection:	TUV, MS
Vials:	QuanRecovery with MaxPeak HPS Vials (p/n: 186009186)

Column(s):	(1) ACQUITY UPLC Peptide CSH C_{18} Column (p/n: 186006938) (2) ACQUITY Premier Peptide CSH C_{18} Column (p/n: 186009489)
Column temp.:	60 °C
Sample temp.:	6 °C
Injection volume:	Blank 10 µL, sample 2–10 µL
Flow rate:	0.2 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
0.00	0.2	99	1	initial
3.00	0.2	99	1	6
78.00	0.2	65	35	6
85.70	0.2	15	85	6
93.00	0.2	15	85	6
100.70	0.2	99	1	6
120.00	0.2	99	1	6

MS Conditions

MS system:	ACQUITY RDa Detector
Ionization mode:	ESI+
Acquisition range:	m/z 50–2000
Capillary voltage:	1.2 kV
Collision energy:	60–120 V
Cone voltage:	20 V

Data Management

Informatics:	waters_connect with Peptide MAM App, LC-MS Tool Kit

Results and Discussion

In this study, we conducted a side-by-side comparison of a conventional stainless-steel flow path BioAccord System vs. the BioAccord System configured with an inert MaxPeak HPS Surfaces ACQUITY Premier LC System and Column (Figure 1). In this comparative evaluation, both BioAccord Platforms included the ACQUITY RDa Mass Detector and used the waters_connect Peptide MAM application workflow to generate results. Analyte recovery, assay sensitivity, and reproducibility of selected CQAs from a digest of the NIST mAb reference material were used to assess system performance and the suitability of the BioAccord System with ACQUITY Premier to support MAM-based assays.

Figure 1. The BioAccord System with ACQUITY Premier. The ACQUITY Premier Technology contains flow paths, frits, and columns with MaxPeak High Performance Surfaces (HPS) to reduced metal surface adsorption of analytes.

Increased Recovery

Deamidation of asparagine to aspartic acid and iso-aspartic acid is a common PTM of monoclonal antibodies (mAbs) that has been shown to impact their efficacy and potency.[8] As part of the development process of mAb-based drug products, sequences susceptible to deamidations are frequently characterized and monitored to maintain the consistent quality of the biotherapeutic. The "PENNY" peptide contains a known sequence with multiple likely deamidation site(s) that is reported to impact antigen binding.[8] As a sequence located in the constant domain (Fc) of the heavy chain, it is a peptide routinely monitored during development and manufacturing of mAb-based therapeutics to monitor process consistency and product quality. For the NISTmAb reference antibody, after enzymatic treatment with trypsin, the HC:T37 "PENNY" peptide already contains four acidic residues (sequence: GFYPSDIAVEWESNGQPENNYK) making it highly susceptible to adsorption to metal surfaces in the LC flow path and column hardware, and making it an ideal candidate analyte to evaluate the MaxPeak Premier HPS Technology.

In this comparison study, two deamidation sites which are commonly monitored for the PENNY peptide were

observed with both systems (Figure 2A, peak 1 & 2), as well as a peak corresponding to the succinimide intermediate (Figure 2A inset). While present in both data sets, the BioAccord System with ACQUITY Premier exhibited improved recovery overall with a 2-fold or greater increase in MS signal intensity for the "PENNY" peptide and its related variants as shown in Figure 2B. Interestingly, while visible, deamidation peak 1 was below the detection limit for the processing method in the conventional data set due to its low recovery and poor peak shape. In this context, the data illustrates how MaxPeak HPS Technology can improve data analysis, particularly in automated workflows that use the same processing method.

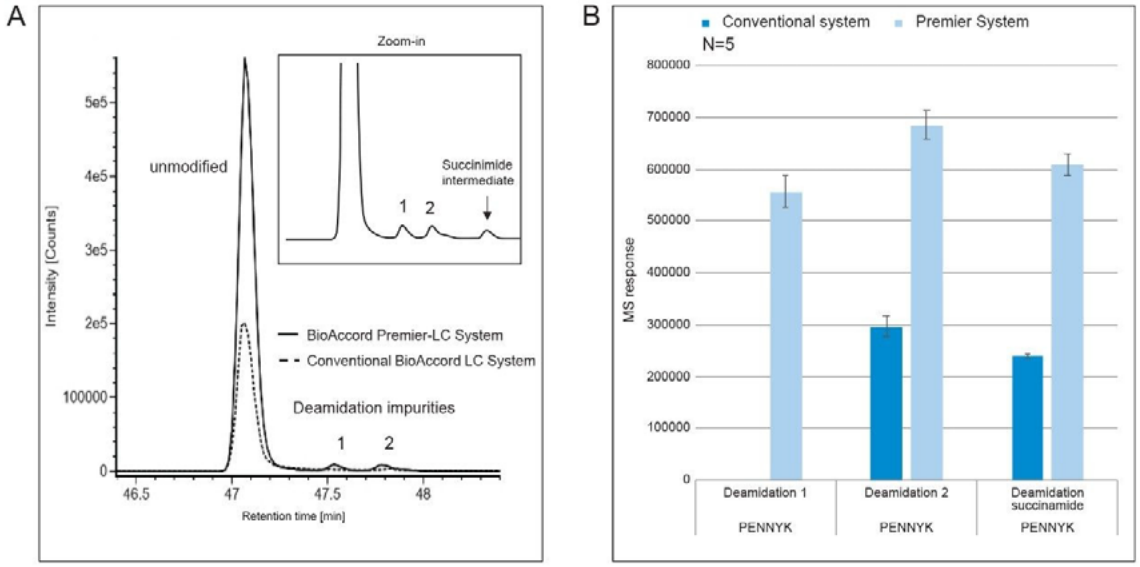

Figure 2. Recovery Comparison. A) Extracted ion chromatograms (XIC) for the HC:T37 peptide fragment (sequence: GFYPSDIAVEWESNGQPENNYK) and associated impurities from a tryptic digest of the NISTmAb reference material separated on a conventional BioAccord System (dashed line) and a BioAccord System with ACQUITY Premier featuring MaxPeak HPS Technology (solid line). B) Total normalized peak area for the HC:T37 impurities were calculated for a set of 5-injections on both systems.

Furthermore, the performance gain exhibited by MaxPeak Premier HPS Technology was observed to directly impact the quality of MS data. As shown in Figure 3, the increased recovery of the PENNY peptide and its associated variants resulted in an increase in the number and intensity of b/y fragment ions (3 vs. 7) for the PENNY peptide. This data demonstrates that ACQUITY Premier Technology can be used to increase recovery of

product quality attribute related peptides for improved confidence in peak assignment in MAM-based assays.

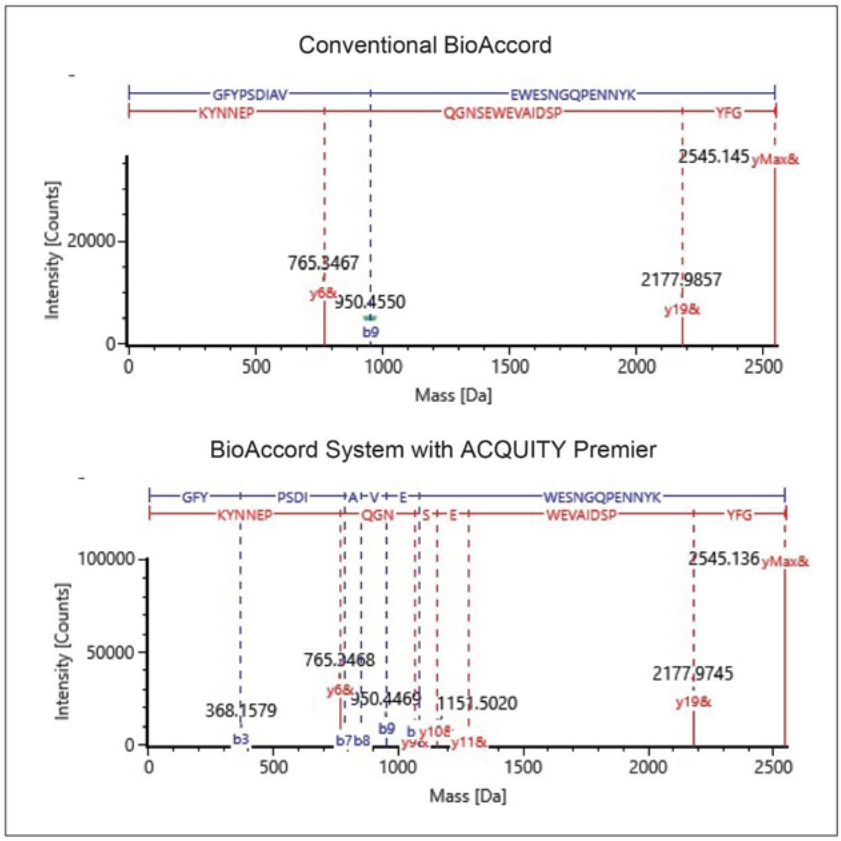

Figure 3. Improving MS data quality. The b/y fragmentation spectra acquired for PENNY deamidation-2 on conventional and ACQUITY Premier Systems. Evaluation of fragmentation data for the HC:T37 deamidated impurity (peak 2) using MS with fragmentation mode (data independent acquisition) exhibited improved intensity for fragment ions on the BioAccord System with ACQUITY Premier (bottom panel) when compared to the conventional BioAccord System (top panel).

Robust Technology

Developing robust methods that can be readily scaled and deployed across an organization is critical to maintain productivity and facilitate easier method transfer between labs. This can be particularly challenging for LC-MS

methods when working with samples across labs that may vary in complexity and concentration. As shown in the previous data, ACQUITY Premier with MaxPeak HPS Technology is able to improve the recovery and MS response of metal sensitive peptides.

To further evaluate the robustness of the BioAccord System with ACQUITY Premier in its ability to support development and manufacturing activity, a panel of product quality associated attributes (Table 1) was monitored over a broad range of concentrations (0.1 µg–2.0 µg). As shown in Figure 4, the BioAccord System with MaxPeak Premier Technology is able to accurately and consistently report area % of attributes over the measured range with %RSD of individual attributes not exceeding 20% for the majority of attributes. An example of this performance stability can be seen with the DTLMISR oxidation modification which consistently responded over the increasing mass load with a calculated %RSD of 6.3%. Higher variability was observed with lower abundant species at lower mass loads, particularly glycopeptides, however this is not entirely unexpected as glycopeptides are poorly ionizing and only present at 1.03% MS response relative to the base peak of the peptide digest. This data illustrates the BioAccord System when coupled to the ACQUITY Premier is a robust LC-MS platform that can be broadly applied in labs to support development and manufacturing activity and is well suited for MAM-based assays which inherently require instruments that can detect multiple analytes across varying abundance levels.

Peptide sequence	Modification	Mean % mod
VVSVLTVLHQDWLNGK	(Base peak)	95.52%
DIQMTQSPSTLSASVGDR	oxidation	0.94%
DMIFNFYFDVWGQGTTVTVSSASTK	oxidation	1.02%
DTLMISR	oxidation	1.60%
GFYPSDIAVEWESNGQPENNYK	Deamidation 1	2.06%
GFYPSDIAVEWESNGQPENNYK	Deamidation 2	1.72%
GFYPSDIAVEWESNGQPENNYK	Deamidation succinamide	1.88%
VTNMDPADTATYYCAR	oxidation	0.74%
VVSVLTVLHQDWLNGK	Deamidation 1	1.01%
VVSVLTVLHQDWLNGK	Deamidation succinamide	2.92%
Glycopeptide		
EEQYNSTYR	(base peak)	1.03%
EEQYNSTYR	G0F	43.30%
EEQYNSTYR	G1F	40.47%
EEQYNSTYR	G2F	9.06%
EEQYNSTYR	G0F-GlcNAc	1.82%
EEQYNSTYR	G1F-GlcNAc	2.82%
EEQYNSTYR	Man5	1.40%

Table 1. A selected list of NIST mAb critical quality attributes measured across different mass loads (0.1 µg–2.0 µg) of the digest on a BioAccord Premier.

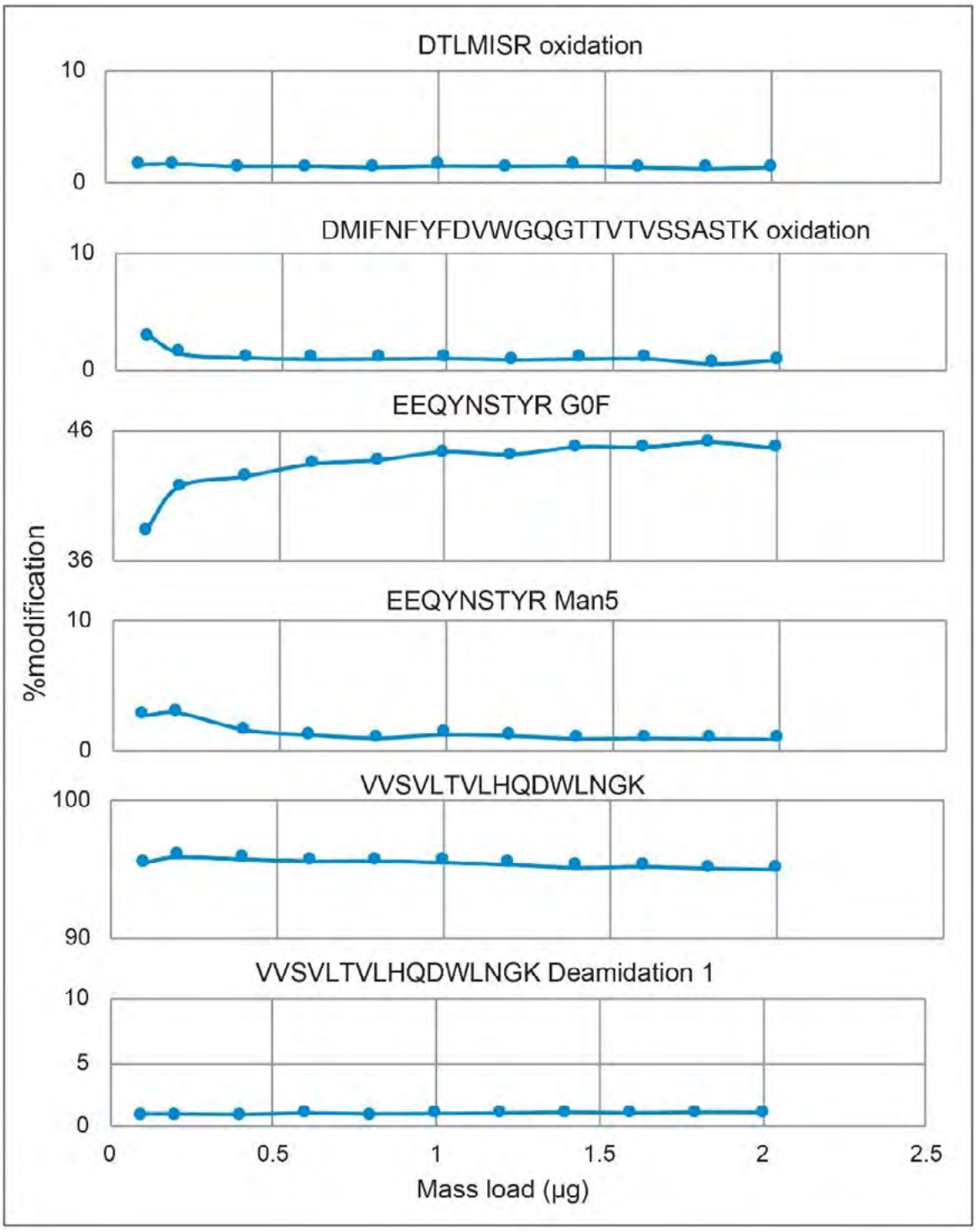

Figure 4. Consistence Performance. Calculated % modification levels for CQAs of the NISTmAb RM 8671 based on % normalized MS response for increasing mass load.

Reproducible and Broad Applicability

As previously shown, the BioAccord System with ACQUITY Premier as an LC-MS platform can deliver consistent and accurate results and is able to support the analytical needs across an organization. However, MAM-based assays contain a particular challenge with respect to linear dynamic range. At a fundamental level, the concept of determining the dynamic range of a simplex assay relies on the accuracy and precision of an assay in response to a reference standard which is used to set the acceptance criteria. This can be more challenging for MAM assays as the dynamic range represents a spectrum of multiple analytes with varying levels of baseline peptide ionization efficiency and relative abundance to their unmodified form. To ensure reproducible and accurate measurement of CQAs in MAM-assays the LC-MS platform must demonstrate a linear response for a given analyte and a broad spectral dynamic range to address complex samples.

To evaluate this practical dynamic range issue, normalized response for the T26 peptide fragment base peak (sequence: VVSVLTVLHQDWLNGK) was plotted against increasing mass load. As shown in Figure 5A, MS source saturation was observed above loadings of 1.2 µg with the linear range spanning 0.1 µg–1.0 µg with an R^2 = 0.99. Using this information, it was determined a mass load of 1 µg would allow for an acceptable mass load with no peaks exceeding upper response limits and maximum sensitivity towards trace-level CQAs. This is demonstrated in Figure 5B where the BioAccord Premier LC was able to detect the Man 5 glycosylation species (0.08% of base peak) of the T25 peptide fragment (sequence: EEQYNSTYR) with a high degree of reproducibility (Response %RSD = 2.78%). More notable is the fact that the in-spectrum detector response represents a spectral dynamic range that spans 3-orders of magnitude. Thus, the BioAccord System with ACQUITY Premier is able to acquire accurate and reproducible data for a diverse set of analyte peptides with a sensitivity that is well suited for routine use MAM-based assays. This is further demonstrated in Table 2 where %RSD for the monitored attributes were observed to be below 4% when performing the MAM assay on the BioAccord System with ACQUITY Premier which was 1.5–2.5-fold lower than the conventional BioAccord System. As expected, the largest differences observed were with acidic residue containing peptides demonstrating the value MaxPeak HPS Technology brings to MAM-based assays.

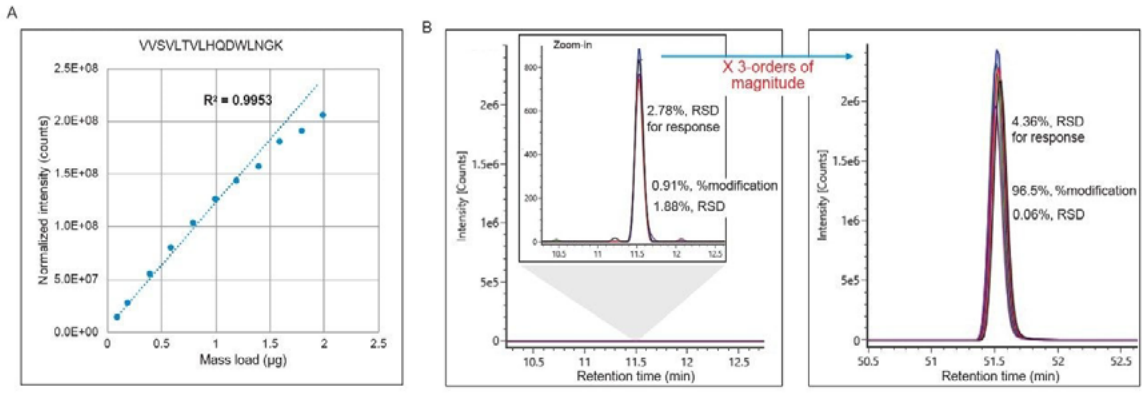

Figure 5. Dynamic range, A) Evaluating the linearity of normalized response across different mass loads. The selected peptide T26:VVSVLTVLHQDWLNGK is the base peak peptide of the MS spectrum. The abundance of lowest measured attribute T25:EEQYNSTYR Glycosylation Man 5 relative to the given T26 peak is 0.08%. B) chromatograms of and VVSVLTVLHQDWLNGK measured at 1 µg optimum loading levels. The data delivered stable MS responses and %modification levels across 5-consecutive injections.

Peptide sequence	Modification	%Modification conventional system	%Modification ACQUITY Premier System	%RSD conventional system	%RSD ACQUITY Premier System
VVSVLTVLHQDWLNGK	(base peak)	95.88	96.55	0.04	0.06
DIQMTQSPSTLSASVGDR	oxidation	0.86	0.86	7	4
DMIFNFYFDVWGQGTTVTVSSASTK	oxidation	1.69	1.06	7.3	1.18
DTLMISR	oxidation	1.33	1.66	3.05	3.45
GFYPSDIAVEWESNGQPENNYK	Deamidation 1	2.1	1.71	7.4	2.81
GFYPSDIAVEWESNGQPENNYK	Deamidation 2	-	2.1	-	1.33
GFYPSDIAVEWESNGQPENNYK	Deamidation succinamide	1.99	1.87	2.68	0.89
VTNMDPADTATYYCAR	oxidation	0.46	0.67	7.45	2.4
VVSVLTVLHQDWLNGK	Deamidation 1	0.92	0.92	3.44	1.48
VVSVLTVLHQDWLNGK	Deamidation succinamide	2.74	2.53	2.15	1.94
EEQYNSTYR	(base peak)	0.68	0.56	4.81	2.41
EEQYNSTYR	G0F	43.81	43.81	0.27	0.46
EEQYNSTYR	G1F	41.51	41.65	0.44	0.31
EEQYNSTYR	G2F	8.17	7.65	0.57	0.93
EEQYNSTYR	G0F-GlcNAc	2.36	2.47	2.68	1.41
EEQYNSTYR	G1F-GlcNAc	2.56	2.85	1.25	1.64
EEQYNSTYR	Man5	0.91	1	3.06	1.88

Table 2. A selected list of NISTmAb critical quality attributes measured at 1 µg mass load with conventional system and a BioAccord System with ACQUITY Premier. The % modification levels determined using waters connect, Peptide MAM application are reported here with respective %RSD levels measured across 5-injections.

Conclusion

To develop a robust peptide MAM assay, an LC system that delivers and unbiased and consistent protein digest separation is a necessity. In this study we evaluated the BioAccord System with ACQUITY Premier featuring MaxPeak HPS Technology against a conventional stainless-steel LC-MS platform for its ability to obtain optimal chromatographic performance for peptides representing product quality attributes in a RPLC-MS based peptide MAM assay. In this comparison, it was demonstrated that the MaxPeak HPS Technology minimizes adsorption of metal sensitive analytes, enabling robust method execution with improved recovery, assay sensitivity, and method reproducibility. In summary, the BioAccord System with ACQUITY Premier represents a robust and flexible LC-MS platform that is ideal for deployment across development, manufacturing, and quality organizations.

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Application Note

Applying Peptide Mapping and Multi-Attribute Method (MAM) Workflow for Biosimilar mAb Drug Products Comparison on the Xevo™ G3 QTof Platform

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Waters Corporation

Abstract

With the increasing emergence of biosimilar monoclonal antibodies (mAbs), streamlined processes for characterizing these products are highly desirable. Instruments that can provide data to confidently identify and quantify peptide attributes, as well as data analysis pipelines that can expedite and automate analysis while maintaining compliance are essential to acquiring results in a fast-paced, growing market. The new Xevo G3 QTof Platform coupled with the ACQUITY™ Premier UPLC™ System enables robust and reliable analysis of biotherapeutic proteins. The app-based compliance-ready waters_connect™ informatics platform enables streamlined data management from acquisition to analysis. These integrated tools enable efficient biopharmaceutical workflows to be developed, including those for characterization and attribute monitoring.

This study demonstrates how waters_connect can be used with the Xevo G3 QTof for biosimilar mAb characterization and monitoring. The peptide maps of four infliximab samples, including the innovator and three biosimilars, were analyzed for product attributes, including deamidation, oxidation, lysine clipping, and

glycosylation. Differences in their relative abundance between mAbs were quantified using the Peptide MAM App within the compliance-ready waters_connect Software. A stress study of targeted attributes was also carried out for the innovator and one biosimilar to identify critical quality attributes (CQAs) from thermal stress for each mAb. The results showcase how the Xevo G3 QTof Platform with integrated waters_connect data acquisition and processing is a suitable platform for comparative peptide mapping workflows to establish mAb biosimilarity.

Benefits

- High-coverage peptide mapping of innovator and biosimilar mAbs with confident identification and quantification of peptide attributes
- Integrated compliance-ready app-based workflow for streamlined acquiring, processing, and reviewing of peptide mapping data
- Reproducible quantification of low-level peptide attributes for establishing biosimilarity

Introduction

As the patents for monoclonal antibodies (mAbs) expire, an increasing number of biosimilars are being approved by regulatory agencies. With this increasingly competitive market for biosimilar drug products, there is a need to streamline processes to characterize and compare biosimilars to the innovator drug products. Characterizing biosimilars requires a workflow that can confidently identify and quantify various product attributes key to safety, efficacy, and stability. Biosimilar products are highly similar to the active ingredient in the innovator product, but their properties can differ due to differences in production methods as long as those differences generate no clinically meaningful impacts. One of the prominent routes to characterizing and comparing biosimilars is using peptide mapping to analyze post-translational modifications. Enzymatically digesting a mAb and analyzing the peptide fragments with liquid chromatography (LC)-mass spectrometry (MS) enables confirmation of the primary sequence and localization of sites of product variation. The Multi-Attribute Method (MAM) has been gaining increasing popularity to quantify targeted attributes over multiple samples with higher throughput than traditional characterization approaches to data analysis. Rather than using multiple orthogonal analytical techniques for individual attributes, MAM offers the ability to directly monitor numerous attributes across samples using LC-MS.[1]

The Xevo G3 QTof Mass Spectrometer operated under the waters_connect informatics platform (Figure 1) provides a streamlined solution for executing biopharmaceutical workflows. With its updated ion optics to optimize peptide transmission and comprehensive quantification capabilities, the Xevo G3 QTof is fit for purpose for both characterization and monitoring of biotherapeutic attributes. The compliance-ready waters_connect platform handled the entire workflow from sample submission through data analysis and review, and the integrated UNIFI™ App (peptide mapping), scientific library (attribute libraries), and Peptide MAM App (targeted attribute monitoring and new peak detection) enabled a seamless harmonization of characterization and monitoring workflows. This study demonstrates the effectiveness of this combined workflow for peptide map characterization and monitoring of infliximab and three biosimilar drug products (Inflectra®, Avsola®, and Renflexis®). Peptide maps were compared across the four mAbs, and critical quality attributes (CQAs) were identified and reproducibly monitored for the innovator and one of the biosimilars in a stress study.

Figure 1. Xevo G3 QTof platform with integrated waters_connect informatics for compliance-ready app-based data acquisition, processing, review, and reporting.

Experimental

Sample Description

Infliximab samples, including innovator (Remicade®) and biosimilar (Inflectra) were incubated at 37 °C for zero weeks (no stress), one week, or two weeks. All samples, including the two other biosimilars (Avsola and Renflexis), were reduced, alkylated, desalted, tryptic digested, and acidified to 0.1% formic acid. The final concentration was measured to be 0.16 µg/µL.

LC Conditions

LC system:	ACQUITY Premier UPLC System–BSM configuration
Detection:	ACQUITY Premier TUV; 10 mm analytical flow cell; λ = 214 nm
Vials:	QuanRecovery™ with MaxPeak™ HPS Vials (p/n: 186009186)
Column(s):	ACQUITY Premier CSH™ 130 Å C18 1.7 µm, 2.1 × 100 mm
Column temperature:	60 °C
Sample emperature:	8 °C
Injection volume:	2 µL
Flow rate:	0.200 mL/min
Mobile phase A:	0.1% Formic acid in water (LC-MS grade)

Mobile phase B:	0.1% Formic acid in acetonitrile (LC-MS grade)
Gradient:	1–35 %B over 50 min gradient (80 min total run time)

MS Conditions

MS system:	Xevo G3 QTof
Ionization mode:	ESI+
Acquisition range:	100–2000 *m/z*
Capillary voltage:	2.2 kV
Collision energy:	Low energy: 6 V High Energy Ramp: 20–50 V
Cone voltage:	20 V
Source temperature:	120 °C
Desolvation temperature:	350 °C
Cone gas:	35 L/hr
Desolvation gas:	600 L/hr
Intelligent data capture (IDC):	Low (5)

Data Management

Data were acquired and processed using the waters_connect informatics platform (version 2.1.1.13) with UNIFI App (version 1.9.12.7) and the Peptide MAM App (version 1.0.0.3).

Results and Discussion

Thorough characterization of biosimilars is critical to ensuring the product's comparability to the innovator product. Here, the suitability of the Xevo G3 QTof operating within the integrated waters_connect platform is demonstrated for rigorous comparative analysis of infliximab and approved biosimilars. Although each biosimilar has an identical amino acid sequence to the innovator, differences in the profile of product variants can impact the safety, stability, and efficacy of the drug.

To gain insight into the differences between biosimilars, the peptide maps of each infliximab product were compared. Trypsin-digested samples were analyzed with an ACQUITY Premier UPLC coupled to the Xevo G3 QTof, using MS^E data independent fragmentation to identify peptides and localize sites of modification. Greater than 95% sequence coverage was achieved for each of the four infliximab products with less than 5 ppm mass error in the UNIFI App peptide mapping workflow within waters_connect. Furthermore, the results demonstrated excellent injection-to-injection reproducibility, with the relative standard deviation of peak intensity below 5%. This remarkable reproducibility can be seen in Figure 2A, where overlaid chromatograms from three replicate injections of Remicade show near perfect overlap.

While the chromatograms for each mAb sample look highly similar, as shown in Figure 2B, notable differences were observed in relative abundances of product variants. Across the four products, 47 modifications were localized, including 6 oxidations, 12 deamidations, 28 N-glycosylations, and C-terminal lysine clipping. Example high energy MS^E spectra are shown in Figure 3 for a peptide in its native and oxidized forms. The high coverage of assigned fragment ions enables both confident identification of the peptide and localization of the modification to the methionine amino acid, as evidenced by the y ion series. The differences in relative abundance of modifications were quantified using the Peptide MAM App in waters_connect. This software enables targeted quantification of a list of product quality attributes identified with the UNIFI App Peptide Mapping workflow across samples while remaining within the waters_connect Ecosystem.

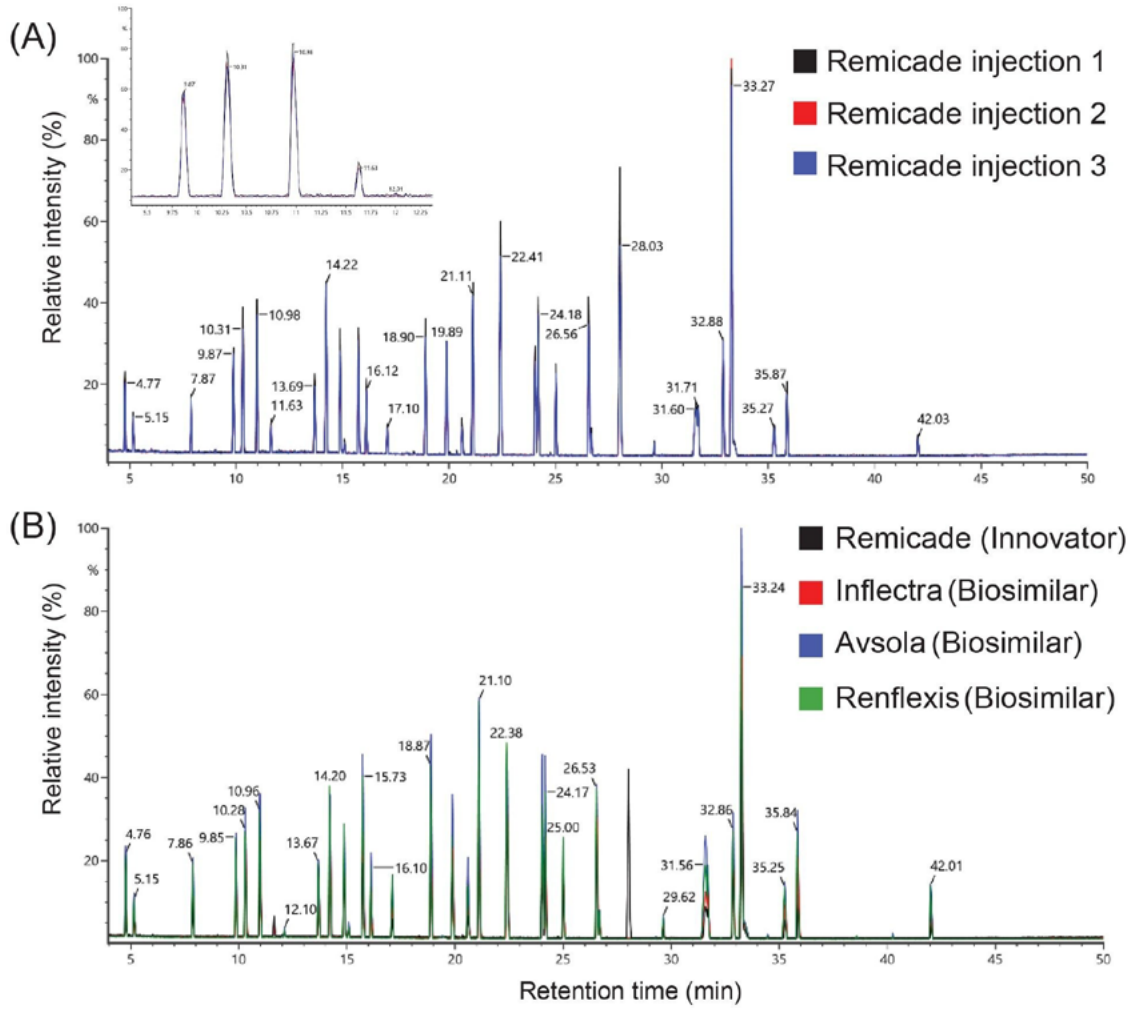


Figure 2. Overlaid base peak ion (BPI) chromatograms of mAb digest, including (A) replicate injections of the innovator (Remicade) sample, showing near perfect overlap of peaks, and (B) injections of the innovator and three biosimilars, indicating similar peptide map profiles.

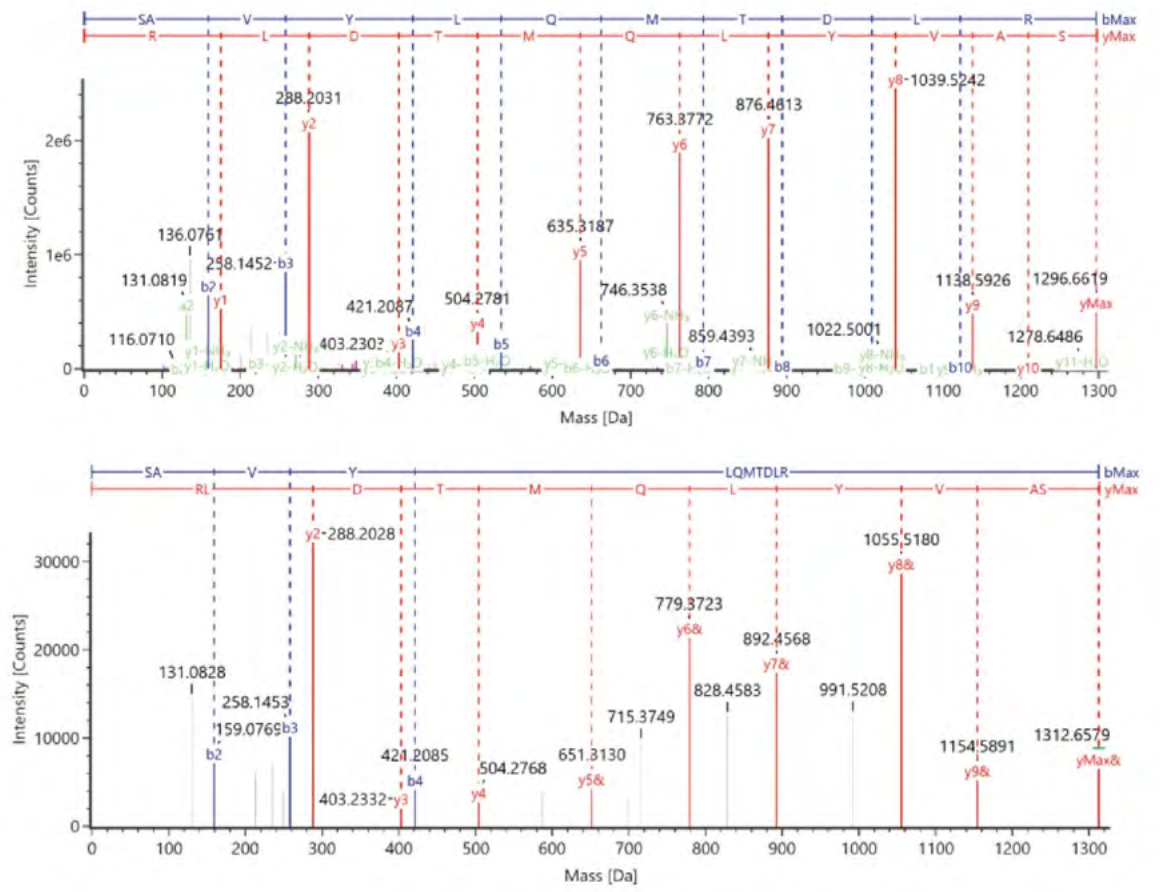
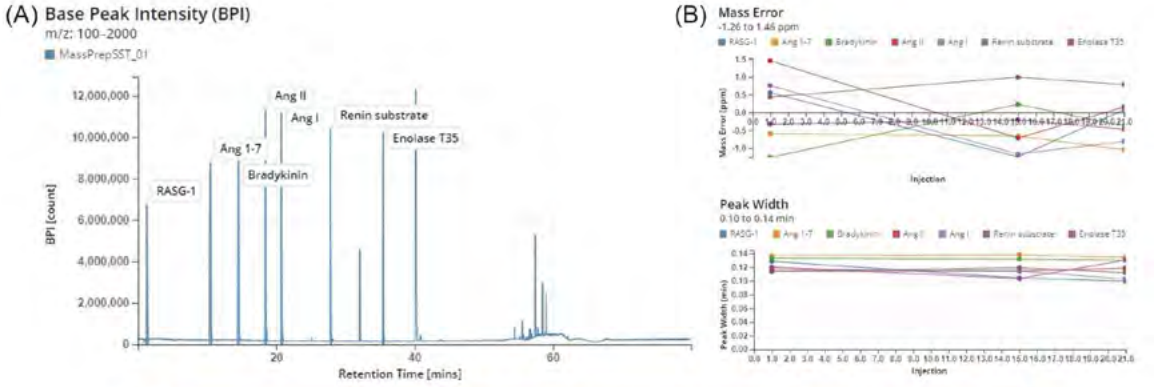


Figure 3. High energy MS^E spectra of HT11 peptide in its (top) unmodified form and (bottom) oxidized form.

Before reviewing quantification results, a system suitability analysis was performed as a part of the Peptide MAM Workflow. By evaluating intermittent injections of a known peptide sample, this step checks to ensure the system is producing acceptable and expected results for the separation and mass detection instrumentation as well as the automated data processing routines. This process is key to instilling confidence in the results, as attribute monitoring requires high analytical rigor. MassPREP™ Peptide Mixture (p/n: 186002337 < https://www.waters.com/nextgen/global/shop/standards--reagents/186002337-massprep-peptide-mixture.html >) was used for system suitability injections. Figure 4B shows two examples of how peptide data are tracked across injections, including mass error and peak width. The visualization of values across replicates enables the user to easily identify anomalies or systemic drifts over time. All four system suitability parameters are shown for each peptide in Figure 4C, reflecting the high mass accuracy (within ±1.5 ppm) and excellent reproducibility of

the platform throughout the duration of the sample sequence. The reproducibility achievable with the Xevo G3 QTof enables subtle trends between samples to be more easily distinguished, while saving time and money by reducing the number of replicates needed to obtain meaningful results for the lowest level product variants.



(C)

Peptide	Mass error (ppm)	RT error (min)	Intensity (×10⁸)	Peak width (min)
Ang 1–7	-0.76 ± 0.20	-0.03 ± 0.02	1.17 ± 0.24	0.137 ± 0.005
Ang I	-0.41 ± 0.84	-0.03 ± 0.03	3.33 ± 0.63	0.107 ± 0.005
Ang II	0.30 ± 0.89	-0.03 ±0.02	3.15 ± 0.62	0.117 ± 0.005
Bradykinin	-0.47 ± 0.61	-0.01 ± 0.03	4.71 ± 0.76	0.130 ± 0.000
Enolase T35	-0.33 ± 0.11	-0.04 ± 0.03	5.47 ± 0.86	0.117 ± 0.012
RASG-1	-0.20 ± 0.76	0.01 ± 0.01	4.94 ± 0.90	0.110 ± 0.014
Renin substrate	0.74 ± 0.23	-0.02 ± 0.04	3.17 ± 0.57	0.113 ± 0.005

Figure 4. System suitability results for MassPREP Peptide Mixture data processed with the Peptide MAM App, including (A) an example chromatogram, (B) examples of values, mass error and peak width, being tracked over injections for each peak, and (C) all suitability parameters for each monitored peak.

The relative quantification of targeted peptide attributes, as measured with the Peptide MAM App, averaged less than 2.0% relative standard deviation for the monitored attributes. An example of how these results are presented within the app is shown in Figure 5, where simplified bar graphs enable rapid evaluation of the data for each targeted attribute.

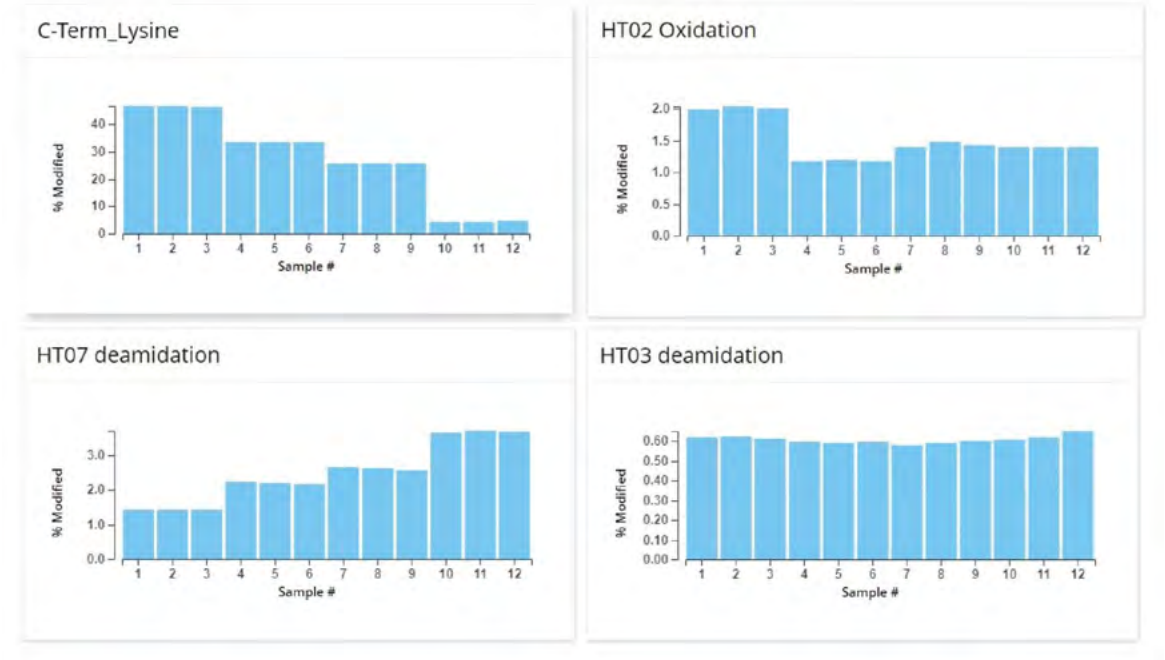


Figure 5. Bar graphs showing results presented in the Peptide MAM App for four monitored attributes, C-terminal Lysine, HT02 oxidation, HT07 oxidation, and HT03 deamidation. Injections are labeled as 1–3 for Remicade, 4–6 for Inflectra, 7–9 for Avsola, and 10–12 for Renflexis.

The quantification results for oxidized and deamidated peptides, as well as C-terminal lysine conjugation, are shown in Figure 6. All six of the oxidation sites monitored showed discernable differences between the infliximab products. For example, heavy chain tryptic peptide (HT) 02, HT11, and one of the sites on HT03 showed greater percentage of oxidation in the innovator, while HT22 showed greater percentage oxidation in two of the biosimilars, Avsola and Renflexis.

Differences in deamidation were less prominent. Of the twelve deamidations monitored, only two showed substantial differences between the biosimilars, including HT07, which had a greater percentage deamidation in the three biosimilars, and one of the sites on HT38, which showed a smaller percentage deamidation in Inflectra and Avsola as compared to Remicade and Renflexis.

The extent of C-terminal lysine clipping was also quantified, as this modification is common during bioproduction and may have an impact on receptor-binding.[4] As shown in Figure 6, the extent of Lysine clipping varies notably between the biosimilars, with Renflexis having the smallest percentage remaining on the peptide.

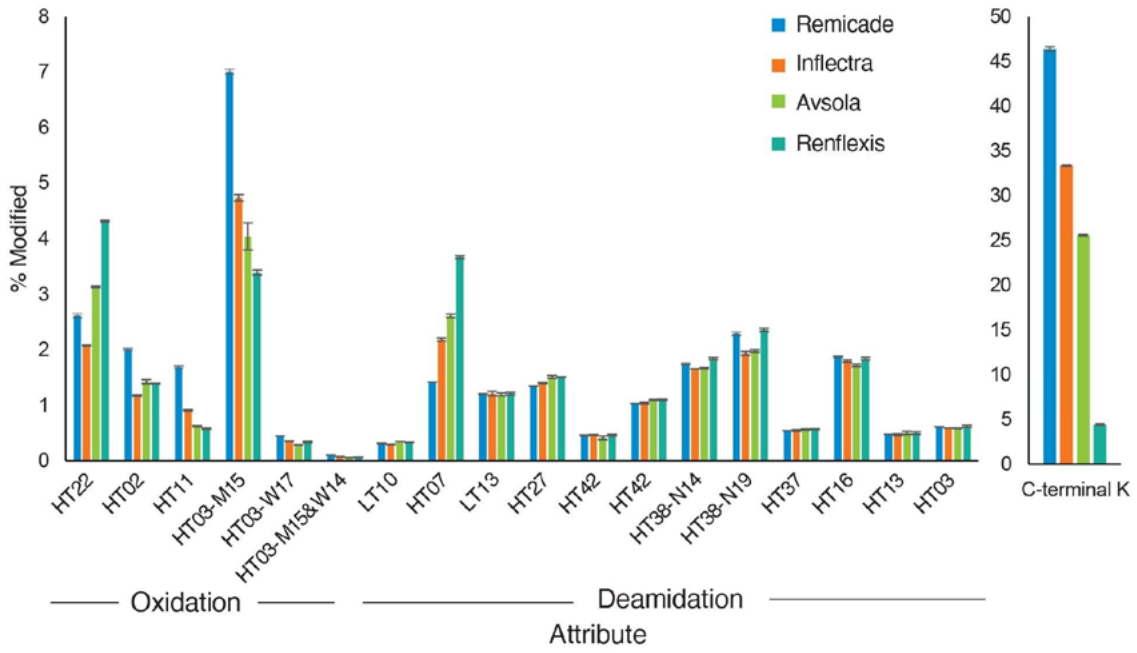


Figure 6. Relative abundances of peptide attributes (oxidation, deamidation, and C-terminal Lysine conjugation) between an innovator and three biosimilars. Error bars indicate standard deviation based on three replicate injections. For peptides with multiple modification sites, the modified amino acid is indicated. H heavy chain; L light chain; T tryptic peptide.

The percentage of various N-glycovariants showed variation between the infliximab products, likely due to manufacturing and cell-line differences.[5] Figure 7 shows the relative abundance of 28 N-glycoforms of the HT26 peptide, categorized into three groups: high-abundance (above approximately 2% relative abundance), low-abundance (below approximately 2% relative abundance), and immunogenic (glycans containing either N-glycolylneuraminic acid (NeuGc) or galactose-alpha-1,3-galactose (alpha-gal)).

In all four mAbs, FA2 and FA2G1 were the most prominent glycoforms, comprising approximately 50% and 30%, respectively, of the total abundance of all forms of the peptide. However, the exact relative amount of each varied between the mAbs, with Avsola having the highest abundance of FA2 and the lowest abundance of FA2G1. Conversely, Inflectra had the lowest abundance of FA2 and the greatest abundance of FA2G2.

The immunogenic glycoforms were in greater abundance in Remicade and Inflectra compared to Avsola and Renflexis, as is expected due to the difference in cell lines, with the former originating from murine cell lines and the latter from Chinese hamster ovary cell lines.

The low-abundance glycoforms showed largely contrasting relative abundances between the four mAbs. For example, A1, M5A1G1, FM5A1, and A1G1 were in the highest relative abundance in Remicade, while A2G1 and M6 showed the highest relative abundance in Renflexis, and A2G1 was highest in Inflectra. Differences in glycosylation are important to note, as they may impact the ability of the drug to provoke a desired or undesirable immune response.

In addition to a comparison of peptide maps, the innovator and one biosimilar (Inflectra) were also subjected to a stress study. Stress testing experiments are important in biotherapeutic development to obtain information pertinent to developing analytical methods, determining dosage forms, identifying impurities, calculating shelf life, and comparing degradation pathways for biosimilar products.[6,7] The stress, either thermal, chemical, or mechanical, is intended to accelerate protein degradation and elevate impurity levels, providing insight into how proteins' efficacy and immunogenicity may be affected. These studies enable identification of CQAs, which are peptide attributes that are critical to the product's potency, efficacy, and safety.

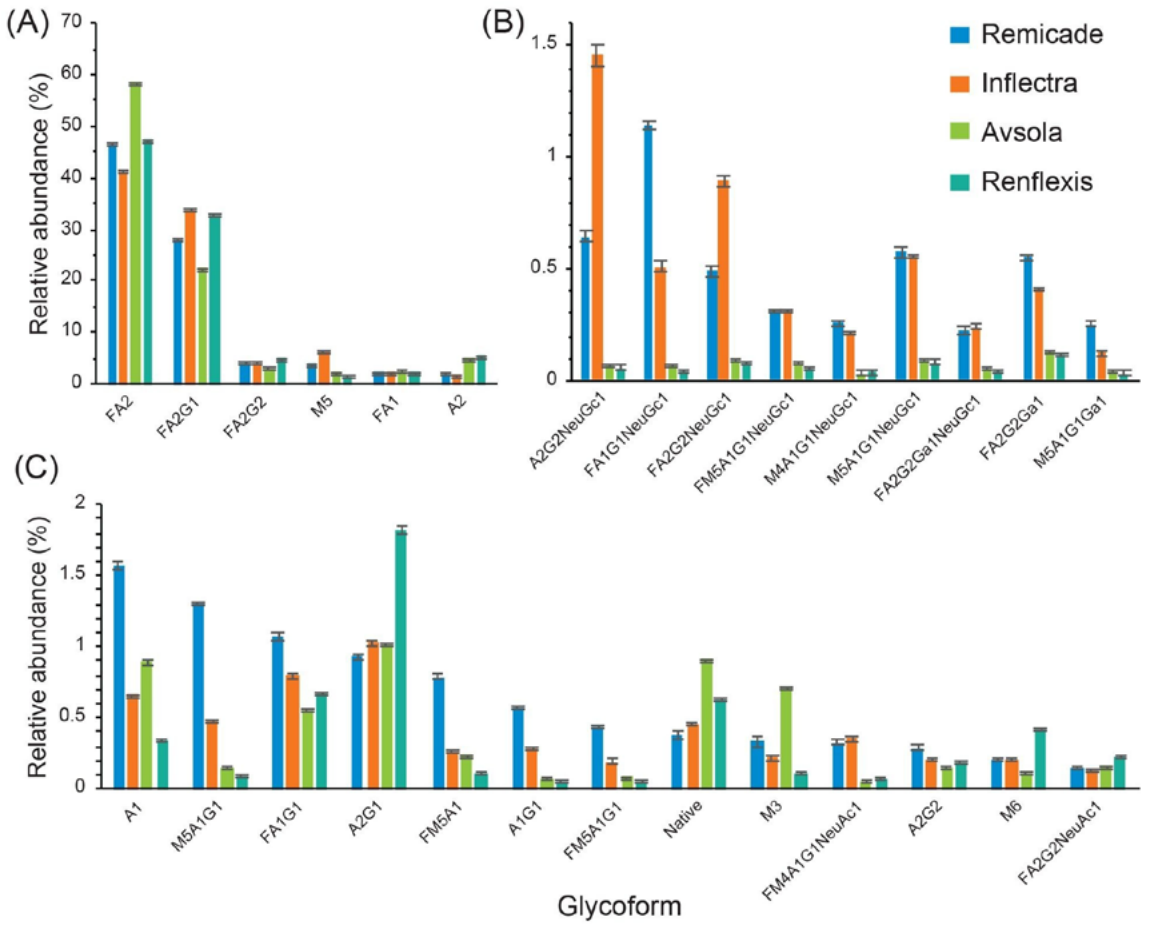


Figure 7. Relative abundances of glycosylated heavy chain peptide HT26 across innovator and three biosimilars, categorized by (A) high-abundance glycoforms, (B) immunogenic glycoforms, and (C) lower-abundance glycoforms. Glycoforms are labeled based on Oxford nomenclature. Error bars indicate standard deviation based on three replicate injections.

Here, the stress experiment was employed to monitor changes in CQAs in Remicade and Inflectra using the Peptide MAM App. Samples were subjected to temperature stress for one or two weeks prior to analysis and compared to an unstressed sample. Figure 8 shows a mirror plot comparing the unstressed (control) and two-week stressed samples of Inflectra vs Remicade. While the chromatograms look similar in the mirror plots with no obvious differences in major peaks, minor changes in several CQAs were identified with the MAM App, as shown in Figure 9. Many of the CQAs showed elevated responses in the stressed samples, though not all showed consistent increases between the innovator and biosimilar. For example, oxidation of HT22 and HT02

decreased in the two-week sample of Remicade but increased in the two-week sample of Inflectra. Others showed consistent results between the two, such as deamidation of HT07 and HT38, which both progressively increased in one- and two-week samples of both mAbs. No notable differences were observed in the relative abundances of glycoforms in the stressed samples or the relative amounts of Lysine clipping (data not shown). Biosimilars are not expected to have identical patterns of product variation, and any differences would be risk assessed to determine potential impacts to the functional and safety profiles of the molecules.

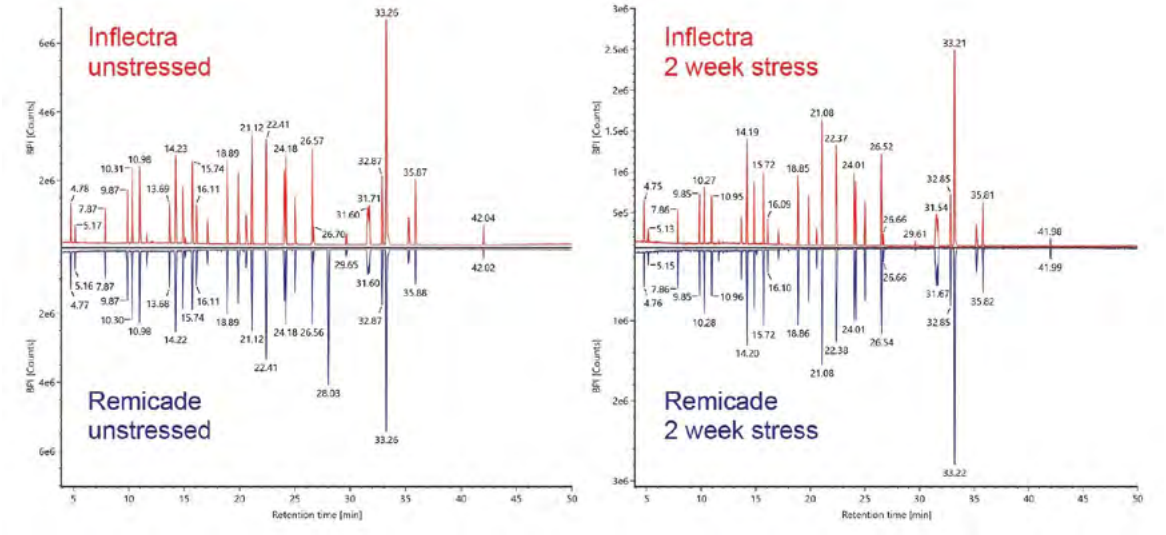


Figure 8. Mirror plots showing the comparison of Inflectra to Remicade for both (left) unstressed samples and (right) samples subjected to two weeks of elevated temperature stress.

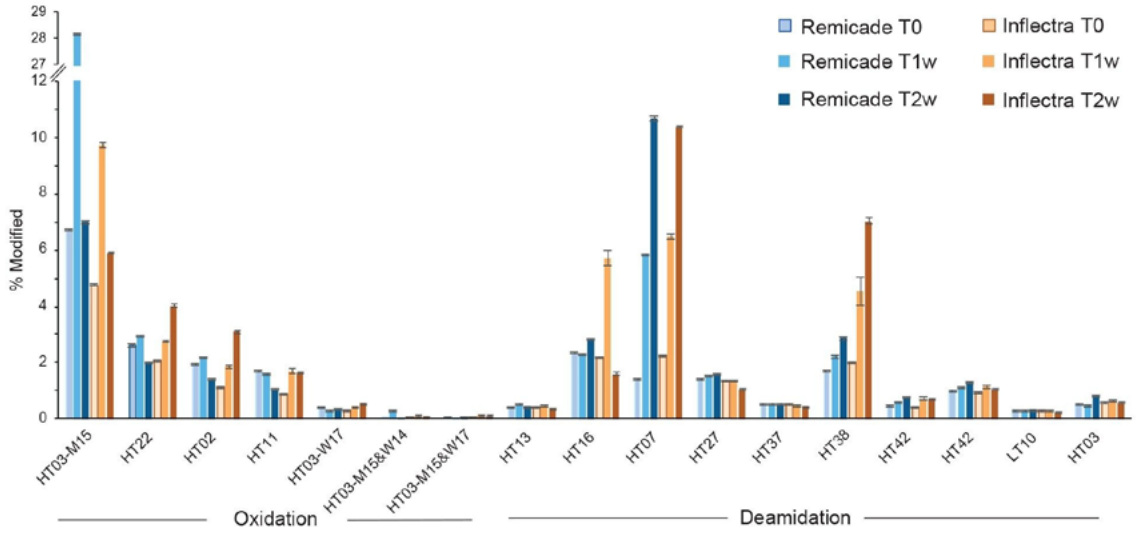


Figure 9. Relative abundance of oxidized and deamidated peptides in Remicade and Inflectra across each stress condition, including unstressed (T0), one week stress (T1w), and two weeks stress (T2w). Error bars indicate standard deviation based on three replicate injections. For peptides with multiple modification sites, the modified amino acid is indicated. H heavy chain; L light chain; T tryptic peptide.

Conclusion

Thorough characterization of biosimilar mAbs is critical to ensuring the safety and efficacy of the products and to relying on the innovator's experience to reduce clinical burdens for the follow-on product. The Xevo G3 QTof Platform with compliance-ready waters_connect Informatics enables robust and efficient analysis of product attributes through streamlined peptide mapping and peptide MAM workflows. Using this platform for primary structure characterization, this study achieved high sequence coverage of innovator and three biosimilar infliximab samples. With the integrated UNIFI App and Peptide MAM App, peptide attributes were identified, relatively quantified, and compared between the four mAb products. Additionally, potential CQAs were identified and quantified in a stress study. These results demonstrate how the Xevo G3 QTof can be used seamlessly with waters_connect data acquisition and processing for biosimilar peptide map characterization and attribute monitoring workflows.

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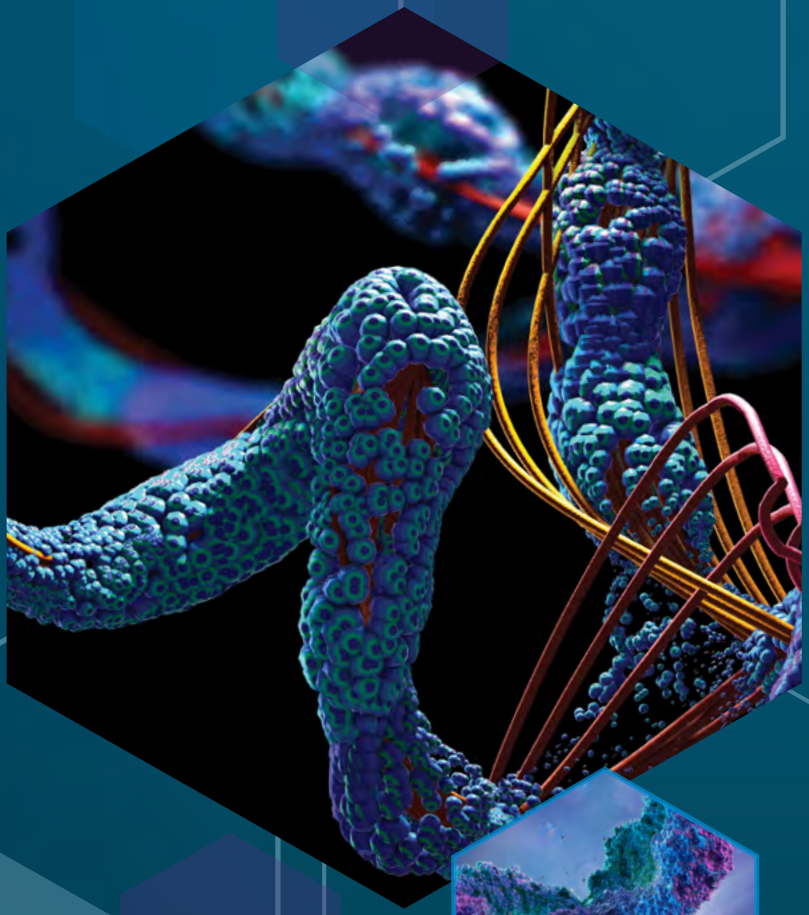

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Application Note

Monitoring Multiple Attributes in a Single Assay Using the ACQUITY QDa Detector for Product Confirmation and Process Monitoring of Product Quality Attributes

Brooke M. Koshel, Robert E. Birdsall, Ying Qing Yu

Waters Corporation

Abstract

This application note reveals a proof of concept strategy for identifying multiple product attributes within a single study using the ACQUITY QDa Detector with compliant-ready chromatographic data software such as Empower.

The ACQUITY QDa Detector provides an efficient and cost-effective solution for monitoring important product attributes in a development or QC environment when characterization has been previously carried out using a high resolution MS instrument. This application note demonstrates that CDR peptides, oxidized and deamidated peptides, and glycopeptides can be identified, quantitated, and reported from a single acquisition using the derived channel and inter-channel calculation functionalities in the Empower software. The list of attributes to be monitored would need to be determined for each respective antibody, but could be readily expanded to meet the individual user's needs.

Benefits

- Multiple attribute monitoring through a single acquisition to enable both product confirmation and routine screening of post-translational modifications
- Empower Software enables an automated and compliant workflow for data acquisition, processing, and reporting of multiple product quality attributes

Introduction

The concept of using a single LC-MS-based analytical method to monitor multiple product quality attributes (PQAs) is a strategy that has started to gain momentum in the biopharmaceutical industry. The idea behind this concept is that a single LC-MS method can be used to assess a product's important quality attributes simultaneously, as opposed to running a panel of optically-based chromatographic methods, which are unable to assess product attributes at a molecular level. The motivation for extending mass spectrometry (MS) beyond characterization into all stages of development and even quality control, is largely due to the complexity of protein-based therapeutics compared to small molecule drugs.[1] Replacing conventional chromatographic methods with a single, more sophisticated LC-MS method ultimately provides greater product and process understanding, which is required to support Quality by Design (QbD) regulatory submissions. Implementation of QbD is encouraged by regulatory agencies as a way of improving product quality and patient safety by offering a systematic and proactive approach to product development.[2,3]

It has recently been shown that a method for identity testing which monitors the complementarity determining region (CDR) peptides of a monoclonal antibody (mAb) has been developed and validated using the ACQUITY QDa Detector.[4] Using this work as the foundation of our study, we consider the need for a single test to be used for both product confirmation and also for monitoring a number of pre-characterized post-translational modifications (PTMs) using the ACQUITY QDa Detector, which provides a cost-effective solution for incorporating mass data into analysis. To align with the published work, a sample of trastuzumab and Waters Intact mAb Standard can be checked against the CDR peptides of a trastuzumab reference standard to verify method specificity through retention time and mass determination. Because a drug product's critical quality attributes (CQAs) would need to be independently determined, the current study is meant to serve as a proof of concept for providing a strategy for identifying multiple attributes within a single study using the ACQUITY QDa Detector with compliant-ready chromatographic data software such as Empower.

Experimental

LC Conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC TUV ACQUITY QDa Detector (Performance Model)
Absorption wavelength:	215 nm
Column:	ACQUITY UPLC Peptide CSH C_{18} 130 Å, 1.7 µm, 2.1 mm x 100 mm
Column temp.:	65 °C
Mobile phase A:	H2O with 0.1% (v/v) formic acid
Mobile phase B:	Acetonitrile with 0.1% (v/v) formic acid
Sample temp.:	10 °C
Injection volume:	10 µL

Gradient

Time (min)	Flow rate (mL/min)	%A	%B	%C	%D
Initial	0.2	97	3	0	0

Time (min)	Flow rate (mL/min)	%A	%B	%C	%D
3	0.2	97	3	0	0
120	0.2	67	33	0	0
127	0.2	20	80	0	0
130	0.2	20	80	0	0
131	0.2	97	3	0	0
150	0.2	97	3	0	0

Detector Settings

Sampling rate:	2 Hz
Mass range:	350–1250 Da
Ionization mode:	ESI+, centroid
Cone voltage:	10 V
Capillary voltage:	1.5 kV
Probe temp.:	500 °C

Data management

Empower 3 CDS, SR2

Results and Discussion

Monitoring Multiple Attributes using Extracted Ion Chromatograms for Identity Confirmation

The variable region of an antibody contains CDR peptides that are unique to that specific antibody, which allows these peptides to be used for identification purposes. To begin the evaluation of the ACQUITY QDa Detector for reporting multiple attributes, we began by collecting a peptide map of trastuzumab. A trypsin digest of reduced and alkylated trastuzumab was prepared and injected at a final concentration of approximately 0.5 mg/mL without further dilution. The peptide mapping method described above was used with the ACQUITY QDa Detector set to collect a full scan so that extracted ion chromatograms (XICs) could be used to identify the attributes of interest. Figure 1 shows a strong correlation between the optical trace and the corresponding mass data. This data suggests that the ACQUITY QDa Detector provides an effective way to incorporate mass measurements into an LC-UV based peptide map assay.

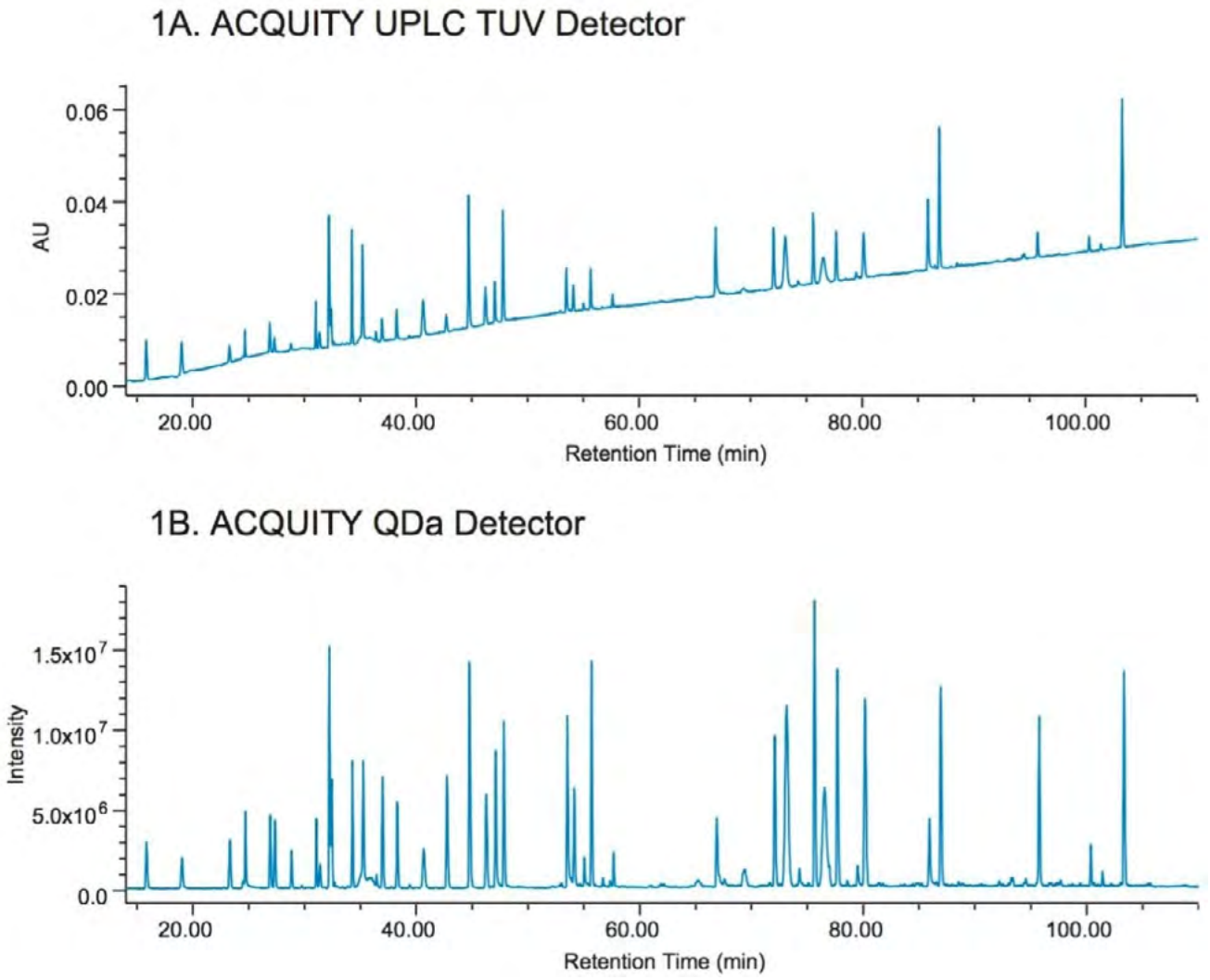

Figure 1. Peptide map detection. 1A) Optical detection of a trypsin digest of trastuzumab. 1B) Corresponding ACQUITY QDa data.

The CDR peptides can be used to confirm the identity of a given antibody by using XICs to extract the *m/z* of each CDR peptide. To avoid manual integration of each of the respective XIC channels, derived channels can be used. The process for creating derived channels has been previously described.[5] In brief, a method set can be created to contain a derived channel containing the *m/z* of each of the CDR peptides. This will essentially extract the *m/z* of each of the desired peptides into a single channel. By linking the derived channel to a processing method with associated retention time and component labels, the result is a single channel containing each of the CDR peptides which are now identified according to mass and retention time. Figure 2A shows a screen

capture from Empower that illustrates how a method set can be created to contain derived channels, where a new derived channel can be created for each of the attributes of interest. In the case of the CDR peptides, the mass of six CDR peptides can be entered into the Formula field of the derived channel as shown in Figure 2B. In this example, a single dominant charge state is used to identify each of the CDR peptides, but the user could sum over additional charge states if desired.

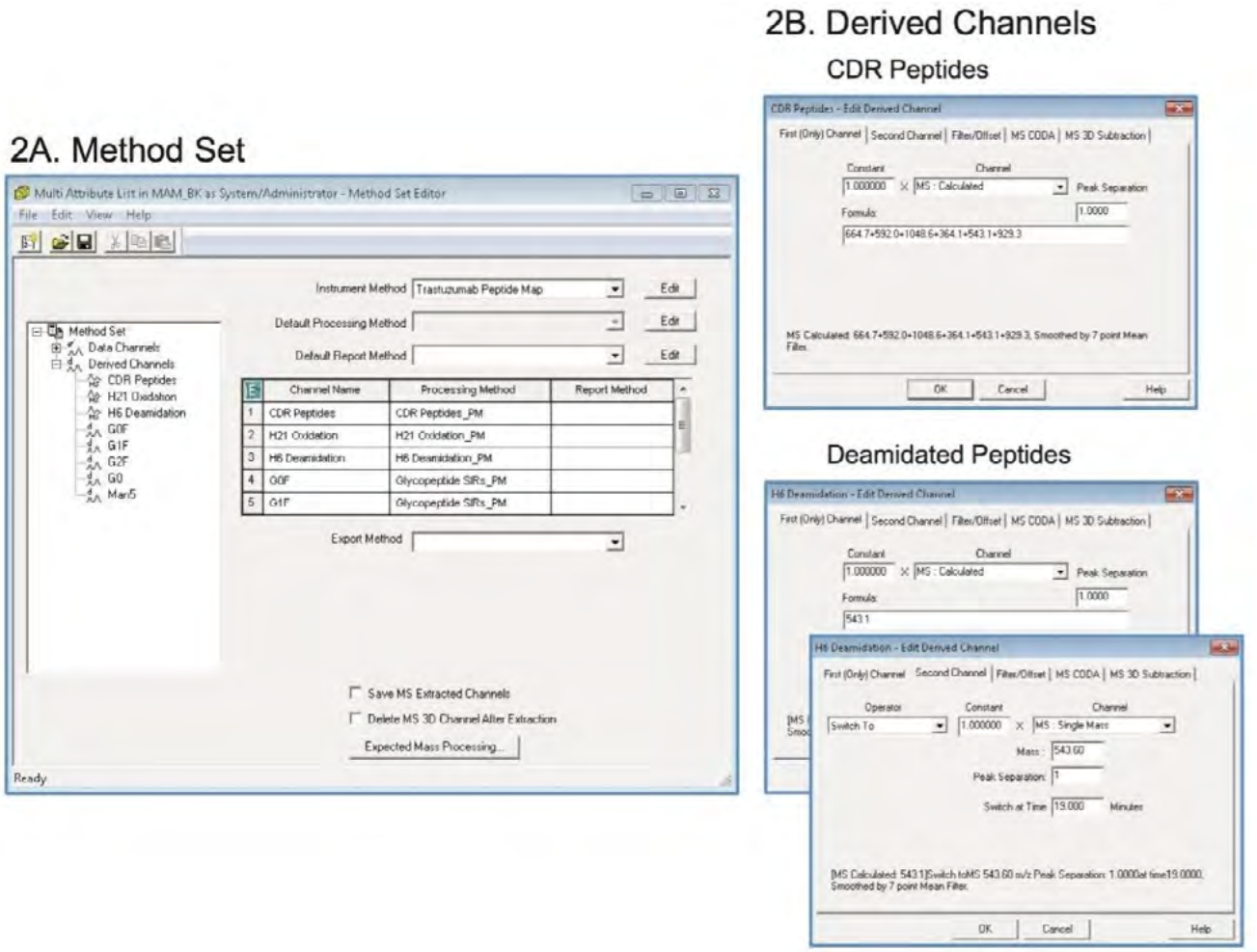

Figure 2. Empower screen captures. 2A) A method set containing derived channels used to determine if previously characterized attributes are present in a sample. The attributes of interest in this case are CDR peptides, an oxidized peptide, a deamidated peptide, and glycopeptides. Each attribute has an independent processing method associated with it. Attributes to be quantitated using XICs can be monitored through a single derived channel for that attribute, while attributes to be quantitated using SIRs must be monitored through individual channels and quantitated through custom calculations. 2B) Examples of derived channels. To create a derived channel for CDR peptides, the m/z of each peptide can be entered into the formula field. Six CDR peptides are monitored in this example. For the deamidated peptide, a time switch can be entered to switch from one m/z to another at a given time. This avoids signal overlap from using XICs having only a small difference in mass between the native and modified peptides.

The average mass for each of the CDR peptides is reported in Table 1 as well as the calculated m/z values used for this study. This table also contains mass information for additional attributes to be monitored as discussed below. The chromatogram resulting from the derived channel can be seen in Figure 3A. In this figure, XICs of each of the six CDR peptides are clearly identified, which confirms sample identity.

Monitoring Multiple Attributes using Extracted Ion Chromatograms for Process Monitoring

A similar approach can be used to quantify chemical modifications, such as deamidation or oxidation. Process changes during manufacturing or storage conditions can affect the rate of modification, which could potentially impact antibody activity or antigen binding.[6] In this example, we consider asparagine deamidation and methionine oxidation. Again, we make the assumption that these modifications would have been characterized using high resolution MS and determined to be important quality attributes.

Because the mass difference of the oxidation modification is large, this modification can be tracked in derived channels in a similar manner as the CDR peptides. The resulting XICs can be seen in Figure 3B. The deamidation event, however, shows a much smaller difference in mass between the native and deamidated peptides, so the data must be treated in a different way to reliably determine the relative abundance of each of the peptides. In this case, because the peaks are chromatographically resolved, a derived channel can be used that switches from one calculated mass to another at a time established by the user. This process is more clearly illustrated in Figure 2B. From the Empower screen captures, the mass to be monitored switches from m/z=543.1 Da (native peptide, z=2) to 543.6 Da (deamidated peptide, z=2) at 19 minutes. Using a second channel avoids signal overlap from the native and deamidated species. The XICs of the native and deamidated peptide can be seen in Figure 3C.

Peptide	Identification/ Modification	Average mass (Da)	Charge state	Calculated (*m/z*)
L3	CDR	1991.17	$[M+3H]^{+3}$	664.7
L5	CDR	1773.04	$[M+3H]^{+3}$	592.0
L7	CDR	4190.48	$[M+4H]^{+4}$	1048.6
H3	CDR	1089.21	$[M+3H]^{+3}$	364.1
H6	CDR	1084.18	$[M+2H]^{+2}$	543.1
H6	Deamidation	1085.17	$[M+2H]^{+2}$	543.6
H12	CDR	2785.01	$[M+3H]^{+3}$	929.3
H21	Native	834.43	$[M+2H]^{+2}$	418.2
H21	Oxidation	850.42	$[M+2H]^{+2}$	426.2
H25	G0F	2634.53	$[M+3H]^{+3}$	879.2
H25	G1F	2796.67	$[M+3H]^{+3}$	933.2
H25	G2F	2958.81	$[M+3H]^{+3}$	987.3
H25	G0	2488.39	$[M+3H]^{+3}$	830.5
H25	G1	2650.53	$[M+3H]^{+3}$	884.5
H25	Man5	2406.28	$[M+3H]^{+3}$	803.1

Table 1. Peptide information for reported attributes.

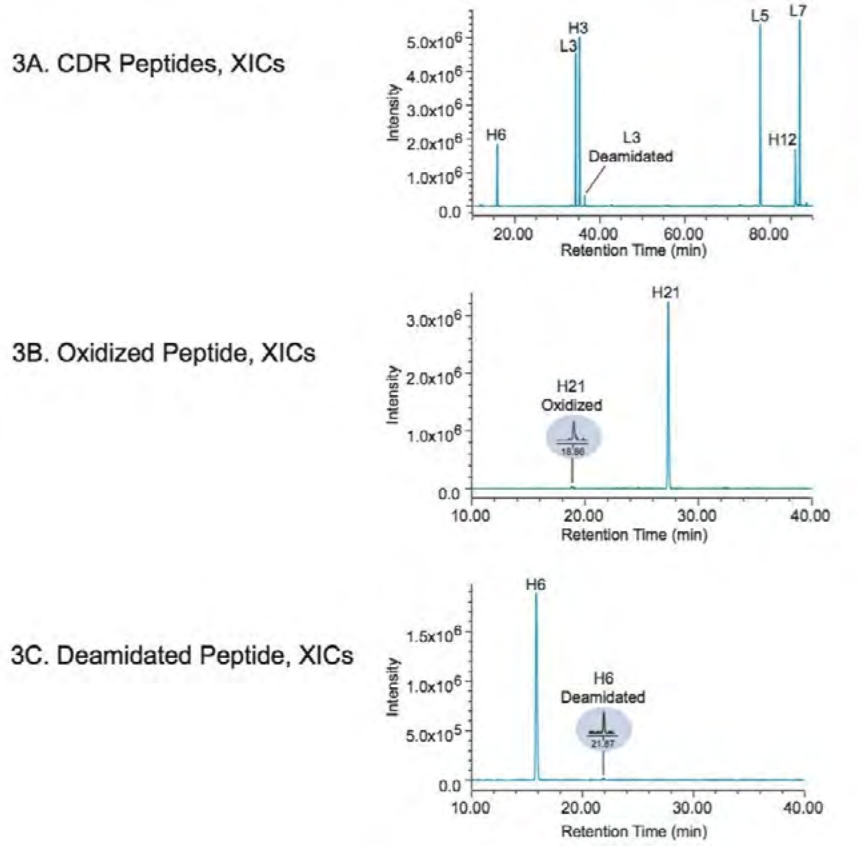

Figure 3. XICs used to identify attributes of interest. Peak labels "H" and "L" refer to heavy chain and light chain peptides, respectively. 3A) The CDR peptides unique to trastuzumab. 3B) A native peptide and its oxidized form. 3C) A native peptide and its deamidated form. Note that the native peptide, H6, is also a CDR peptide. This peptide can function to establish identity but can also be used along with its deamidated form to quantitate the percent modification independent of the CDR channel. Insets in 3B. and 3C. show a 10X zoomed in image of the oxidized and deamidated peptides, respectively. Signal-to-noise is well above minimum requirements to reliably quantitate these low level modifications.

Monitoring Multiple Attributes using Selected Ion Recording for Process Monitoring

When additional specificity and sensitivity are needed for peak monitoring, selected ion recording (SIR) can be used. By incorporating SIRs, a single *m/z* is selected and passed through to the detector. To demonstrate the utility of SIRs, the dominant charge state of the five most abundant glycopeptides (G0F, G1F, G2F, G0, and Man5) was previously determined. Figure 4 shows an overlay of the five SIR channels.

For monitoring glycopeptides, the additional sensitivity afforded by the SIRs is required for accurate quantitation. Because each SIR is associated with its own channel, custom calculations can be used to calculate the relative abundance of each glycopeptide.

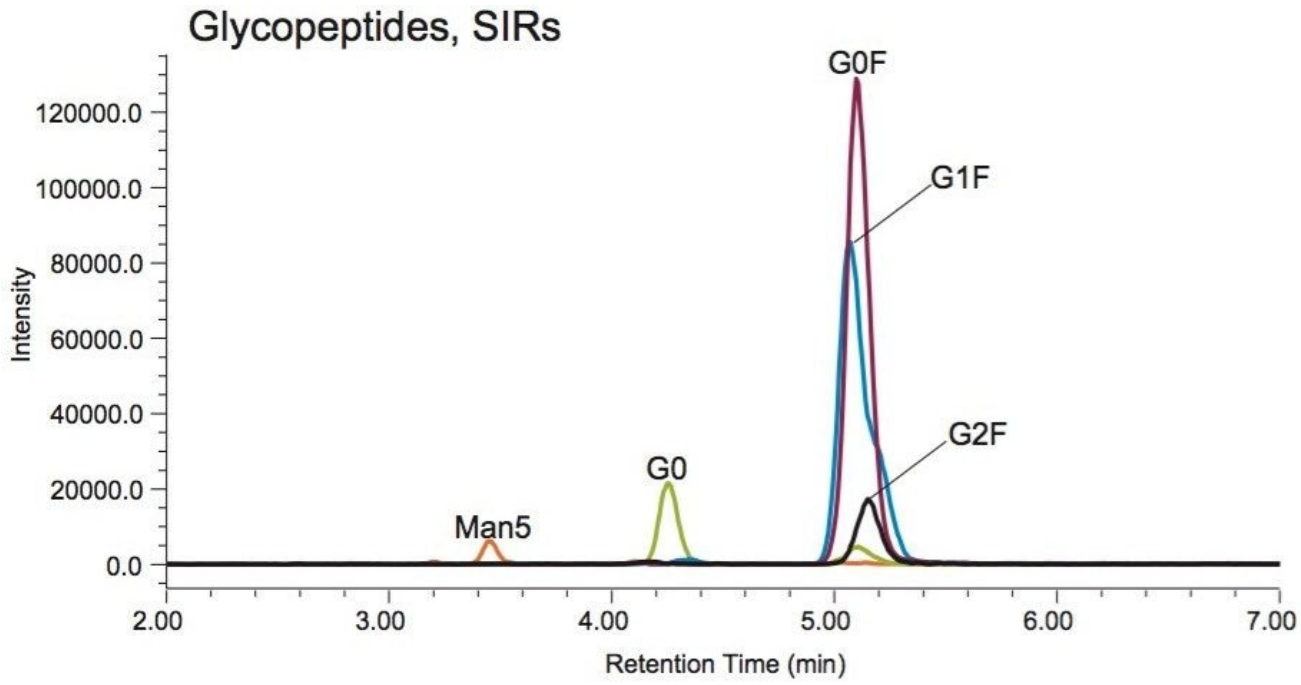

Figure 4. Overlay of five SIR channels used for determining relative abundance of five glycopeptides. Because each SIR is collected in an individual channel, custom calculations must be used to automate the process of determining relative abundance of each glycopeptide.

Empower Software Enables Automated Reporting of Multiple Attributes

We have just shown how a method set can be created to contain derived channels used to monitor attributes of interest, each of which can have an independent processing method associated with it. This same method set can be used to associate independent reporting methods for each of the attributes of interest, which aids to further automate the monitoring process. Should a user wish to import all results into a single report, Empower

can accommodate this functionality as well. Figure 5 shows screen captures of Empower reports generated to monitor each of the attributes previously discussed.

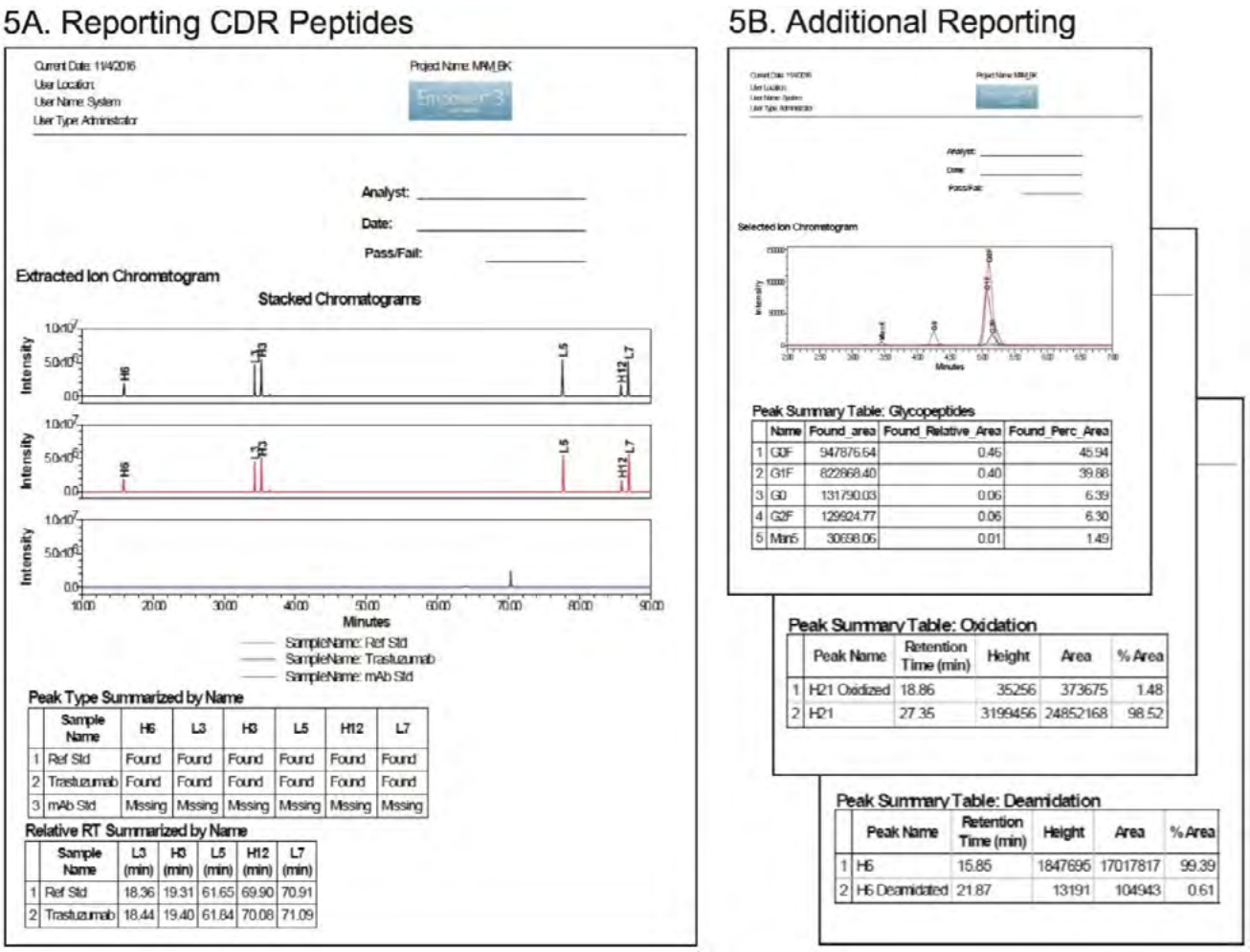

Figure 5. Empower reporting. 5A) Comparing a reference standard, a sample of trastuzumab, and an intact mAb standard (negative control). Peak tables summarize whether the peaks of interest were detected and the relative retention time for each CDR peptide. In lieu of an internal standard, H6 was used to calculate relative retention time. 5B) Additional reporting for a sample of trastuzumab. Each of the items reported is the result of designing a processing method and derived channel for that specific attribute. In reporting relative abundance of the glycopeptides, Found_area, Found_Relative_Area, and Found_Perc_Area are defined by the custom calculations functionality in Empower. These fields are created so that peak area from individual channels can be reported relative to one another.

Conclusion

The ACQUITY QDa Detector provides an efficient and cost-effective solution for monitoring important product attributes in a development or QC environment when characterization has been previously carried out using a high resolution MS instrument. This application note demonstrates that CDR peptides, oxidized and deamidated peptides, and glycopeptides can be identified, quantitated, and reported from a single acquisition using the derived channel and inter-channel calculation functionalities in the Empower software. The list of attributes to be monitored would need to be determined for each respective antibody, but could be readily expanded to meet the individual user's needs.

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720005919, February 2016

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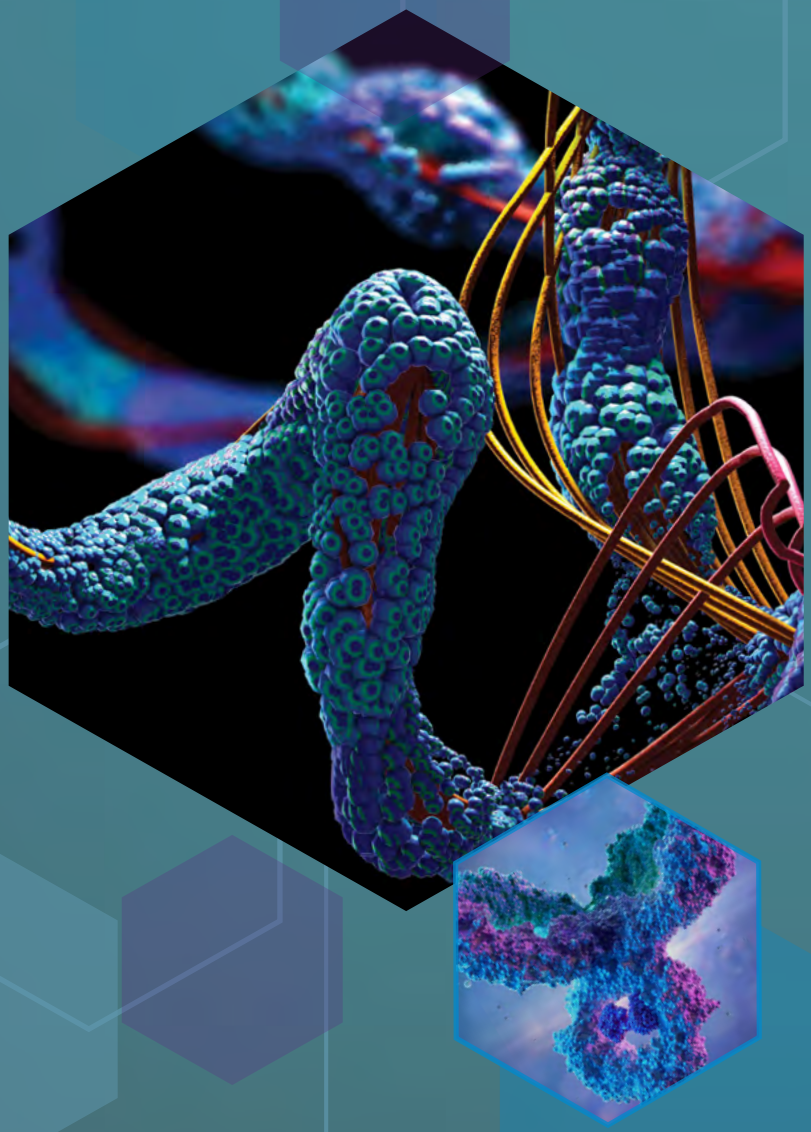
Subunit-based MAM Using HRMS and waters_connect Informatics

Application Notes



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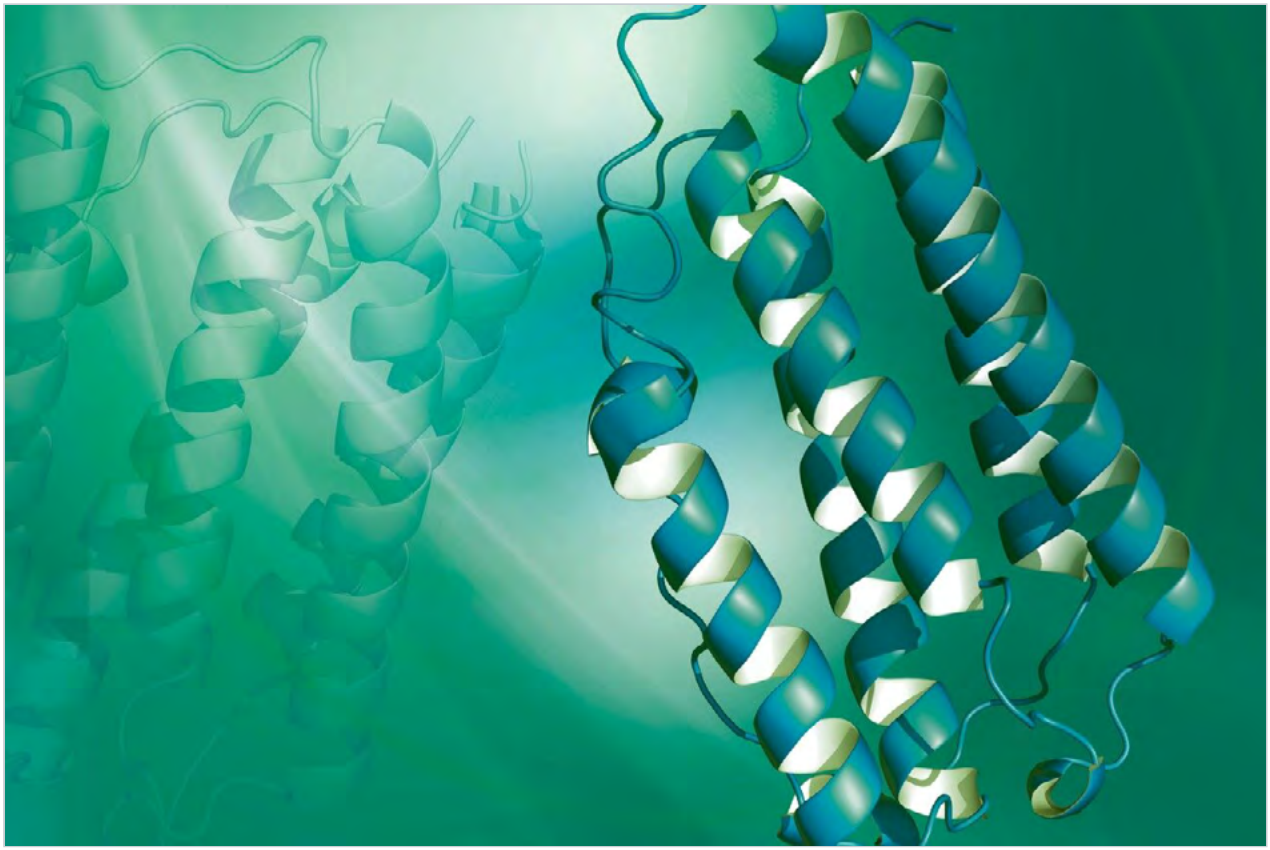
Application Note

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

Samantha Ippoliti, Ying Qing Yu, Nilini Ranbaduge, Weibin Chen

Waters Corporation



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)

LC Conditions

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
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.

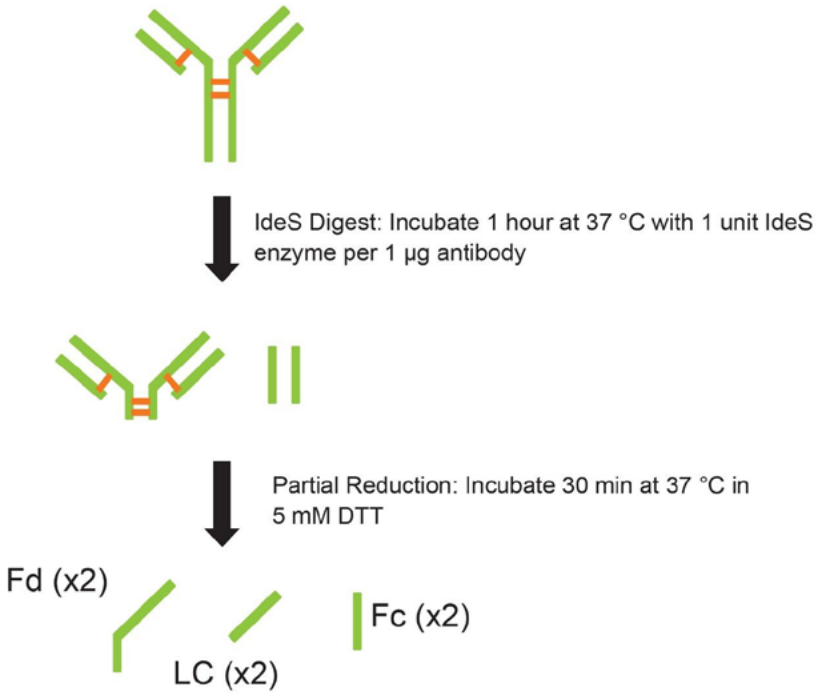


Figure 1. Sample preparation for mAb Subunit MAM, including IdeS digestion and partial reduction.

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.

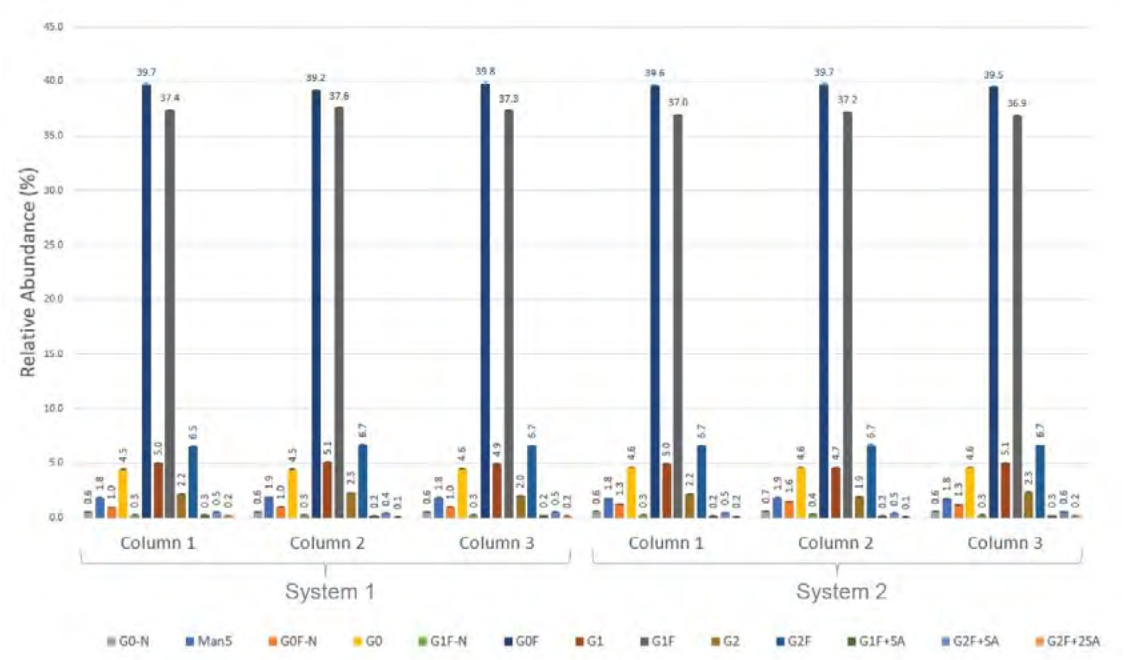


Figure 2. Trastuzumab Fc N-glycosylation species analyzed via subunit MAM method on two BioAccord systems, three columns each, triplicate injections. For all species >0.5% relative abundance, %RSD is <5%.

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.

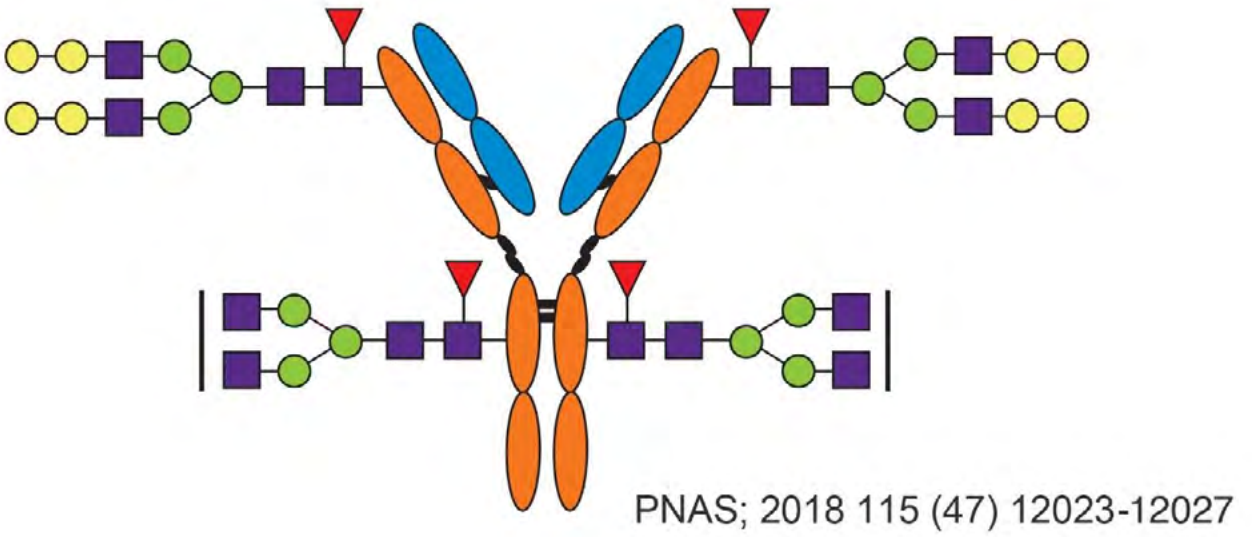


Figure 3. Depiction of cetuximab showing the N-glycosylation sites in the Fd in addition to the typical sites in Fc.

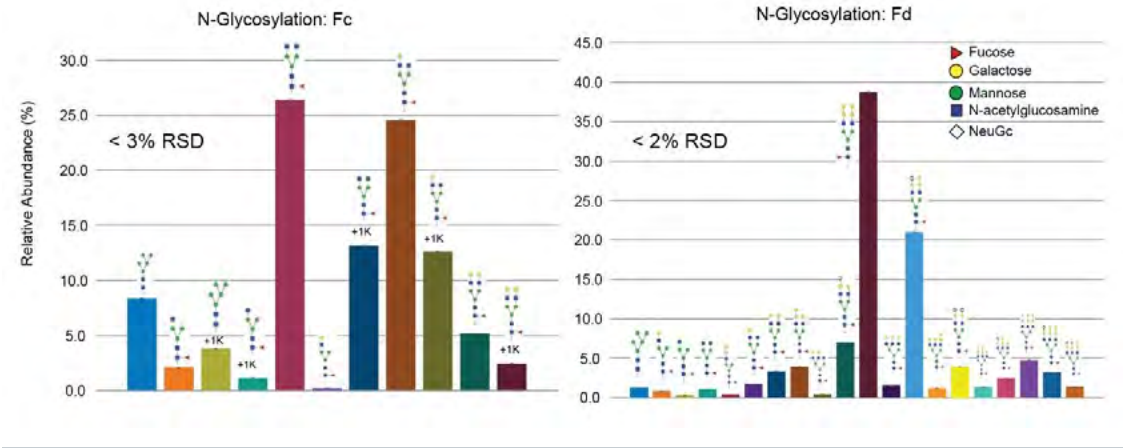


Figure 4. Cetuximab Fc and Fd N-glycosylation species.

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).

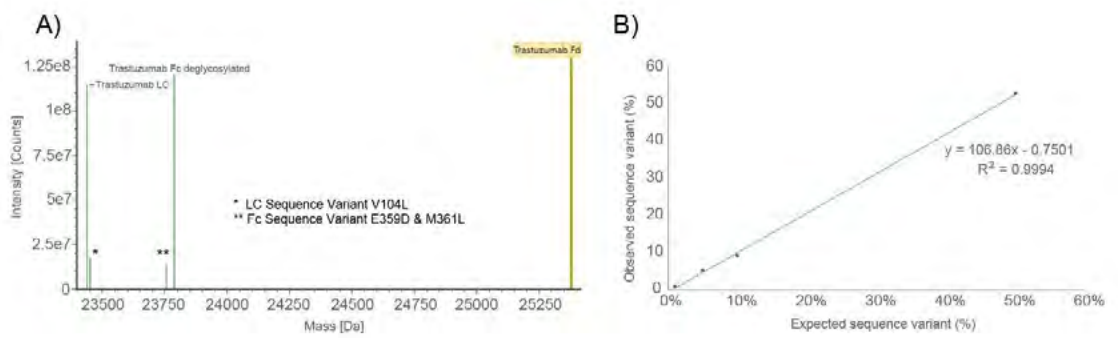


Figure 5. Trastuzumab sequence variant analysis.
(A) Component plot for 10% sequence variant spiked sample with automatically labelled LC, Fd, Fc, and the two sequence variant species. LC sequence variant is labelled with (*) and Fc sequence variant species is labelled with (**). (B) % observed sequence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

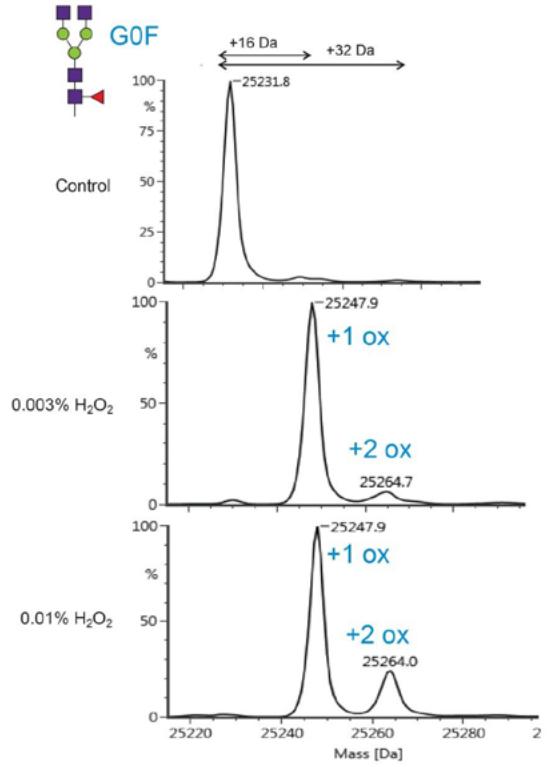


Figure 6. NIST mAb forced oxidation study, Fc species. MaxEnt1 deconvolution (zoom of G0F species), showing +1, +2 oxidation following incubation in 0.003% and 0.01% hydrogen peroxide (H_2O_2).

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

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

Biopharmaceutical Platform Solution with UNIFI <https://www.waters.com/10195515>

waters_connect <https://www.waters.com/waters/nav.htm?cid=135040165>

720007129, January 2021

Waters™

Application Note

INTACT Mass™ - a Versatile waters_connect™ Application for Rapid Mass Confirmation and Purity Assessment of Biotherapeutics

Henry Shion, Patrick Boyce, Scott J. Berger, Ying Qing Yu

Waters Corporation

Abstract

In recent years, liquid chromatography-mass spectrometry (LC-MS) intact mass analysis has become an indispensable tool for attribute characterization and monitoring of biotherapeutics across all stages of their development and commercialization. Functions such as discovery screening, process development, and formulations/stability can generate a large number of samples, and analytical solutions that can achieve high-throughput intact mass analysis and impurity profiling are advantageous to matching these requirements. Here, we report the development of a new versatile software application to streamline LC-MS mass confirmation and quantitative monitoring of biomolecules, suitable for deployment in a compliance-ready environment.

Benefits

- Automatic and efficient workflow for high throughput mass confirmation and purity analysis
- Purity determination using optical chromatogram, total ion chromatogram (TIC) or intra-mass spectra

- Automatically determination of input and output mass ranges and deconvolution parameters, yielding monoisotopic or average deconvoluted mass results
- Methods can now be applied as platform methods for wide range of biomolecule classes

Introduction

Intact mass analysis can provide a global profile of biotherapeutics, confirming predicted mass, profiling product heterogeneity, and assigning impurities. In recent years, LC-MS intact mass analysis has become an indispensable tool for attribute characterization and attribute monitoring of biotherapeutics across all stages of their development and commercialization. Functions such as discovery screening, process development, and formulations/stability can generate a large number of samples, and analytical solutions that can achieve high-throughput intact mass analysis and impurity profiling are advantageous to matching these requirements.

Here, we report the development of a waters_connect application that streamlines LC-MS data acquisition, data intact mass processing and reporting of results suitable for a compliance-ready environment. The INTACT Mass Application can be applied for simple mass confirmation, high-throughput intact mass screening, and product impurity profiling. In this application note, the key stages of the workflow for high throughput targeted mass confirmation will be demonstrated. Experimental results obtained from six commercial monoclonal antibodies (mAbs), and NISTmAb IdeS digested subunits will be discussed.

Figure 1. The BioAccord™ LC-MS System with ACQUITY™ Premier, comprised of an ACQUITY Premier binary UPLC™ with detection by Tunable UV detector (TUV) coupled in-line with the ACQUITY RDa™ accurate mass detector, operated under the waters_connect informatics platform.

Experimental

Materials and Sample Preparation

LC-MS grade acetonitrile (ACN) was purchased from Honeywell - Burdick & Jackson. LC-MS grade formic acid was purchased from Thermo Fisher Scientific. Commercially available mAbs, Trastuzumab (Genentech), Infliximab (J&J), Bevacizumab (Abbott), Rituximab (Biogen Idec), and Omalizumab (Genentech) were purchased from Besse Medical (www.besse.com <http://www.besse.com/>). All the mAbs were stored at -80 °C before they were thawed and then diluted in Milli-Q water to 0.5 µg/µL for LC-MS analysis. In addition, Waters™ humanized mAb (NISTmAb) Mass Check Standard (Waters p/n: 186009125 < https://www.waters.com/nextgen/global/shop/standards--reagents/186009125-humanized-mab-mass-check-standard.html> ,) and Waters mAb (NISTmAb) Subunit Standard (Waters p/n: 186008927 < https://www.waters.com/nextgen/global/shop/standards--reagents/186008927-mab-subunit-standard.html>)

were used for this study. For the intact NISTmAb sample, 160 µL of Milli-Q water was added to the sample vial (containing 80 µg of protein material) to produce a solution of 0.5 µg/µL before analysis (with 2 µL injection). For subunit analysis, 125 µL of water was added to the sample vial (containing 25 µg of subunit mAb material) to produce a solution of 0.2 µg/µL before analysis (with 2 µL injection).

BioAccord System with ACQUITY Premier

System:	ACQUITY Premier UPLC BSM, ACQUITY Premier UPLC FTN with column heater, ACQUITY RDa Mass Detector, ACQUITY Premier TUV Optical Detector, waters_connect v2.1.2.4

Intact Mass Analysis – LC-MS Method Setup

Column:	ACQUITY Premier Protein BEH™ C_4 Column, 300 Å, 1.7 µm, 2.1 mm x 50 mm (Waters p/n=186010326)
Column temp.:	80 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
TUV optical detection:	UV 280 nm

LC Gradient Table for Intact mAb Analysis

	Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
1	0.00	0.40	95.0	5.0	Initial
2	1.00	0.40	15.0	85.0	6
3	1.20	0.40	5.0	95.0	6
4	1.50	0.40	95.0	5.0	6
5	2.50	0.40	95.0	5.0	6

Total run time: 2.5 minutes.

MS Conditions for Intact Mass Analysis

Acquisition Settings

Mode:	Full scan
Mass range:	High (400–7000 *m/z*)
Polarity:	ESI Positive
Scan rate:	2 Hz
Cone voltage:	Custom (70 V)
Capillary voltage:	Custom (1.50 kV)
Desolvation temp.:	Custom (550 °C)
Intelligent data capture (IDC):	On

Subunit Mass Analysis LC-MS Method Setup

Column:	ACQUITY Premier Protein BEH C_4 Column, 300 Å, 1.7 µm, 2.1 mm x 50 mm (Waters p/n=186010326)
Column temp.:	80 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
TUV optical detection:	UV 280 nm

LC Gradient Table for the Analysis

	Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
1	0.00	0.40	80.0	20.0	Initial
2	0.25	0.40	75.0	25.0	6
3	1.75	0.40	60.0	40.0	6
4	2.00	0.40	5.0	95.0	6
5	2.25	0.40	80.0	20.0	6
6	3.00	0.40	80.0	20.0	6

Total run time: 3.0 minutes.

MS Conditions for Subunit Analysis

Acquisition Settings

Mode:	Full scan
Mass range:	High (400–7000 *m/z*)

Polarity:	Positive
Scan rate:	2 Hz
Cone voltage:	Custom (50 V)
Capillary voltage:	Custom (1.00 kV)
Desolvation temp.:	Custom (450 °C)
Intelligent data capture (IDC):	On

Results and Discussion

The INTACT Mass Application, residing within the waters_connect informatics platform HUB (Figure 2) can be used to confirm the mass and assess the purity for a wide range of biomolecule classes. Untargeted mass analyses can be performed as well to support discovery studies. Additionally, purity assessments can be produced using either optical chromatogram, TIC or mass spectra.

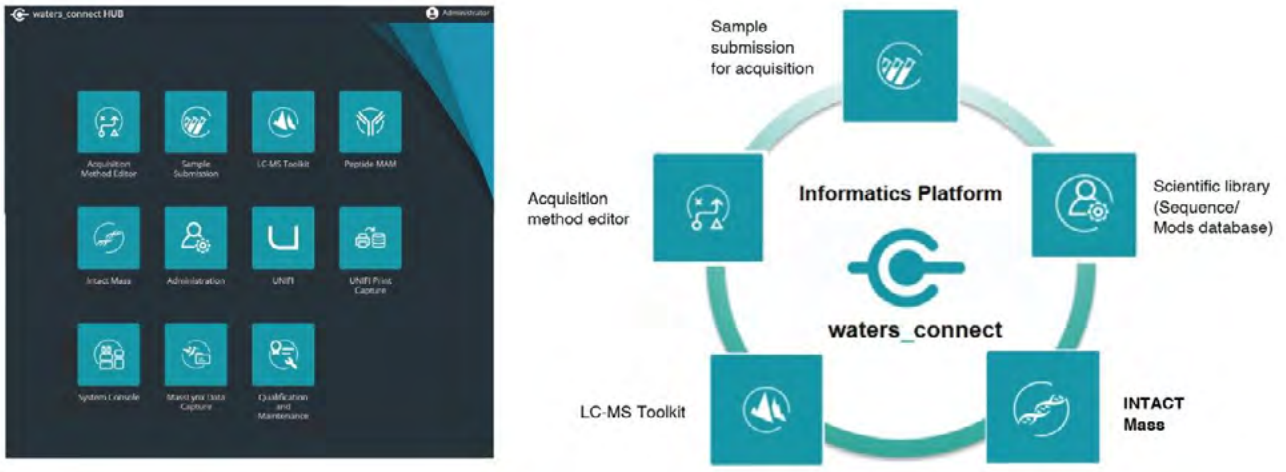

Figure 2. waters_connect HUB contains the application icons that are associated with intact mass analysis workflow, including the Acqusition Method Editor, Sample Submission, Scientific Library, INTACT Mass, and LC-MS Toolkit.

The waters_connect informatics platform HUB contains the applications utilized for the intact mass analysis workflow. The Acquisition Method Editor App is used to define the data acquiring conditions, such as LC gradients and MS settings. The INTACT Mass Application operates the integrated workflow for data acquisition, processing, review and reporting. The Sample Submission App can be launched independently, but when the users selects the option to acquire and process, it is executed within the INTACT Mass application. The Scientific Library contains an extensive list of variable modifications for various biomolecule types that can be selected to search for product variants, impurities, and MS adducts. The LC-MS Toolkit App can be used to manually examine the data when needed for result confirmation or troubleshooting.

Creating the Acqusition Method

To facillitate acquisition of intact mass LC-MS data, a method is defined using the Acqusition Method Editor App in the waters_connect HUB. This Acquisition method includes settings for the solvent manager, sample manager, column compartment, tunable UV (TUV), and ACQUITY RDa MS detectors. To facillitate a higher throughput analysis, a 2.5 minutes total runtime fast LC desalting method was defined, featuring the gradient table and chart (Figure 3A), and MS settings (Figure 3B) indicated. The “scheduled lockmass” was selected in the MS method for this experiment to shorten the total acquisition time, by reducing inter-injecton system check activities from a per injection basis. With the “scheduled lockmass” selected in the INTACT Mass Application, it takes 136 minutes to finish the LC-MS data acquisition and data processing for 48 sample runs using a 2.5 minutes fast LC method.

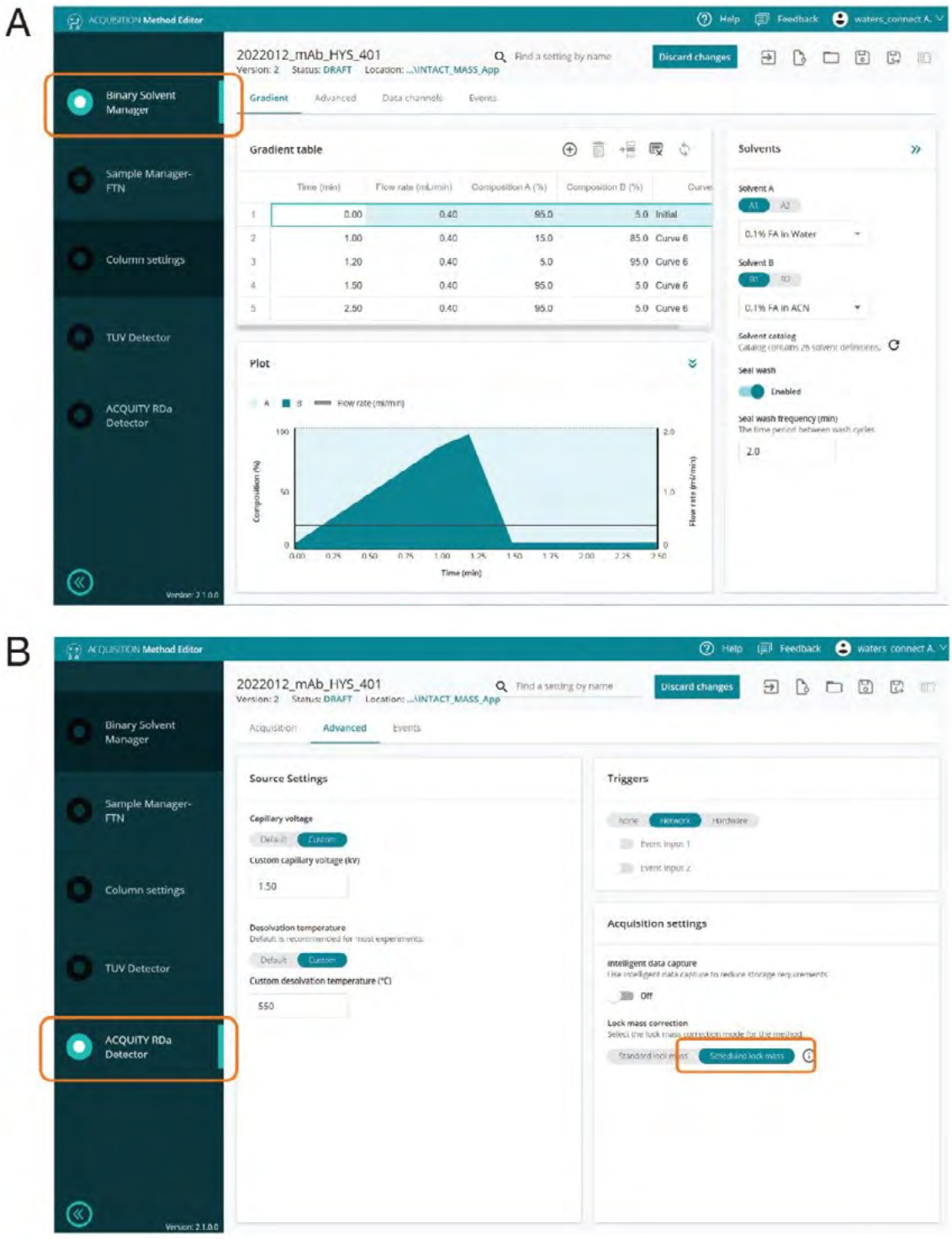

Figure 3. The Acquisition Method Editor was used to generate a higher throughput intact mAb LC-MS acquisition method. It shows a 2.5 minutes total runtime, as indicated on the gradient table and chart (A), and selecting the scheduled lockmass under the advanced MS settings (B).

Creating the Processing Method

The Data Processing method is created within the INTACT Mass Application. It can be cloned from an existing method, or newly generated. The welcome page of the INTACT Mass Application (Figure 4) allows the user to generate a process only analysis for an existing data set or combine acquiring and processing for a new data set.

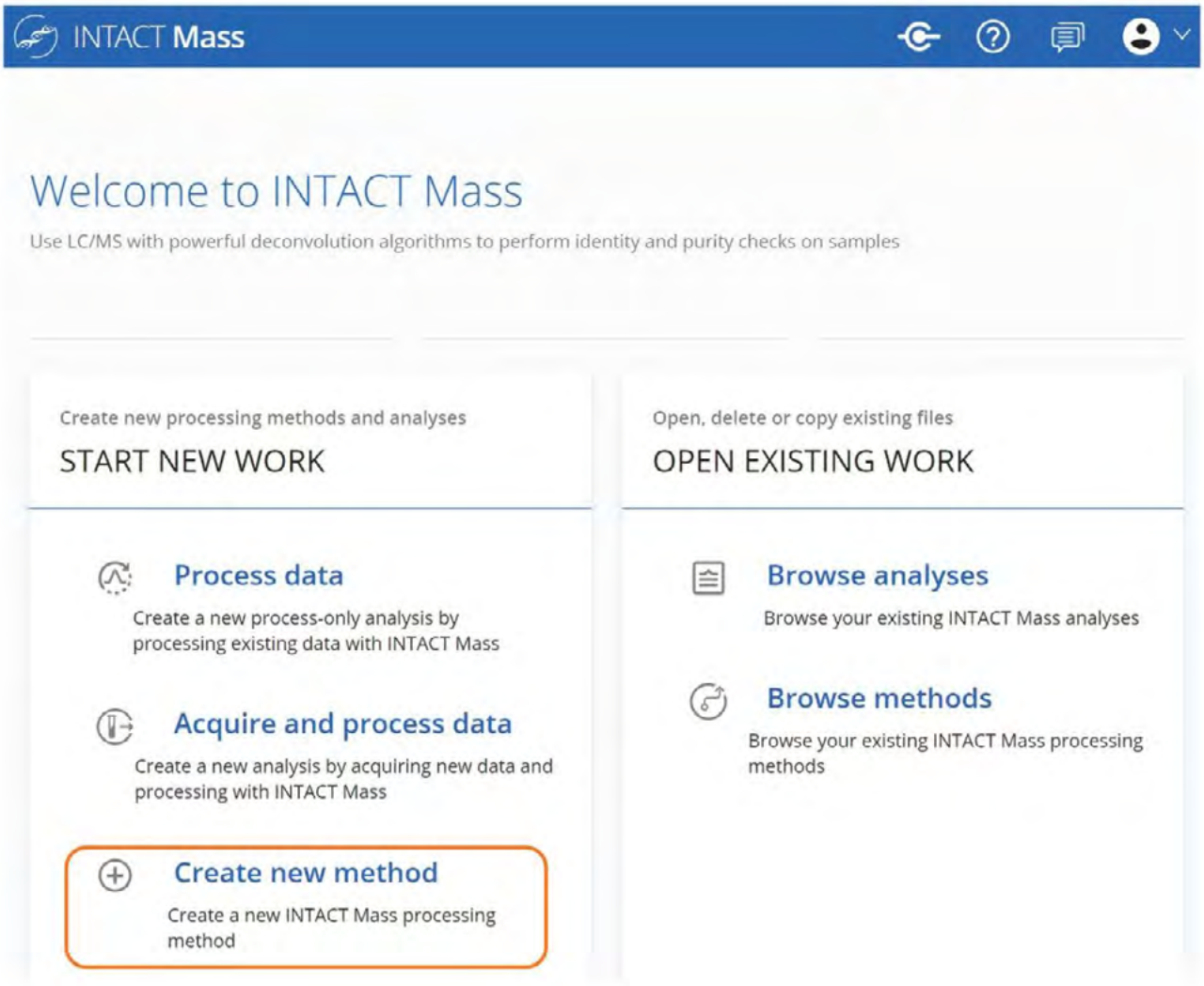

Figure 4. The main page of the INTACT Mass Application. Processing methods can be executed on exisiting data, executed as part of a combined acquire and process selection, or created a new data processing.

In building the processing method for intact mAb screening (Figure 5A), user has the flexibility to choose either the latest BayesSpray[1] or the traditional MaxEnt1[2] algorithm for data deconvolution. BayesSpray can be applied to generate either average or monoisotopic spectra, while MaxEnt1 delivers average mass.[3] When selecting the automated peak deconvolution settings, the mass range of the raw spectra and deconvolved spectra and other deconvolution parameters will be self-optimized, with the results of this optimization available for locking down future methods, if desired. In this study, the MaxEnt1 deconvolving agerithm was selected for processing. The user can choose the molecular type (Protein was selected for this study) from the pull down list (Figure 5B), allowing the deconvolution algorithm to model a proper elemental composition to obtain optimal results, or select a custom elemental composition to support atypical classes of molecule. This feature gives optimum charge deconvolution results with little user intervention, and, enables the creation of platform methods that can be applied to sample sets containing a diverse range of molecules. As an example, an oligo-focused analysis using the INTACT Mass Application titled "LC-MS Analysis of Single Guide RNA Impurities Using the BioAccord System with ACQUITY Premier and Automated waters_connect INTACT Mass Application" is listed in the references.[4]

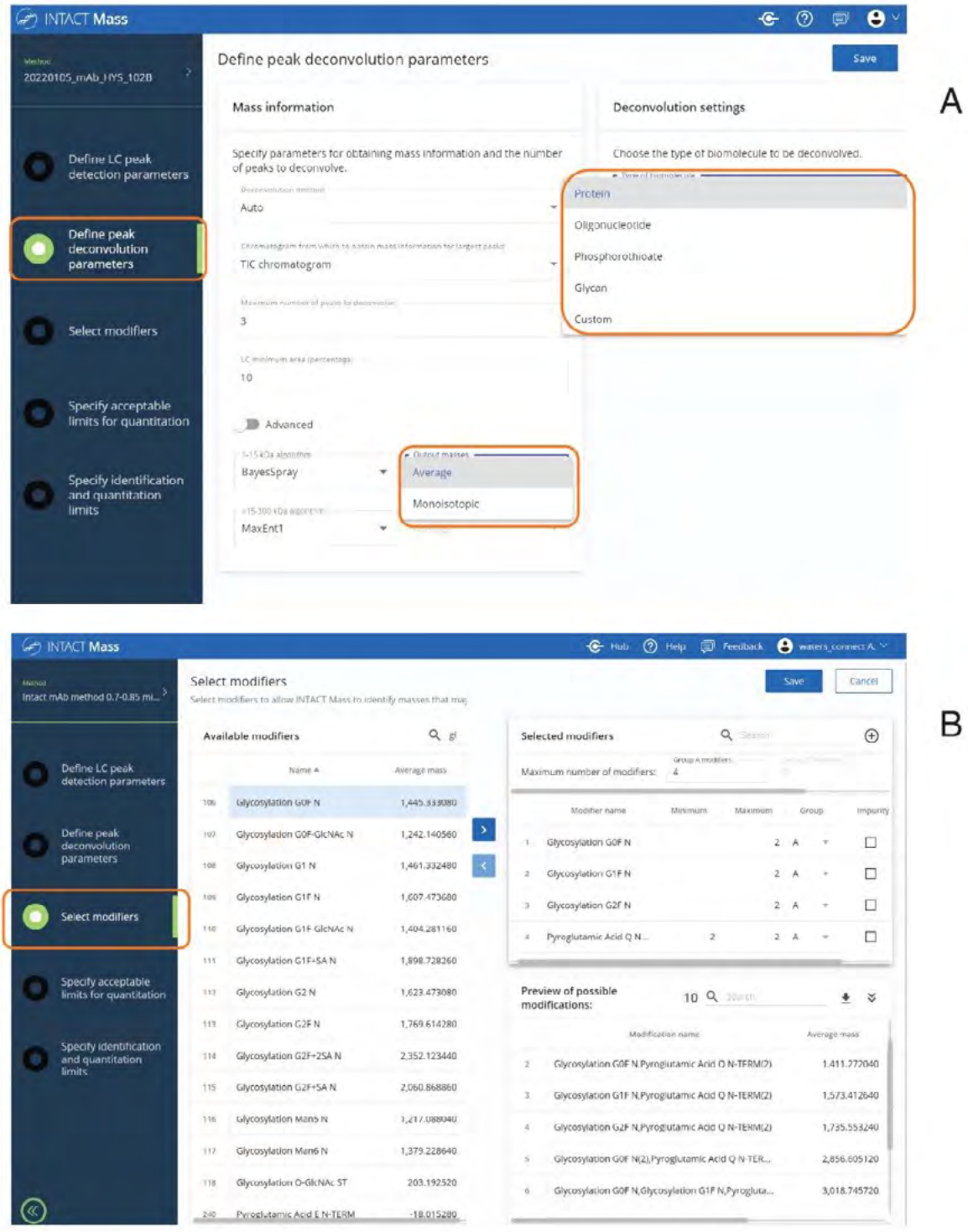

Figure 5. Selecting Processing Parameters. The App supports both the BayesSpray or traditional MaxEnt1

algorithms for data deconvolution: the BayesSpray output can be average or monoisotopic mass while MaxEnt 1 provides average mass. Using the default automated setting enables the mass range of the raw and deconvolved spectra and other deconvolutuion settings to be intelligently self-optimized during data processing (Figure 5A). Furthermore, various sample types can be selected, optimizing the elemental composition model for more optimum charge deconvolution (Figure 5B).

Executing the Analysis

Selecting the methods following selection of the Acquire and Process option, opens a Sample List (Figure 6) where sample information, such as targeted masses, sample, and molecule types, any pre-sample treatment (***e.g.,*** IdeS digestion) can be specified.

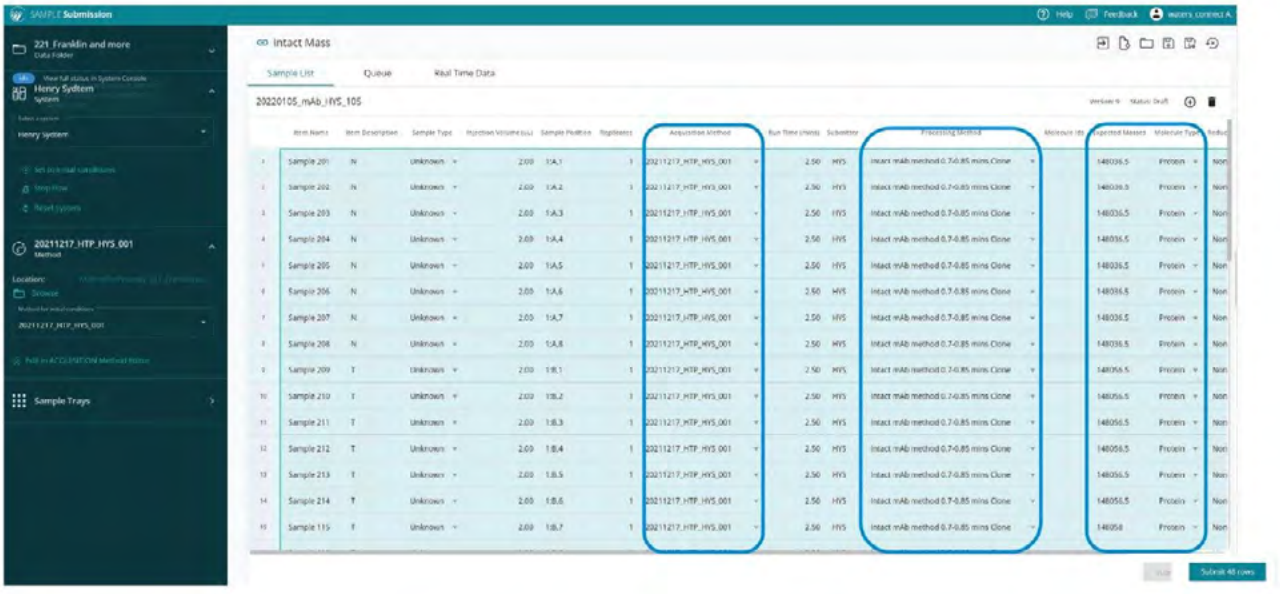

Figure 6. Acquire and Process Sample List. Instrument system, acqusition method, processing method can be selected accordingly. In additional, sample specific information can be added as needed to enable intelligent acquisition and processing on a sample-by-sample basis.

When the analysis is submitted, the automated data acquistion and processing will start. Data processing will take place in parallel with subsequent data acquisition, with deconvolution of up to five peaks from one or more

samples occuring concurrently. Users can examine these results in real-time to make decisions on continuing acquisition, or determine any needed method enhancements.

Review and Reporting of Results

The dashboard view of the results (Figure 7) from an intact mAb screening experiment summarizes the results for a plate of 48 injections. This represents six antibodies, prepared as described in the Experimental, and analyzed with eight replicate wells per antibody. In the processing method, each antibody is searched against its appropriate target average mass.

The color flags displayed on the sample plate reflect the status of the samples analyzed, and reflect whether targted masses were confirmed, and if any product purity specifications were exceeded. In these experiments no purity criteria were specified.

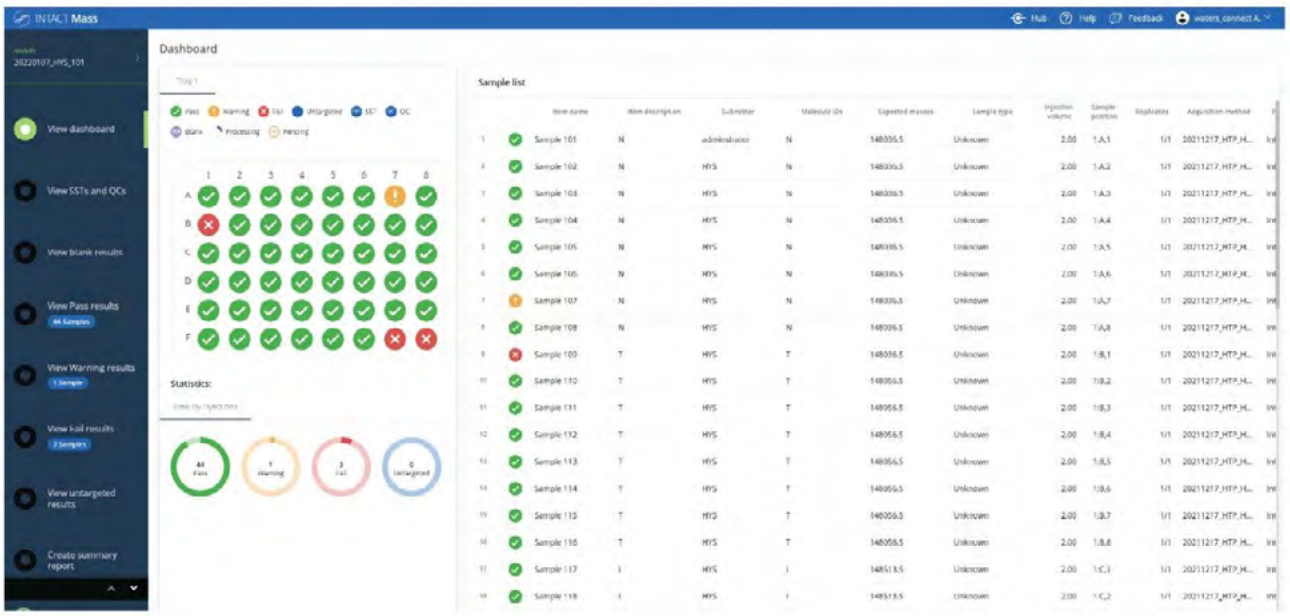

Figure 7. The dashboard results view. Results are from an intact mAb screening experiment with 48 injections representing eight replicates of six different antibodies. The color codes on the sample plate reflect the result status of the samples analyzed. The detailed individual sample results can be reviewed by clicking within the sample list.

A green color indicates a pass for the sample when measured against the threshold settings for the mass accuracy matched to the targeted masses, and for the expected purity of the sample. An orange color indicates a warning is issued, either the mass error is above the threshold, or the purity of the sample is lower than expected. A red color indicates that either mass accuracy measured against the targeted masses is too large or unmatched, and/or the purity is below thresholds.

Table 1 summarizes the mass error measured against the expected masses of a 48 injections representing eight replicates of six different antibodies (NISTmAb, Trastuzumab, Infliximab, Bevacizumab, Rituximab, and Omalizumab). The mass accuracy for all the injections were found to be around 10 ppm, and the standard deviation of the replicate injections for each antibody are less than 5 ppm. The data in this table indicate that the INTACT Mass Application can produce consistent and useful results for fast intact mass screening projects.

mAbs name	Expected mass (Da)	Sample mass error (ppm)								Average mass error (ppm)	Mass error st. dev. (ppm)
		1	2	3	4	5	6	7	8		
NISTmAb	148036.5	8.8	9.8	11	7.3	11.2	10.7	9.2	13.2	10.15	1.79
Trastuzumab	148056.5	9.7	12.1	14.5	11.9	14.8	15.7	16.3	5.0	12.5	3.75
Infliximab	148513.5	6.7	0.5	6.5	-3	3.2	4.3	10.7	7.7	4.58	4.33
Bevacizumab	149197.5	7.8	8.8	7.6	8.2	9.4	10.8	7.8	9.1	8.69	1.08
Rituximab	147075.5	9.2	7.6	7.7	8.8	8.8	6.1	6.1	6.3	7.58	1.29
Omalizumab	149171.5	1.5	-0.6	-3.8	-2.5	-1.8	-1.5	-0.3	-1.9	-1.36	1.59

Table 1. Mass accuracy summary for 48 injections that representing six antibodies with eight replicate injections each.

Using a NISTmAb sample (Sample 5 in the overview) as an example, we can access summarized individual sample information and its automatically generated report (Figure 8).

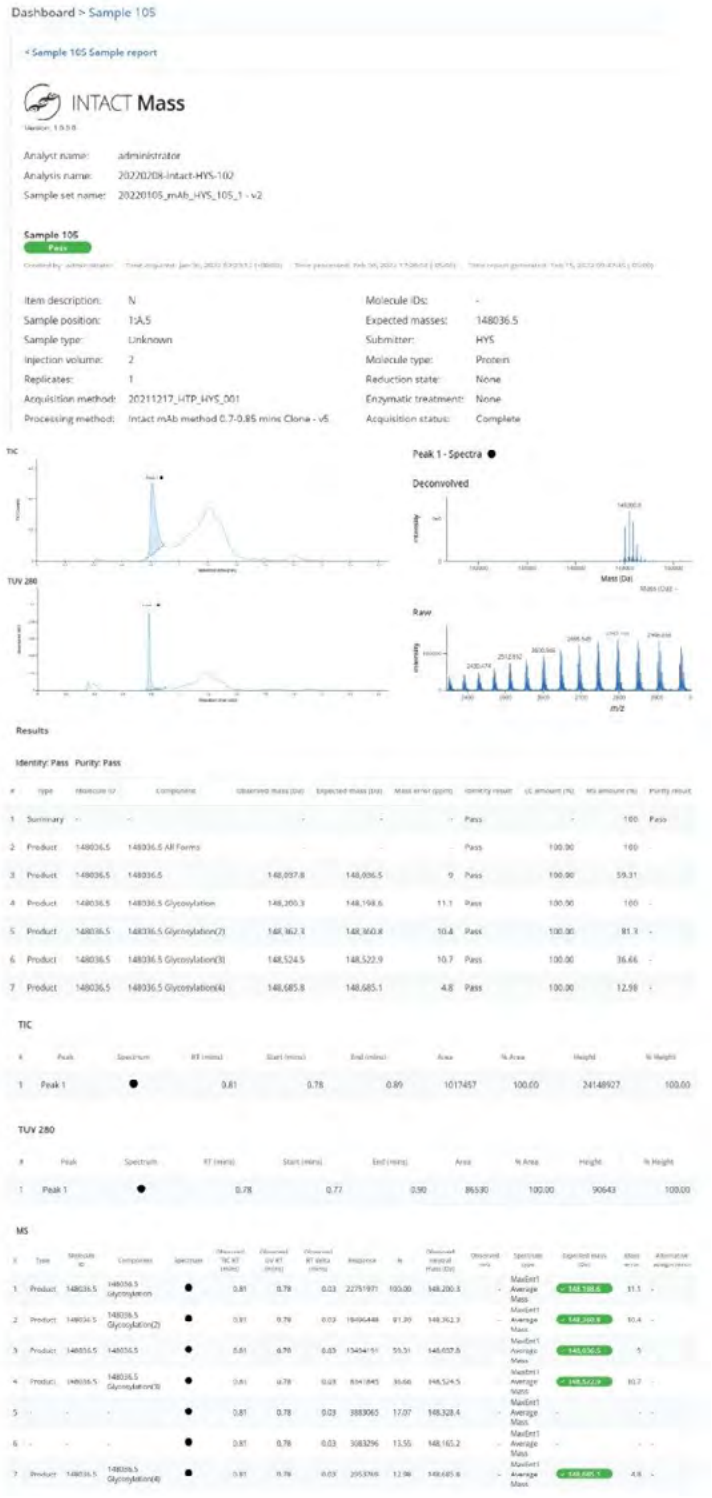

Figure 8. Report for Sample 5 (NISTmAb) from the 48-well sample plate. The report includes the experimental information, TIC, and TUV chromatograms with the integrated peaks noted, the identified components with their assigned modifications, and the calculated mass accuracy and purity information. The raw and deconvolved spectral data are also visualized in the formatted report.

The experimental results for sample 5 (a NISTmAb injection) from the 48-well plate sample set are presented in Figure 8. The easy to read standardized report template includes information such as TIC and TUV chromatograms with the integrated peaks, the identified targeted components with their assigned modifications, the calculated mass accuracy and sample purity, as well as the raw and deconvolved spectra. The use of the automated MaxEnt1 setting facilitated a single platform acquisition and processing method that could be applied to the different samples. For NISTmAb sample 5, the measured mass accuracy for the top five major glycoforms were about 10 ppm. Similar glycoform mass accuracies were observed for the Trastuzumab, Infliximab, Bevacizumab, Rituximab, and Omalizumab samples (data not shown).

The relative percentage of the indentified components (against the base peak) was also calculated and was found to be consistent across replicated samples (data not shown). Additional quantitative information derived from the TIC, TUV, and MS data is also reviewable in the tables of the sample centric report.

In addition to the intact mAb high throughput analysis experiment, we also conducted an IdeS digested mAb subunit analysis experiment using the Waters mAb (NISTmAb) Subunit Standard as the test sample. This provides the opportunity to display the capabilities of the automated chromatographic peak detection, and spectral deconvolution, in the context of a multi-analyte sample.

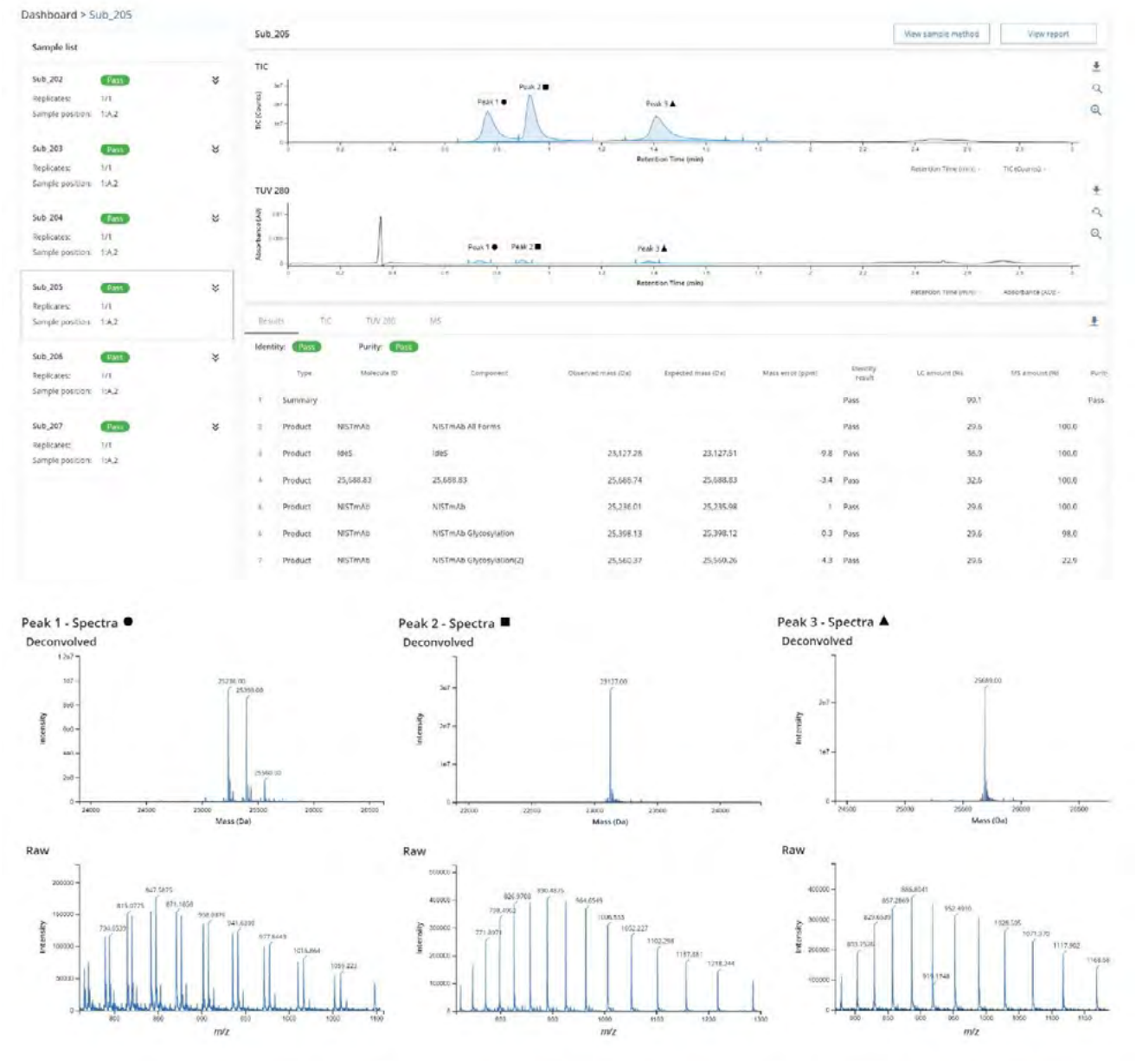

Figure 9. Waters mAb (NISTmAb) Subunit Standard results. The TIC and TUV chromatograms show that the three major peaks of the scFc, LC, and Fd subunits were separated in a 3-minute gradient LC-MS run. The mass accuracies for the assigned 25 kD subunits and their modifications were measured within about 5 ppm). Peak purity was calculated by combining the three subunit TIC peaks and found to be 99.1%. The raw and deconvolved subunit spectra are shown at the bottom panel.

The analysis of the Waters NISTmAb subunit Standard was acquired with a 3-minute LC gradient on a newly launched ACQUITY Premier Protein BEH C4 Column (300 Å, 1.7 µm, 2.1 mm x 50 mm, Waters p/n: 186010326). The three major peaks of the scFc, LC, and Fd subunits were separated using 0.1% formic acid in water and 0.1% formic acid in ACN as the mobile phases, and average mass errors were found to be about 5 ppm. The peak purity was calculated using the combined TIC peak areas of the three subunits and found to be 99.1%.

The ability to automate the processing of intact protein LC-MS data is key to drive the efficiency of laboratories using this methodology to screen samples or monitor attributes, particularly when these studies are conducted with large number of samples. The ability to create platform methods that can be used to analyze diverse sets of samples within one analysis will benefit not only discovery organizations, where this challenge is common, but also those core labs looking to simplify operations by grouping of a multitude of samples for bulk analysis. The combination of efficient separations, robust and simple detectors, and automated acquisition/processing on a compliance-ready platform should enable this approach to see wider deployment, particularly in manufacturing and quality organizations that have been challenged to deploy such methods previously.

Conclusion

- The waters_connect INTACT Mass Application facilitates a streamlined integrated workflow for data acquisition, processing and reporting of deconvoluted mass data for biotherapeutics, deployable in regulated and non-regulated environments.
- The analysis workflows supported by this app include targeted mass confirmation, untargeted profiling, and purity assessments using optical, TIC, or mass spectral approaches.
- Automation of chromatographic peak detection along with the MaxEnt1 and BayesSpray deconvolution algorithms has enabled the development of platform- based methods to look across a diverse set of molecules within a single sample set while preserving the ability to compare against sample-specific specifications.
- In this work, the waters_connect INTACT Mass Application provided low ppm mass confirmation data for assessment of six commercial mAbs analyzed in replicate on a 48-sample plate using a quick desalting LC-MS method, and for a multi-chromatographic peak IdeS digested and reduced NISTmAb subunits sample subjected to a fast gradient separation.

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2. Skilling, J., *et al*. Beyond MaxEnt Deconvolution: Increasing the Fidelity and Universal Applicability of Mass Spectral Deconvolution Routines for Biomolecules with the Application of Bayesian Probability Theory Analytical Chromatography, Waters Poster 720003756 < https://www.waters.com/waters/library.htm?lid=10180413> , 2010.

3. Berger. S. Boyce, P., Beyond INTACT Mass Application within waters_connect, *Waters Vblog*, 2022. https://blog.waters.com/automating-intact-mass-deconvolution-its-about-time < https://blog.waters.com/automating-intact-mass-deconvolution-its-about-time>

4. Doneanu, C., *et al*. LC-MS Analysis of Single Guide RNA Impurities Using the BioAccord System with ACQUITY Premier and Automated waters_connect INTACT Mass Application, Waters Application Note, 720007546, 2022.

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720007547, February 2022

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Application Note

Automating Rapid High-Throughput LC-MS mAb Subunit Screening of Microbioreactor Cell Culture Samples

Alireza Aghayee, Yamin Htet, Stephan M. Koza, Lindsay Morrison, Henry Shion, Ying Qing Yu

Waters Corporation

Abstract

Monoclonal antibodies (mAbs) remain one of the fast-growing classes of biopharmaceuticals and are significantly improving the quality of life for patients all around the world. Discovery and development of successful mAb therapeutics requires sophisticated analytical technologies that can rapidly measure the critical product attributes that have profound impact on the safety and efficacy of these drugs. Therefore, high-throughput analytical platforms for the monitoring of proteins produced at the clone selection stage and during process development have been increasingly in demand.[1]

Due to their production in host cell cultures, large sizes, and heterogenous structures monitoring mAb production raises many analytical challenges. Selection of an appropriate clone typically requires parallel incubation of tens to hundreds of transfected lines, requiring many samples to be analyzed. The optimization of cell culture conditions typically also requires parallel cultures with analytics needed for each culture at least once per experiment. This requires high-throughput methods with fast sample preparation and robust analytical instrumentation, along with facile and straightforward data interpretation. As an integral part of the method, automated sample preparation protocols can increase the sample throughput and improve the efficiency of analytical workflows in biopharmaceutical development.[2]

Here, we present a fully automated workflow for sample preparation and LC-MS analysis of mAbs obtained directly from complex samples such as spent cell culture media including host cell protein. The method includes a mAb purification step using Protein A followed by FabRICATOR® (IdeS) digestion and subsequent DTT reduction to yield mAb subunits suitable for high-throughput LC-MS analysis using a Waters™ BioAccord™ LC-MS System.

Benefits

- Rapid mAb subunit analysis with fully automated Protein A and Ides digestion protocol with Andrew+™ Pipetting Robot
- High-Throughput LC-MS Analysis using BioAccord™ LC-MS System with waters_connect™ Informatics Solution/INTACT Mass™ App[3]
- Reduced subunits analyzed by reversed-phase UPLC™ using the BioResolve™ RP mAb Polyphenyl column for optimal peak shape and resolution with this solid core particle with a 450 Å pore size

Introduction

The development and manufacturing of therapeutic mAbs require close monitoring of multiple product quality attributes. N-glycosylation, oxidation, C-terminal lysine clipping, and glycation are some of the product quality attributes evaluated. Numerous samples are often required to be analyzed during clone selection and throughout process development to ensure a successful final product. This may create a bottleneck which can either slow down the development of new therapeutics or limit the extent of process optimization. Ultimately rapid LC-MS methods along with the automation of mundane sample preparation and sample analysis can help to relieve this bottleneck. We have already demonstrated that the analysis of mAbs at the intact level can be fast and be performed with minimum sample preparation.[4] However, intact mAbs analysis can limit the protein attributes that can be tracked without the use of high-resolution LC-MS instrumentation.

Rapid and high-throughput screening of mAbs in the form of subunits can potentially enhance development and improve overall quality of biopharmaceutical products, while reducing manufacturing costs. In this work, mAb

subunits (23–25 kDa in size) are generated using FabRICATOR (IdeS) protease to cleave antibody at a specific site below the hinge followed by DTT reduction of the disulfide bonds between the light chain and the heavy chain. Subunits are more homogeneous than the intact mAb and of low enough mass to allow for the acquisition of adequate-quality spectra with more moderately priced MS instruments while still providing considerably short analysis time and simplified data interpretation. Here, we present a fully automated workflow for purification and digestion of therapeutic mAbs harvested directly from cell culture media. This method is based on purification using magnetic protein A beads followed by a combined digestion/reduction. An Andrew+ Pipetting Robot was used to automate the entire sample preparation process which yielded subunits ready for analysis by Waters BioAccord LC-MS System. In order to develop a procedure more amenable for use with micro-bioreactors small volumes (20 µL to 100 µL) of the mAb media samples were evaluated for this study either directly (using unpurified media) or as Protein A purified samples for intact, subunit level analyses.[5]

Sample Description

Non-transfected Chinese hamster ovary (CHO) cells culture media was commissioned from Syd Labs, Inc.. Briefly, 6 x 10^6 CHO-K1 cells/mL per were seeded in flask on day 1 and were incubated in 120 mL culture media. On day 2, 100 mL of spent media was collected from flask and was 0.2 µm filtered. This was repeated every day to day 15. All collected media was pooled and stored at 4 °C. Cell viability and numbers were recorded accordingly throughout cell culture process (Figure 1a). Then, trastuzumab (T-mab) was added to collected cell culture to prepare samples at 0.5 µg/µL to create a mock media sample of known concentration (Figure 1b–c). The lower cell viability aliquots observed in the later days of this cell culture were included in the mock sample to provide a greater challenge for the sample purification step.

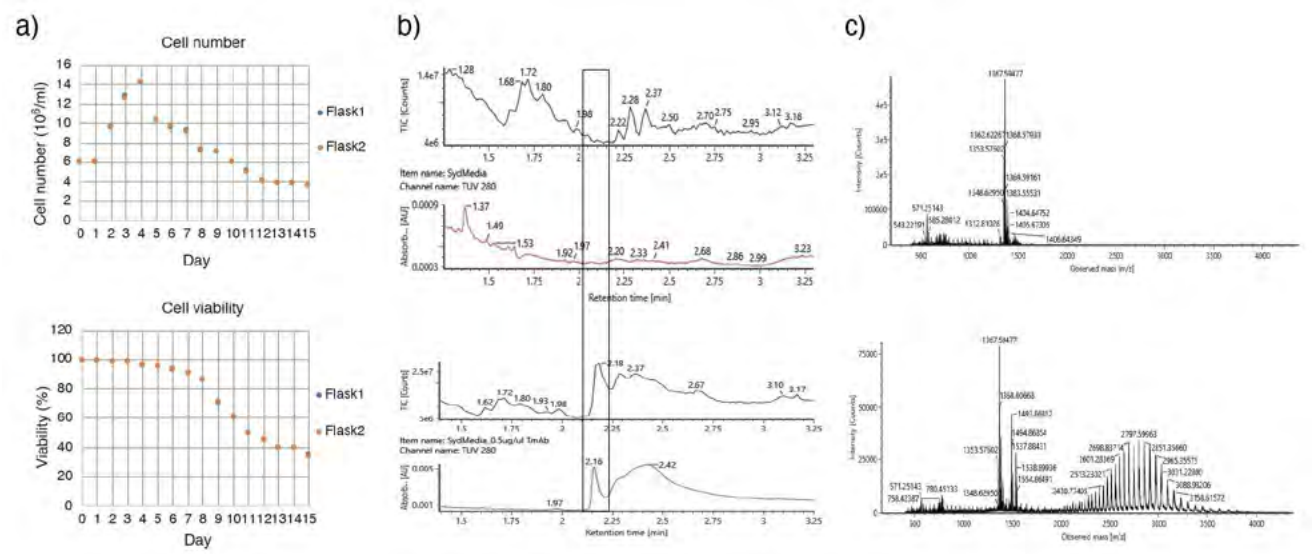

Figure 1. a) Cell count, and viability of non-transfected CHO cells cultured for 15 days. b–c) Direct LC-MS analysis (b: TIC traces, c: MS spectra) of CHO cell culture media (top) and after spiked addition of trastuzumab (bottom).

Sample Preparation

Using an Andrew+ Pipetting Robot, a fully automated workflow was developed for sample preparation. Purification of the mAb from cell culture media was performed by incubating 100 µL of sample (with the indicated concentration) with Magne® Protein A Beads (Promega, 50 µL slurry per sample) followed by capturing of the magnetic beads on the Andrew+ Robot in a 96-well plate format using the Magnet+ device. After washing, purified mAb was eluted in 50 µL Glycine-HCl (2M, pH 2.5) two times (100 µL total) and were added to 60 µL neutralization buffer comprised of MES (100 mM) and Tris-HCl (900 mM) at pH 7.5.

Afterwards, an aliquot of 20 µL purified mAb (with <0.5 µg/µL concentration) were digested and the disulfide bonds reduced to yield three mAb subunits (Fc/2, light chain and Fd') by adding 10 µL FabRICATOR (Ides) at 2 units/µL and 30 µL dithiothreitol (DTT) at 40 mM to each sample and incubating for 60 minutes at 37 °C. The detailed protocols for the Andrew+ robot can be downloaded from the OneLab Library (onelab.andrewalliance.com). All reagents and chemicals used were shown in the table below (Table 1).

Protocol specification	Materials	Volume
Cell culture media	Filtered CHO cells culture spent media	100 µL/well
Magnetic beads	Promega Magne™ protein G magnetic affinity beads	50 µL/well
Equilibration buffer	1x Phosphate buffer saline (PBS), pH 7.4	3 × 150 µL/well
Binding buffer	1x PBS pH 7.4	3 × 150 µL/well
Wash buffers	1x PBS pH 7.4 and water	3 × 150 µL/well
Elution buffer	Glycine-HCl, 200 mM, pH 2.5	2 × 50 µL/well
Neutralization buffer	MES 100 mM and Tris-HCl 900 mM pH 7.5	60 µL/well
Fabricator	Ides 2 unites/µL in water	15 µL/well
DTT in buffer	DTT in Guanidine-HCl 6 M and Tris-HCl 200 mM	35 µL/well
Sample platform	twin.tec PCR Plate 96, skirted, green, Eppendorf	200 µL/well

Table 1. List of all reagents and materials used for mAbs purification and digestion.

Andrew+ Automation

Andrew+ Pipetting Robot can provide a streamlined fully automated protocol for protein purification and digestion, as shown in (Figure 2). In comparison, other automated liquid handlers might provide a semi-automated protocol that requires one to five manual intervention steps for a similar procedure.[5]

Position	Dominos and connected devices
1,2,3,4,5	Tip insertion systems (10–300 µL)
6,7	Microplate domino
8	Plate heater-shaker+
9	96-PCR plate magnet+
10, 11	Deepwell microplate

Figure 2. Andrew+ Domino and connected device configuration for automated Protein A purification and subunit analysis of up to 48 samples.

LC-MS Analysis

The LC-MS analysis of the mAb subunits was performed using a 4.5 minute reversed-phase LC-MS method with 0.1% formic acid and acetonitrile mobile phase on a Waters BioAccord System according to the parameters in Table 2. All data was acquired and processed using UNIFI v2.1.2.14.

LC Conditions

LC system:	ACQUITY™ UPLC I-Class PLUS
Detection:	TUV Detector
Sample collection:	twin.tec PCR Plate 96, skirted, green, Eppendorf, p/n: 951020443
Column:	BioResolve RP mAb Polyphenyl Column 450 Å, 2.7 µm, 2.1 mm x 100 mm p/n: 176004157
Column temp.:	80 °C
Sample temp.:	10 °C
Injection volume:	3 µL
Flow rate:	0.4 mL/min
Run time:	4.5 minutes
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile

Gradient Table

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Curve
0	0.4	80	20	Initial
2	0.4	59	41	6
2.2	0.4	15	85	6
2.3	0.4	15	85	6
2.5	0.4	80	20	6
3.5	0.4	59	41	6
3.6	0.4	15	85	6
3.65	0.4	15	85	6
3.8	0.4	80	20	6
4.5	0.4	80	20	6

MS Conditions

MS system:	ACQUITY RDa™
Ionization mode:	ESI+
Acquisition range:	50–2000 *m/z*
Capillary voltage:	1.50 kV
Scan rate:	2 Hz

Cone voltage:	30 V
Lock-mass:	waters_connect Lockmass Kit (p/n: 186009298)

Data Management

Data acquisition and processing software:	waters_connect with INTACT Mass App

Results and Discussion

Fully automated sample preparation using low volumes (20–100 µL) of cell culture media for purification and digestion were performed in a 96-well plate format by the Andrew+ Pipetting Robot. Affinity purification of mAb from crude samples such as cell culture is a standard procedure during the process development and manufacturing of therapeutic antibodies. This is usually performed using an affinity ligand such as Protein A, which binds specifically to IgG. Protein A purification can be a tedious process as it involves numerous equilibrations and washing steps and uses a significant amount of an analyst's time (Figure 3a). At the intact level, both unpurified and purified samples generated comparable MS results. However, the Protein A purified samples additionally resulted in higher quality chromatograms that were very similar to those of the intact mAb in formulation buffer (Figure 3b).

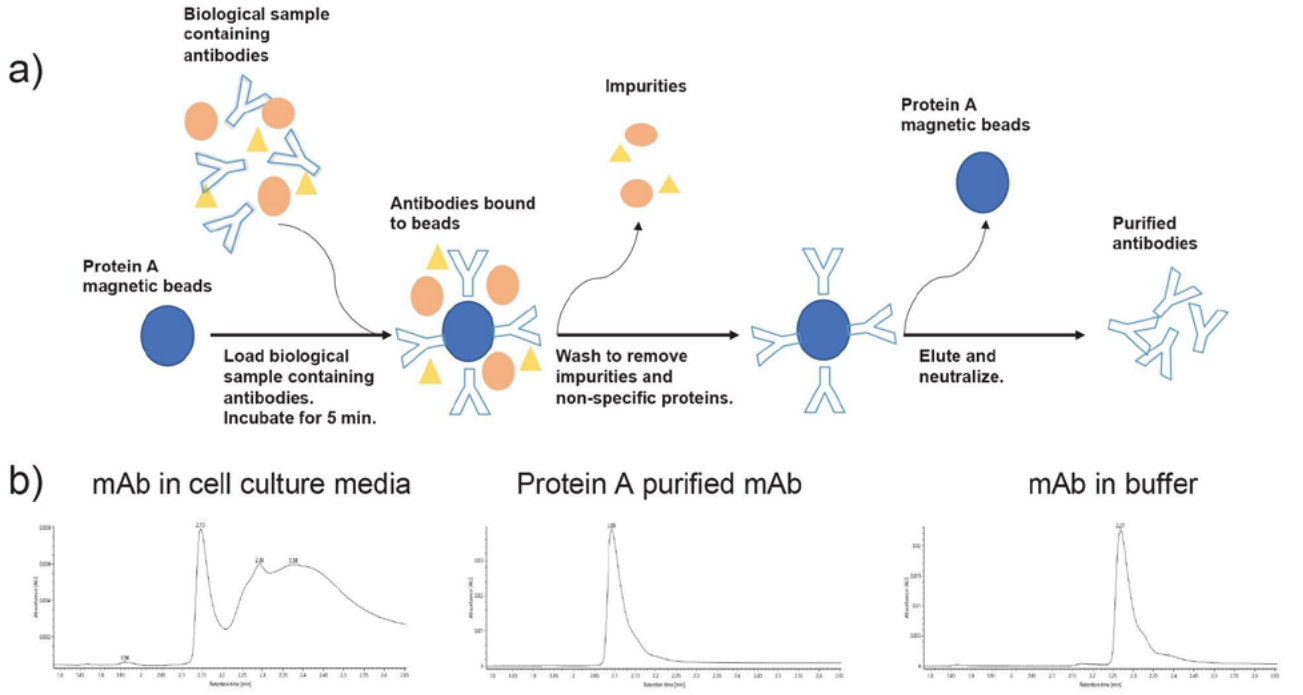

Figure 3. a) Schematic of antibody purification using Protein A-coupled magnetic beads. b) Chromatograms of mAb in cell culture media, after Protein A purification and mAb In buffer.

A highly reproducible, scalable, and fully automated 96-well plate protocol was designed to be used with cell culture samples using the Andrew+ Pipetting Robot. Samples were incubated with magnetic Protein A beads on the Shaker+ device followed by magnetic capture of the protein A beads using the Magnet+ device to allow for removal of the flow-through fractions. The magnetic Protein A beads with the captured mAb were washed, and purified mAb was eluted using a low pH wash. After neutralization of the pH, the pure mAb samples were digested using FabRICATOR (Ides) in the presence of DTT, a disulfide-bond reducing agent. This yielded reduced mAb subunits on a 96-well plate which were suitable for direct transfer to the autosampler of a BioAccord LC-MS System. Analysis of fractions demonstrated effective capture of the mAb from cell culture media, followed by elution of pure mAb and digestion into subunits using FabRICATOR (Ides) and DTT (Figure 4).

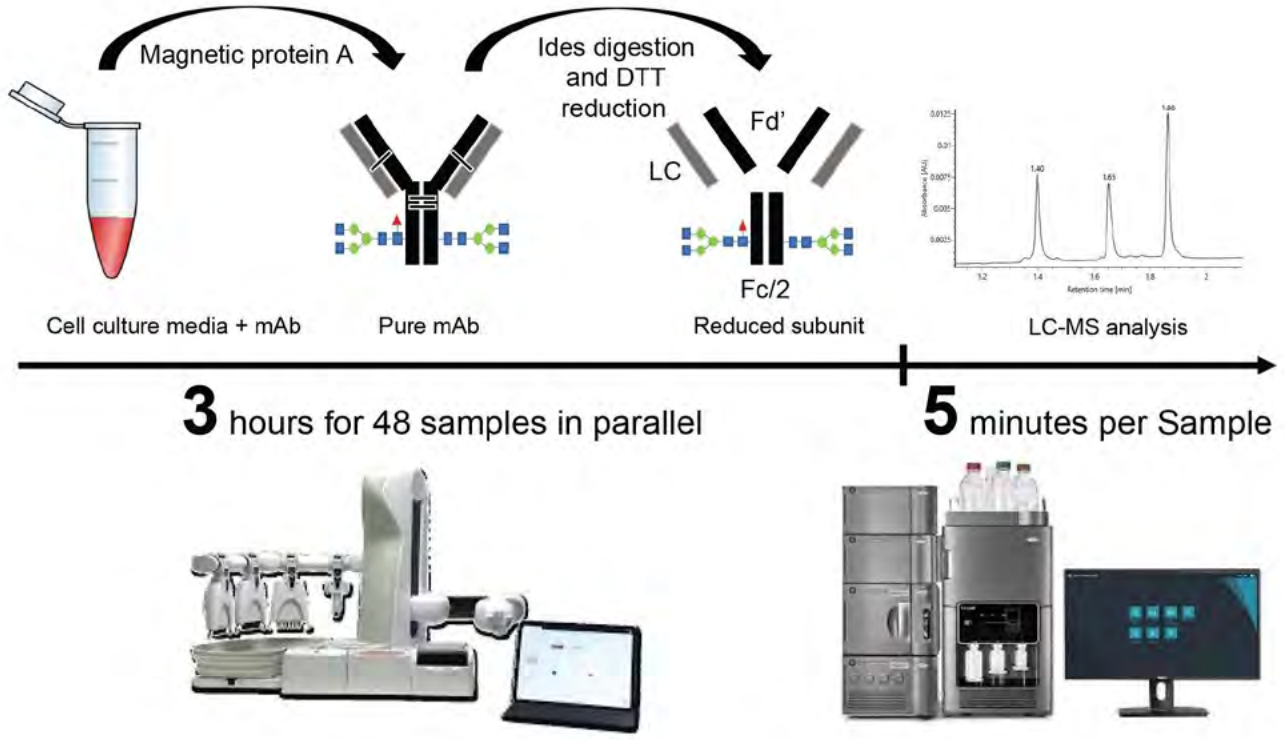

Figure 4. Workflow of automated antibody purification and digestion with Andrew+ and rapid LC-MS analysis using Waters BioAccord System.

Reduced subunits were separated by reversed-phase UPLC using a BioResolve RP mAb Polyphenyl Column on the BioAccord LC-MS. Waters mAb Subunit Standard (p/n: 186008927 < https://www.waters.com/nextgen/global/shop/standards--reagents/186008927-mab-subunit-standard.html>) was used to benchmark System performance. The mass accuracy of RDa Detector for the subunit analysis was determined to be within specification at <20 ppm. LC-MS of the purified samples yielded high-quality spectra of all three subunits (Figure 5 a–c), allowing for the relative quantification of different modifications such as Fc glycosylation. The direct LC-MS analysis of unpurified cell culture samples did not yield subunits suitable for LC-MS analysis (Figure 5d). This may be due to inefficient enzymatic reaction in unpurified cell culture media and presence of host cell proteins.

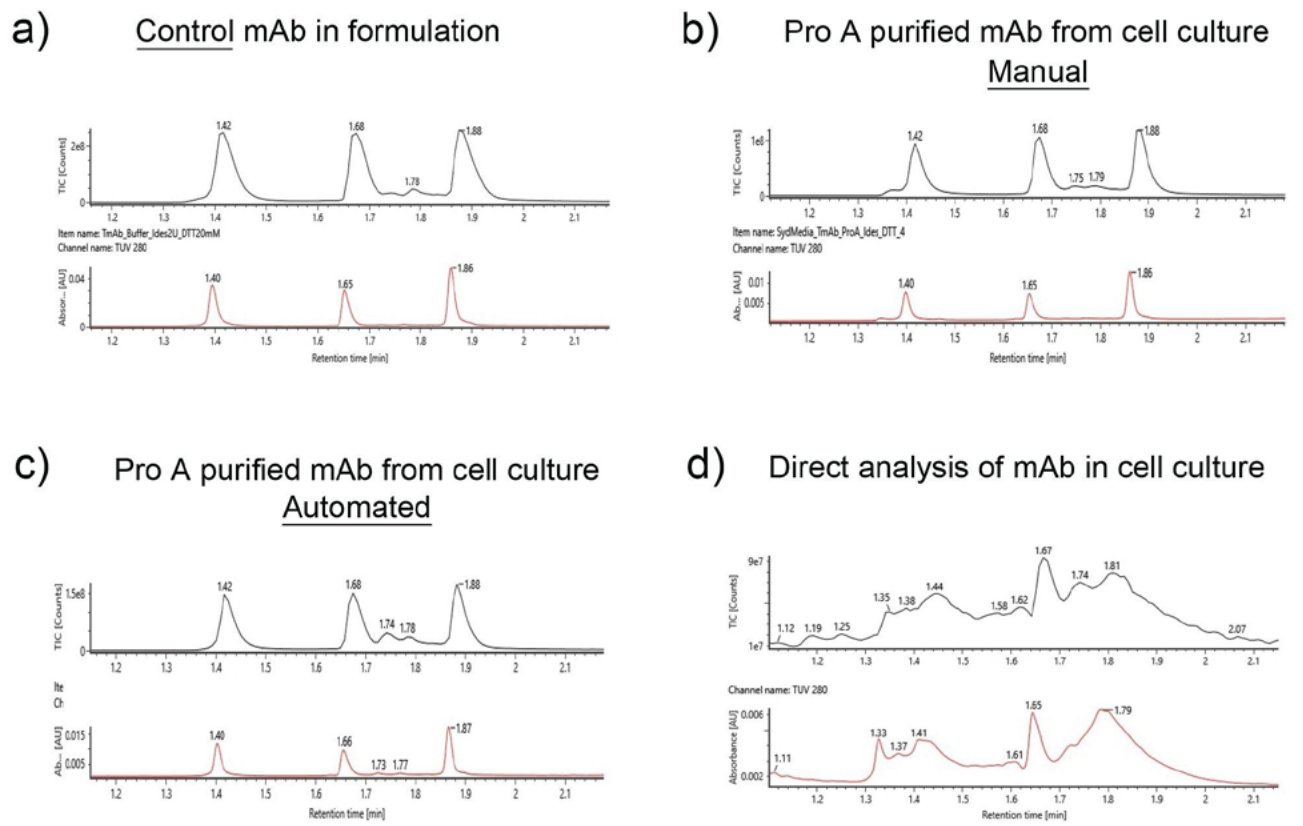

Figure 5. a-c) LC-MS analysis of subunits from purified and digested mAbs. d) unpurified and digested mAbs.

The LC-MS results of protein A purified and digested mAb generated by both manual and Andrew+ Robot were similar and all results were comparable to that of a control sample (Table 2). These subunit level analysis facilitated readily interpreted experiment results for LC, Fd', and Fc/2, and also provided information on mAb Fc glycosylation (Figure 6).

Subunits	Control average subunit mass (Da)	Manual average subunit mass (Da)	Andrew+ average subunit mass (Da)
LC	23443.26 ± 0.03	23443.18 ± 0.05	23443.19 ± 0.05
Fd'	25383.39 ± 0.07	25383.13 ± 0.09	25383.59 ± 0.07
Fc/2			
▪G0	25089.67 ± 0.08	25089.30 ± 0.16	25089.04± 0.13
▪G0F	25236.05 ± 0.05	25236.07 ± 0.11	25236.02 ± 0.09
▪G1F	25398.37 ± 0.06	25398.30 ± 0.12	25398.46 ± 0.08
▪G2F	25560.82 ± 0.12	25560.64 ± 0.26	25560.14 ± 0.26

Table 2. Subunits LC-MS analysis comparison between control (TmAb in Buffer, No Pro A, Digested), manual (TmAb in Cells Media, Pro A Purified, Digested), and automated (TmAb in Cells Media, Pro A Purified, Digested) protocols. N=8 for each condition.

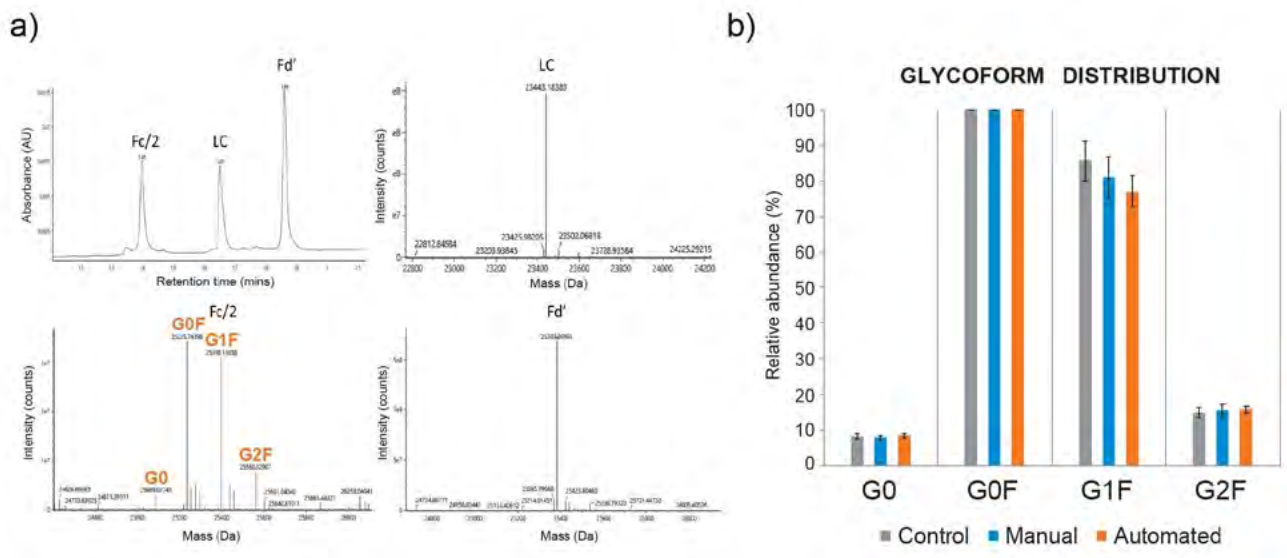

Figure 6. a) Subunit level analysis interpreted results for LC, Fd', and Fc/2 b) Fc N-glycosylation profile from deconvoluted mass spectrum of the Fc/2 fragment generated by manual (blue) and Andrew+ assisted (orange), compared to control (grey). N=8 for each condition.

Conclusion

Developing and implementing analytical methods to facilitate recombinant protein process development can be challenging as efficient and robust analytics are crucial. With effective automated sample preparation and LC-MS screening, we have shown mAbs can be analyzed directly from complex media for the determination of several important product quality attributes.

This fully automated combined protocol for purification and digestion takes approximately two hours for eight samples and three hours for 48 samples and can consistently and reliably generate results similar to the manual execution of the procedure. In addition, due to the low minimum amount of sample required and the scalability of the magnetic bead purification step, this procedure can be deployed for the preparation of limited sample amounts as low as 0.5 µg and up to 10 µg of mAb and is therefore amenable to be use with both microbioreactors and larger scale bioreactor setups.

Further more, the ease of use of the BioAccord LC-MS System with the waters_connect Informatics Solution/INTACT Mass App can improve analyst efficiency with automated data acquisition and processing for high-throughput mass confirmation of mAb subunits.

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