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

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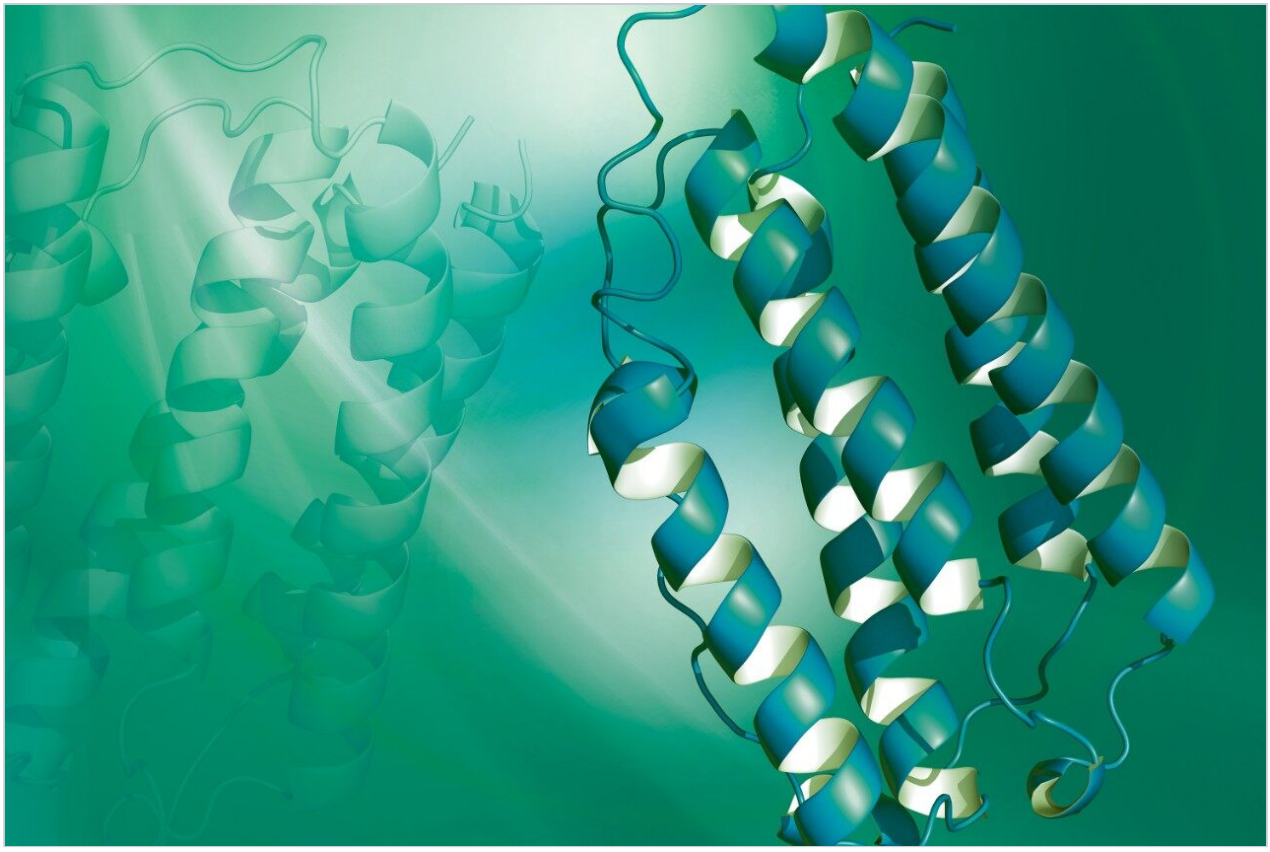

Abstract

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.

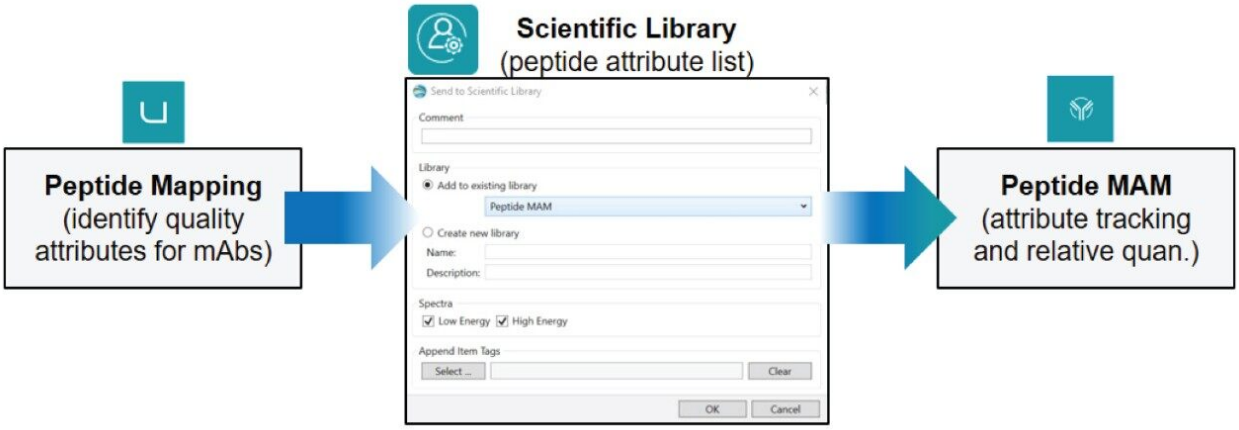

Figure 1. The Scientific library is available for managing attribute peptide information between characterization and monitoring applications. Attribute peptides assigned in peptide mapping experiments can be exported into a library that is available to the Peptide MAM App for building a target list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

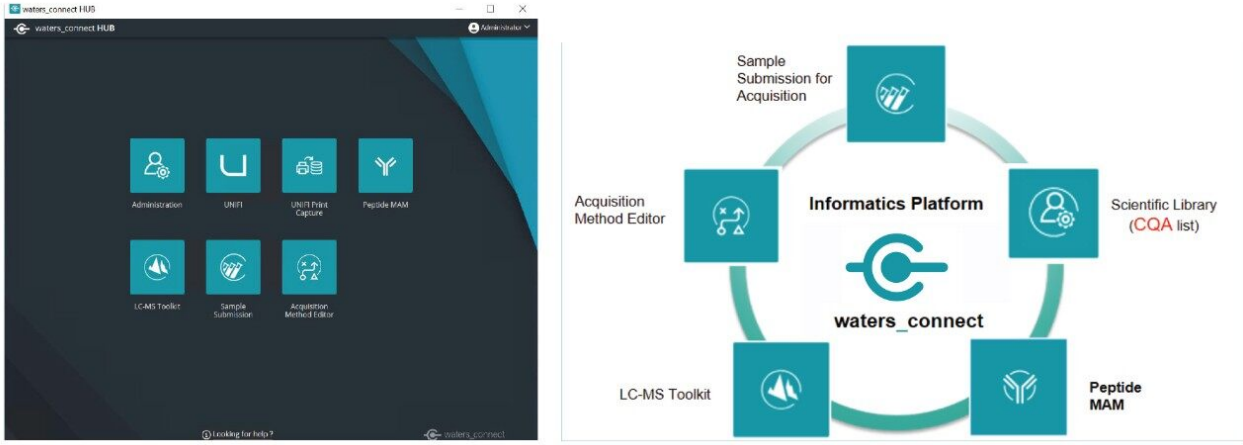

Figure 2. The waters_connect hub (A) enabled rapid navigation between Apps. The workflow for peptide MAM (B) comprises several integrated functions: The ACQUISITION method editor App for method generation, The SAMPLE submission App for data acquisition, The Scientific library for storage and management 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.

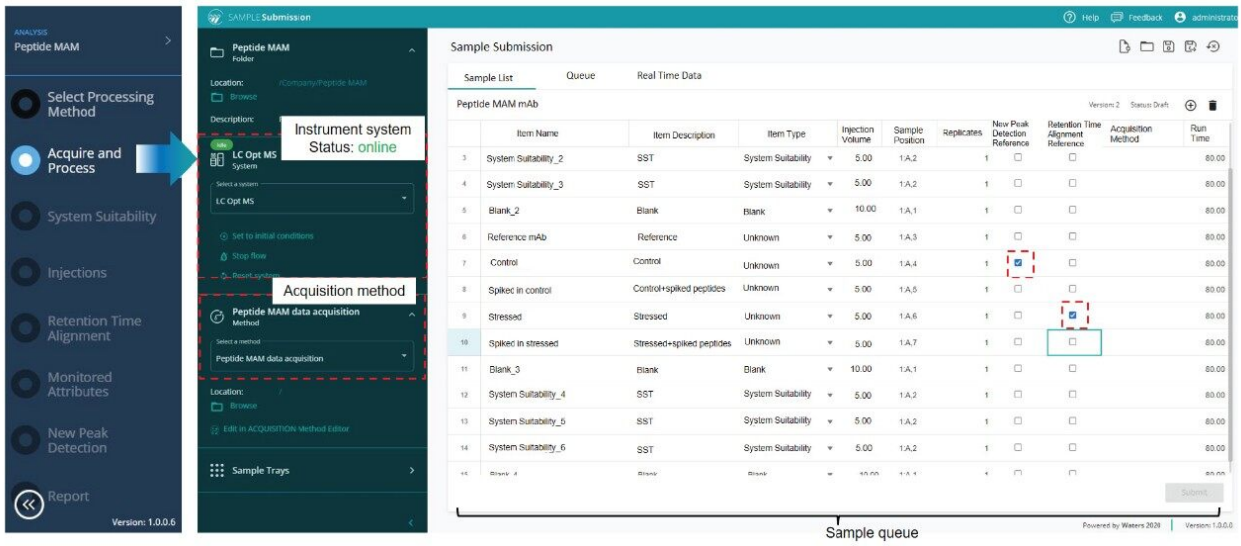

Figure 3. The sample submission functionality is accessed within the Peptide MAM App when creating a streamlined acquisition and processing method for Peptide MAM studies. The central panel contains information on file location, the acquisition system, and sample locations, while the main table to the 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.

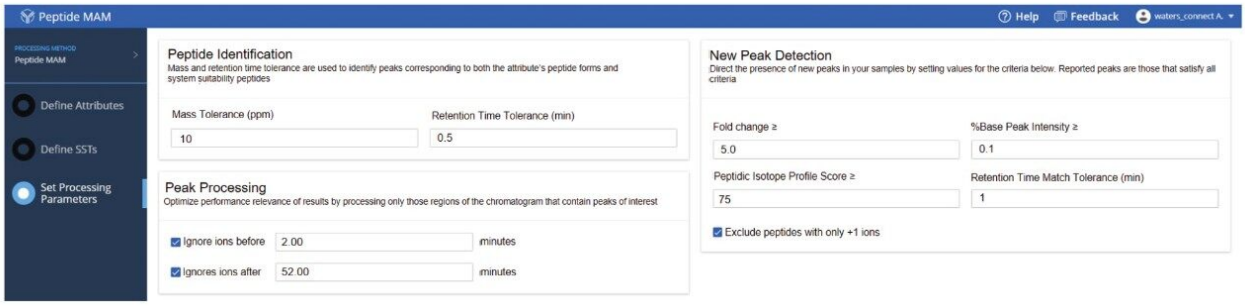

Figure 4. Setting parameters for targeted attribute monitoring and new peak detection. Peptide MAM processing uses fixed mass and retention time tolerances and can be restricted to a user defined retention time range. The new peak detection criteria comprise five potential parameters: minimum fold change, 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.

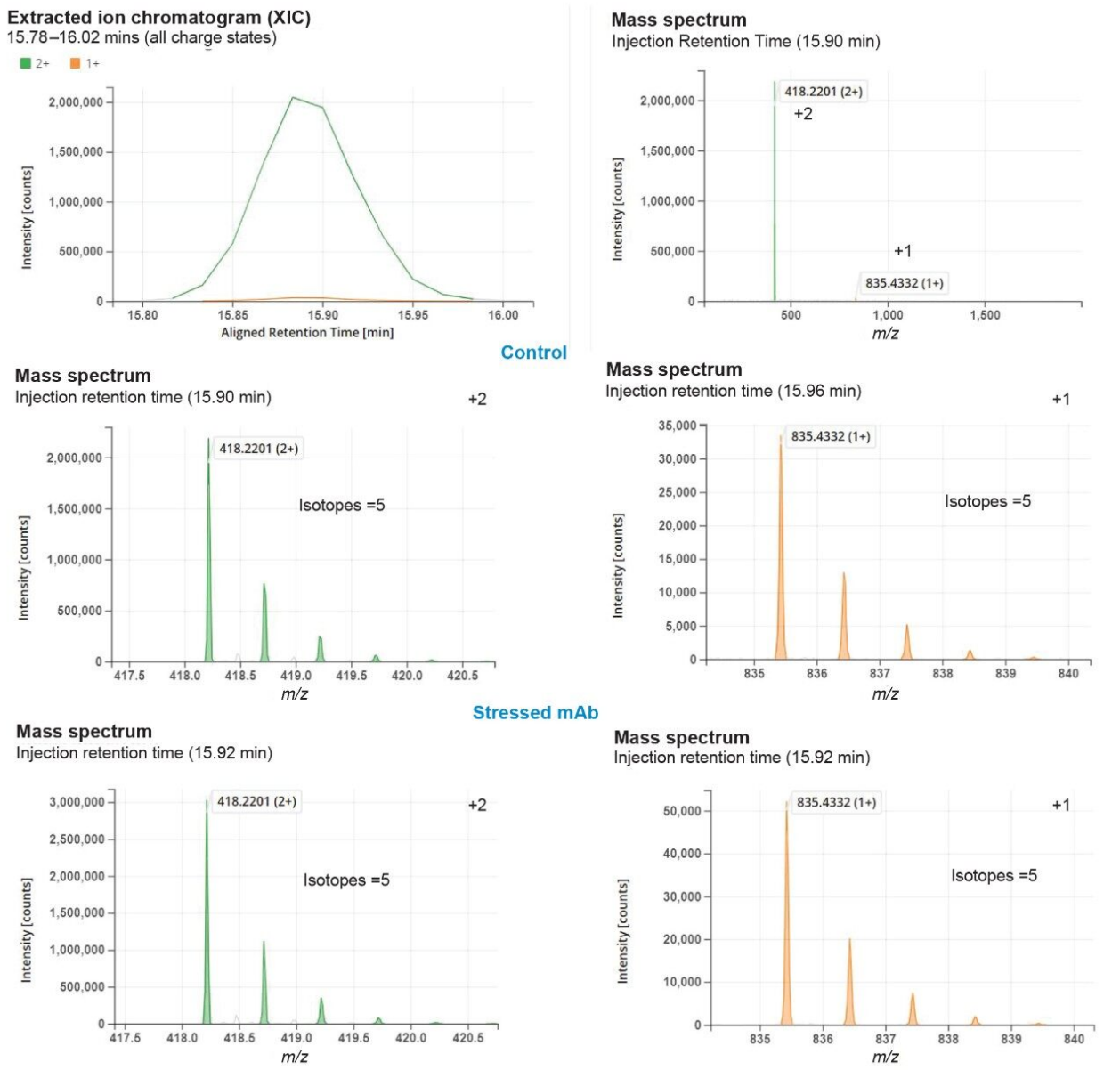

Figure 6. The XIC (top left) and the MS spectrum (top right) for DTLMISR oxidation peptide demonstrate the charged ions and peaks used for quantification of the attribute. DTLMISR has two charge states used for peak area calculation: +2 (the most abundant form) and a +1 minor form ion. The isotope patterns 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).

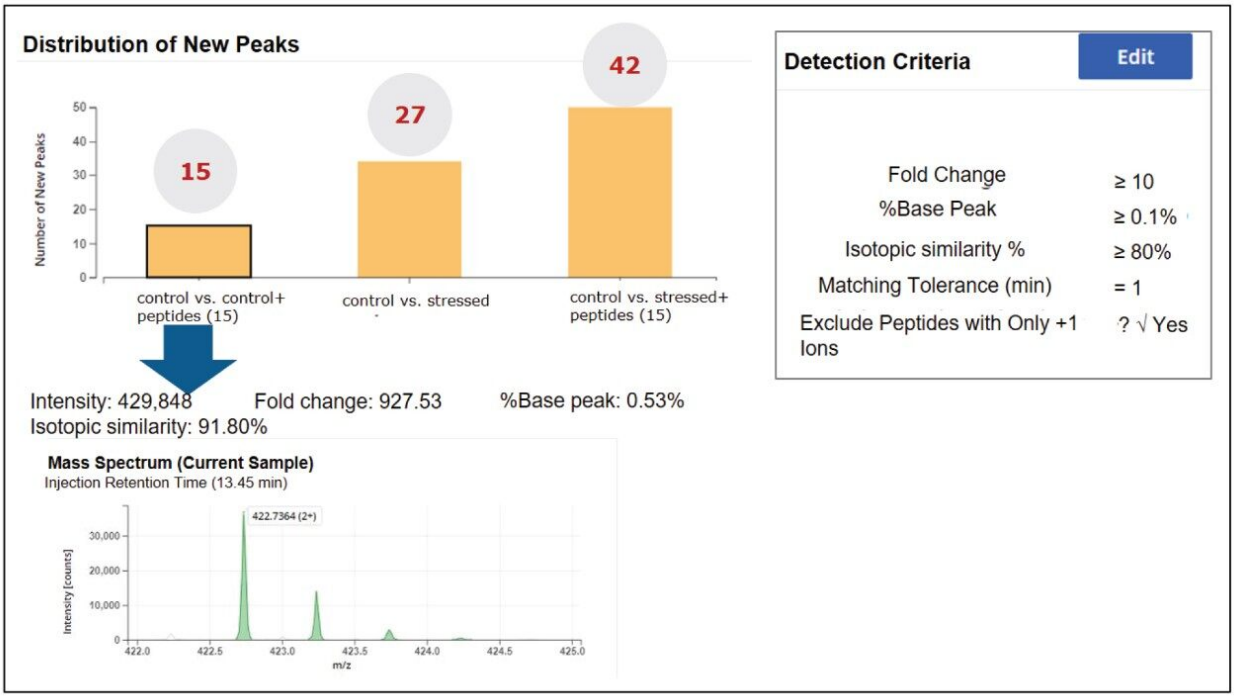

Figure 7. New Peak Detection performed with defined criteria based on fold change, %base peak level, %isotopic similarity and retention time tolerance. Each peak passing the criteria (Right Side) is displayed for review. The peaks can be further examined using MS spectra to manually verify the NPD results.

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.

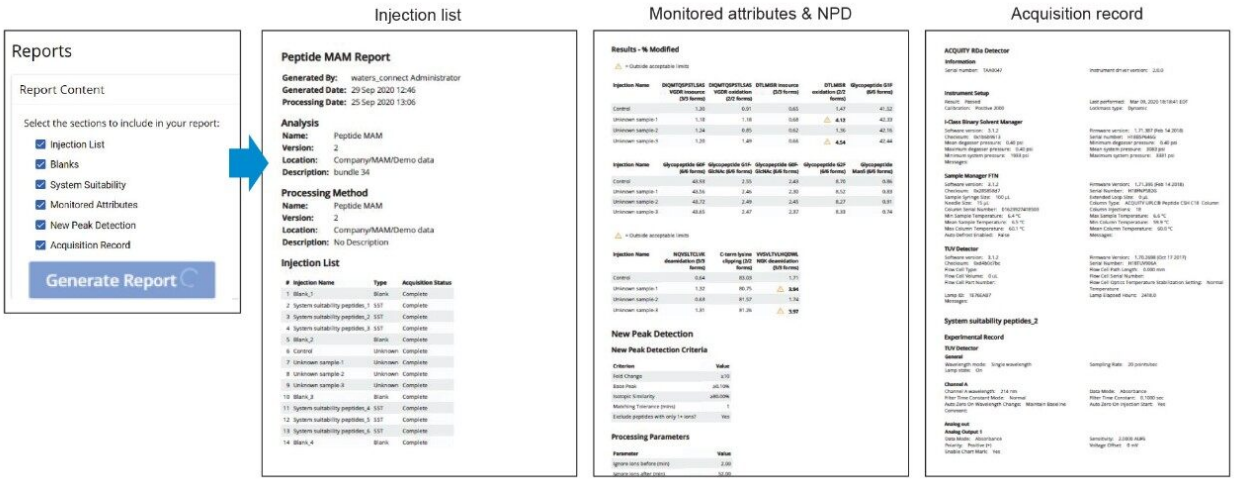

Figure 8. Selectable report template chapters (left panel) are available for configuring peptide MAM data reporting. This report comprises a summary of injections, system suitability test data, peptide attribute tracking data, NPD, and LC-MS method criteria.

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.

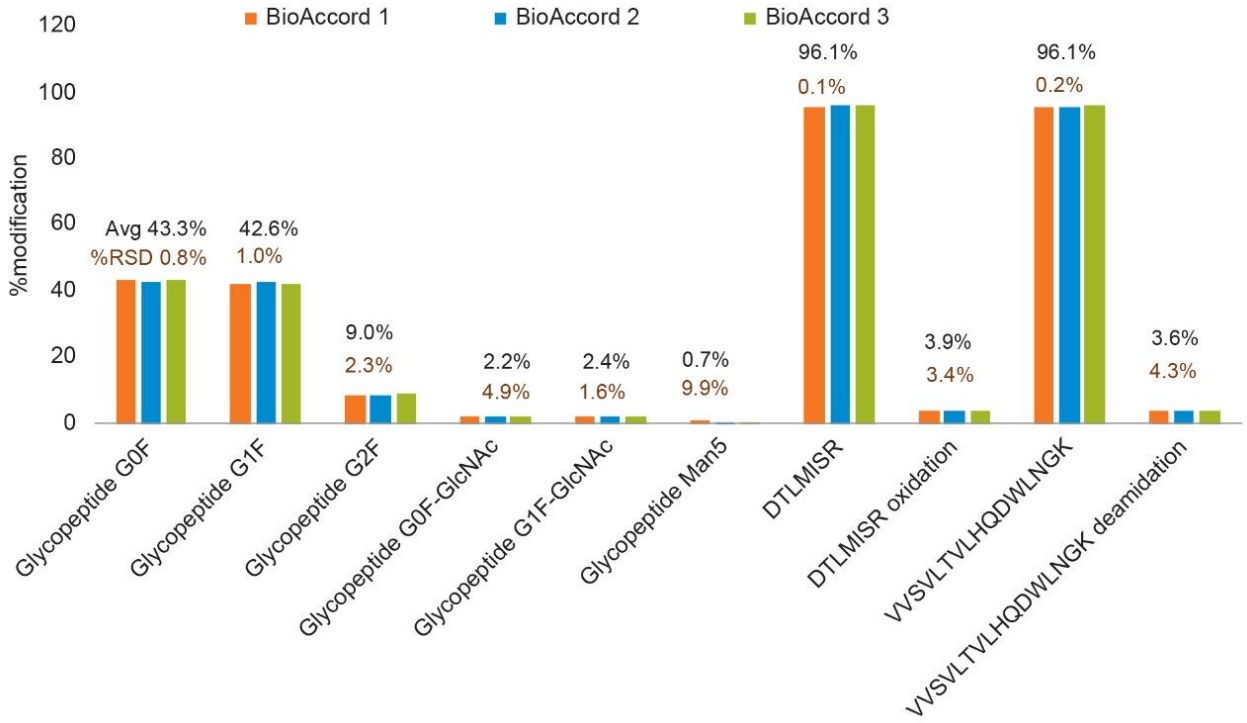

Figure 9. Attribute monitoring data acquired on three BioAccord systems indicate low %RSD variability for attribute-based measurements between replicate experiments on a common instrument and across instruments.

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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