Best Practices for Mass Spectrometry-Based Multi-Attribute Method (MAM) for Therapeutic Proteins and Introduction to MAM Knowledge Hub

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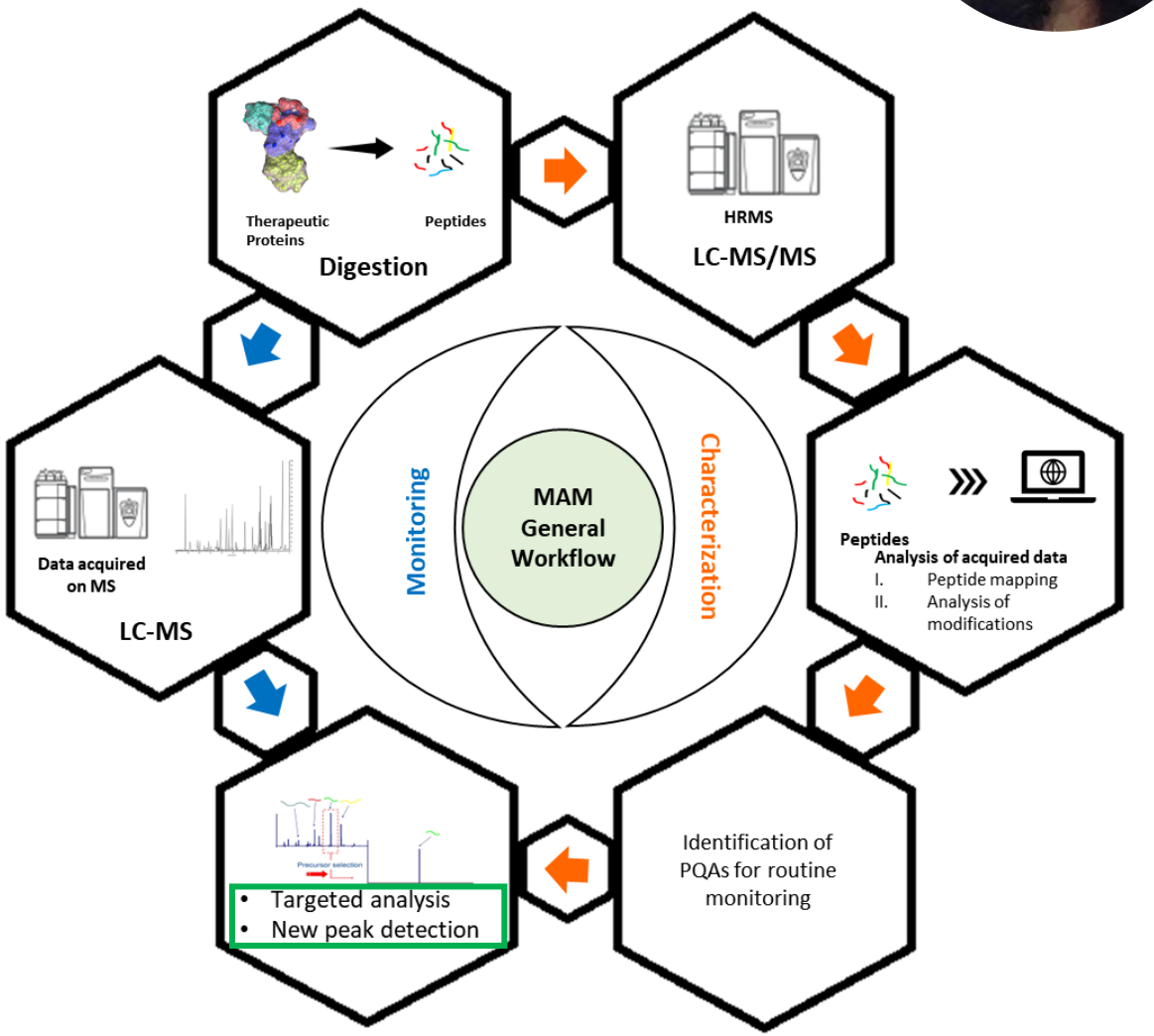
Agenda

- General Chapter <1060> Multi-Attribute Method for therapeutic proteins
  - Overview of MAM
  - Considerations for sample preparation
  - Considerations for system readiness
  - Considerations for non-targeted analysis (new peak detection)
  - Case studies
- MAM knowledge hub

Introduction to Multi-Attribute Method

- A multi-attribute method (MAM) could use any technology that allows a scientist to investigate multiple quality attributes at the same time
- LC-MS-based peptide mapping approach has emerged as the most mature and widely used platform for MAM
  - 2015 publication by Rogers et al. (Amgen) first described LC-MS-based MAM method for mAbs
- Advantages of MAM
  - Ensure drug product quality
  - Align with QbD principles & increase process development and manufacturing efficiencies
    - Meaningful product quality specifications
    - Enhanced product and process understanding
    - Replacing multiple conventional technologies



MAM Expert Panel Membership

Name	Organization
Edward Chess (Chair)	Consultant
Rachel Chen	Biogen
Disha Dadke	Aurobindo Biologics
Andrew Dawdy	Pfizer
Anita Krishnan	Biocon Biologics
Zhirui Lian	Eli Lilly
Benjamin Moore	Genentech
Yuko Ogata	Just-Evotec Biologics
Da Ren	BioTherapeutics Solutions
Lei Wang	Takeda
Christopher Yu	Genentech
Sarah Rogstad	FDA liaison
Xiaoshi Wang	FDA liaison

Chapter Design Strategy

WORKFLOW COMPONENTS

Overview of MAM Method
- MAM workflow
- MAM vs. conventional methods

Sample Preparation
- Strategies to reduce variability and artifacts
- Manual vs. automated techniques

Instrumentation
- Considerations for MAM instrumentation

Software
- Core components of data processing software
- Requirements for targeted and non-targeted analysis

TECHNICAL CONSIDERATIONS

System Readiness
- Metrics, standards, and methodology
- Establishing and employing system readiness criteria

Characterization
- Method and data analysis
- Selection of product quality attributes (PQAs)

Targeted Analysis
- Strategy for monitoring known quality attributes

Non-targeted Analysis (New Peak Detection)
- Parameters and optimization
- Interpretation and significance of new peaks

REAL-WORLD APPLICATIONS

Product Development
- Case studies: bridging MAM and conventional technologies on analysis of: charge variants; protein clipping; glycation; and glycosylation

Production
- Aligning MAM with control strategy
- Setting specifications when transitioning from conventional methods

Qualification and Validation
- Validation and robustness
- Establishing system suitability
- Method transfer and lifecycle management

Alternate Applications and Workflows
- PAT
- Intact and Subunit Workflows

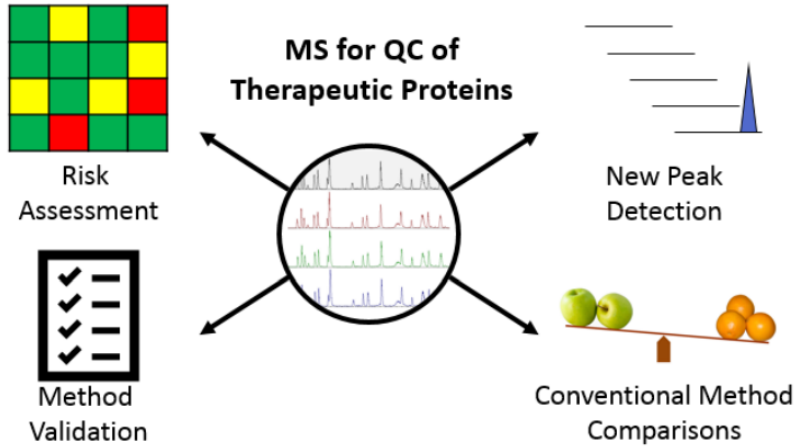
Benefits and Considerations of MAM

Benefits

- Testing multiple attributes at once
- More detailed information of site-specific modifications
- Differentiate between species that may overlap using conventional approaches
- New peak detection allows for control of unexpected new modifications

Considerations

- Risk assessment
- Method validation
- Capabilities of new peak detection
- Comparison to conventional methods



Picture form *S Rogsad et al Analytical Chemistry* **2019** *91* (22), 14170-14177 DOI: 10.1021/acs.analchem.9b03808

Comparison of Common PQAs measured by MAM *vs.* Conventional Methods

mAb Product Quality Attribute		MAM	Conventional Method				
		Pep Map LC-MS	SEC	IEX/cIEF/ icIEF	rCE-SDS	nrCE-SDS	Glycan by HILIC
Identity		+	-	+/-	-	-	-
Soluble aggregates		-	+	-	-	+/-	-
Fragments/Clips		+	+/-	-	+	+	-
Amino acid mutation/Mis-incorporation		+	-	-	-	-	-
Cys related modifications	Unpaired Cys	+	-	+/-	-	-	-
	Disulfide isoform	+	-	-	-	-	-
	Thioether	+	-	-	+/-	-	-
Glycosylation	*N*-linked glycosylation	+	-	+/-	-	-	+
	Non-glycosylated	+	-	-	+	-	-
	O-Linked glycosylation (Ser, Thr)	+	-	+/-	-	-	-
Isomerization (Asp)		+	-	+/-	-	-	-
Oxidation (Met, Trp)		+	-	-	-	-	-
Hydroxylysine		+	-	-	-	-	-
Charge variants	Deamidation (Asn, Gln)	+	-	+	-	-	-
	Glycation	+	-	+	-	-	-
N-Terminal modifications	Signal peptide	+	-	-	-	-	-
	N-Terminal pyroGlutamate	+	-	+	-	-	-
C-Terminal modifications	Lys deletion	+	-	+	-	-	-
	Amidation	+	-	+	-	-	-

Key

"+" application can be used;

"-" application not commonly used;

"+/-" application may be used

Considerations for MAM Sample Prep

- A typical MAM workflow uses a reduced peptide mapping workflow for the relative quantitation of PTMs such as oxidation, deamidation.
- Other modes of MAM involving non-reduced peptide mapping or reduced peptide mapping with a differential alkylation strategy are employed according to the choice of attributes that can be potentially targeted in a single method.

MAM (non-reduced)

Denaturation To perturb protein structure and increase accessibility	**Alkylation (optional)** To prevent disulfide scrambling and aggregation	**Desalting** To remove salts and chemical reagents that impact digestion efficiency	**Digestion*** Enzymatic proteolytic digestion to generate peptides

MAM (reduced)

Denaturation To perturb protein structure and increase accessibility	**Reduction** To break cysteine-cysteine sulfur linkages and release subunits	**Alkylation** To prevent disulfide scrambling, avoid disulfide bond reformation and prevent aggregation	**Desalting** To remove salts and chemical reagents that impact digestion efficiency	**Digestion*** Enzymatic proteolytic digestion to generate peptides

* A combination of enzymes can be used for digestion in the non-reduced condition due to the generation of longer disulfide-bonded peptides

Sample Preparation: Options and Technical Considerations

Considerations

1. Denaturation
2. Reduction
3. Alkylation
4. Desalting
5. Choice of Protease
6. Digestion pH and Temperature
7. Protease: Protein Ratio
8. Digestion Time

6. DIGESTION pH AND TEMPERATURE
   Digestion buffer, and more importantly the pH, is very critical to avoid sample preparation induced protein modifications such as deamidation. A lower pH buffer decreases the rate of induced deamidation. If a pH-resistant enzyme is used, then it is possible to lower the digestion pH below 7.0. Common digestion buffer includes Tris-HCl, ammonium bicarbonate, and ammonium acetate. Commercial digestion kits are available that contain the digestion buffer. The digestion temperature is normally 37°.

7. PROTEASE:PROTEIN RATIO
   The typical ratio of protease to protein can range between 1: 10 to 1:100. The amount of enzyme can be increased to shorten digestion time, decrease missed cleavages, and improve sequence coverage.

8. DIGESTION TIME
   Typical digestion time ranges from 30 minutes to overnight. The combination of higher protease amount and shorter digestion time has the best outcome in terms of lowering artificial deamidation.

System Readiness

Common Metrics

- Total Ion Chromatogram (TIC)
- Mass Accuracy
- MS Resolution
- Retention Time
- Chromatographic Resolution
- Integrated Peptide Area
- Met Oxidation (a measure of artifactual oxidation)
- In-Source Fragmentation
- MS/MS Fragment Ion Intensity (if applicable)
- MS/MS Fragment Ion Mass Accuracy (if applicable)

Table 7. Considerations for MAM System Readiness Standards

MAM Standard	Advantages	Disadvantages
Commercial Peptide Mix	• Universally available across industry • Easy sample preparation / no enzymatic digestion involved • Simpler and may be more consistent measure • May have associated CoA (Certificate of Analysis) • Application across multiple projects – facilitates large body of system readiness data	• No measure of sample preparation quality • May not be as representative of the final sample (e.g., *N*-glycosylation, oxidation hotspots, deamidation hotspots) • Cost
Commercial Protein Standard	• Universally available across industry • May have associated CoA • May be more representative of sample (e.g., *N*-glycosylation, oxidation hotspots, deamidation hotspots) • Opportunity to access quality of enzymatic digestion along with system • Application across multiple projects – facilitates large body of system readiness data	• Requires additional sample handling which can increase variability • Not molecule-specific • Cost
In-House-Manufactured Protein Standard	• May be more accessible than commercial standards • Opportunity to access quality of enzymatic digestion along with system • Application across multiple projects – facilitates large body of system readiness data	• Requires additional sample handling which can increase variability • Does not allow for evaluation of the exact data processing method used for the project-specific samples • May require a different LC-MS method than that used for the project-specific MAM assay • Not universally available across industry • No vender CoA - burden of quality assurance is on user
Project-Specific Reference Material	• May be more accessible than commercial standards • Provides most complete assessment of the exact MAM assay, including quantitation of the project-specific attributes • Opportunity to access quality of enzymatic digestion along with system	• Requires additional sample handling which can increase variability • Not universally available across industry • No vender CoA - burden of quality assurance is on user

Considerations for Validation of MAM

Specificity	Specificity can be accomplished through evaluation of mass spectrometric and chromatographic resolving power, sample and matrix complexity, and potential injection carryover. Isobaric or near-isobaric interferences can be eliminated or controlled by combining the m/z with a specific retention time.
Linearity	As a quantitative method, a linear response is expected and commonly observed across the desired range for each attribute. Materials with different amount of product variants or impurities can be prepared (through enrichment or forced degradation) and used to create a set of samples for demonstration of linearity.
Accuracy	The same set of samples used to demonstrate linearity can often be used for evaluation of accuracy for each attribute. A common approach would be to generate samples at five or more levels of attribute abundance by mixing two standard samples with known values that represent the low and high end of the target range. Accuracy is evaluated using the percentage of recovery at each level.
Precision	In general, precision performance is expected to be comparable with conventional purity methods. Based on development experience, product- or attribute-specific considerations may be appropriate for analytical method validation. Similarly, attribute-specific acceptance criteria, including product specification, can be used to support the demonstration of precision for the intended purpose of the method.
Quantitation Limit	The quantitation limit (QL) would be attribute-dependent due to the difference in recovery from sample preparation and HPLC separation, as well as difference in ionization. While there are several ways to estimate the lower QL, including signal to noise ratio and standard deviation of the response and slope, it may be necessary to verify such estimates using samples that are at or near the estimated QL
Range	As with conventional methods, range is established where adequate analytical method performance is demonstrated for linearity, accuracy, and precision.

Non-Targeted Analysis (New Peak Detection, NPD)

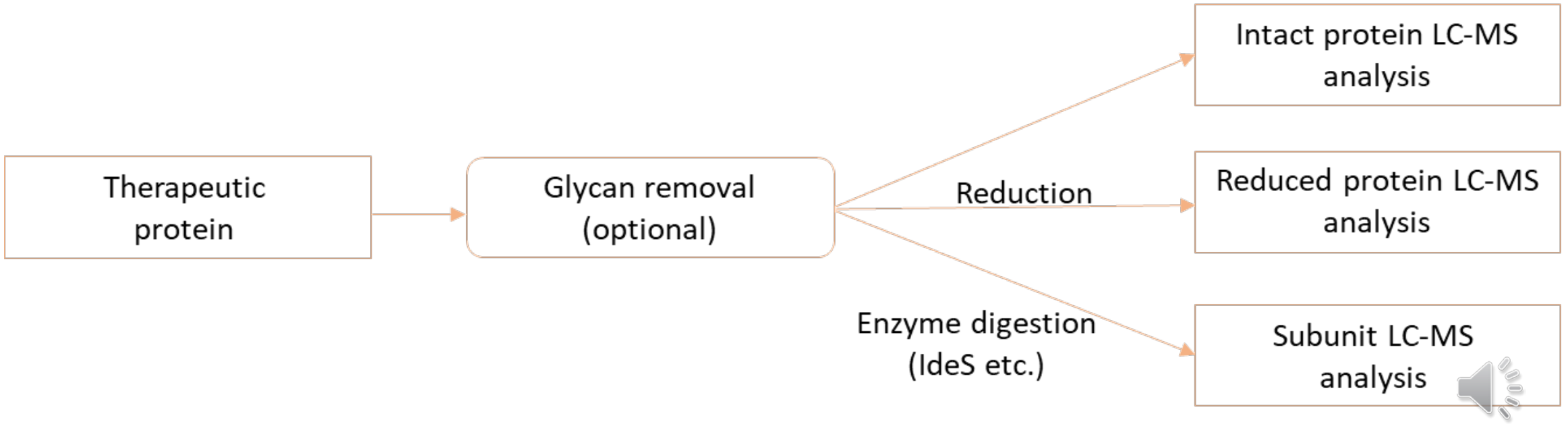
- Targeted analysis in MAM focuses on pre-defined attributes
- In conventional methods, NPD is typically performed manually by analyst using visual comparison of data
- When use MAM to replace conventional assays, NPD function is necessary
- MAM NPD workflows rely on automated comparison of the mass signal data in the three-dimensional space of: retention time, *m/z*, and intensity
- MAM can identify new peaks co-eluted from LC
- The reference sample used in NPD is typically a well-characterized reference standard

Considerations and attributes for non-Targeted Analysis (New Peak Detection, NPD)

- MS signal intensity
- MS signal intensity fold change
- Number of isotopes and Isotope distribution pattern
- Number of charge states
- Molecular weight and m/z value
- Retention time
- XIC peak shape
- MS/MS fragmentation (if LC-MS/MS system is used for NPD)

Intact and Subunit Workflow Using MAM

General Sample Preparation Workflow for Intact/Subunit Mass Measurements



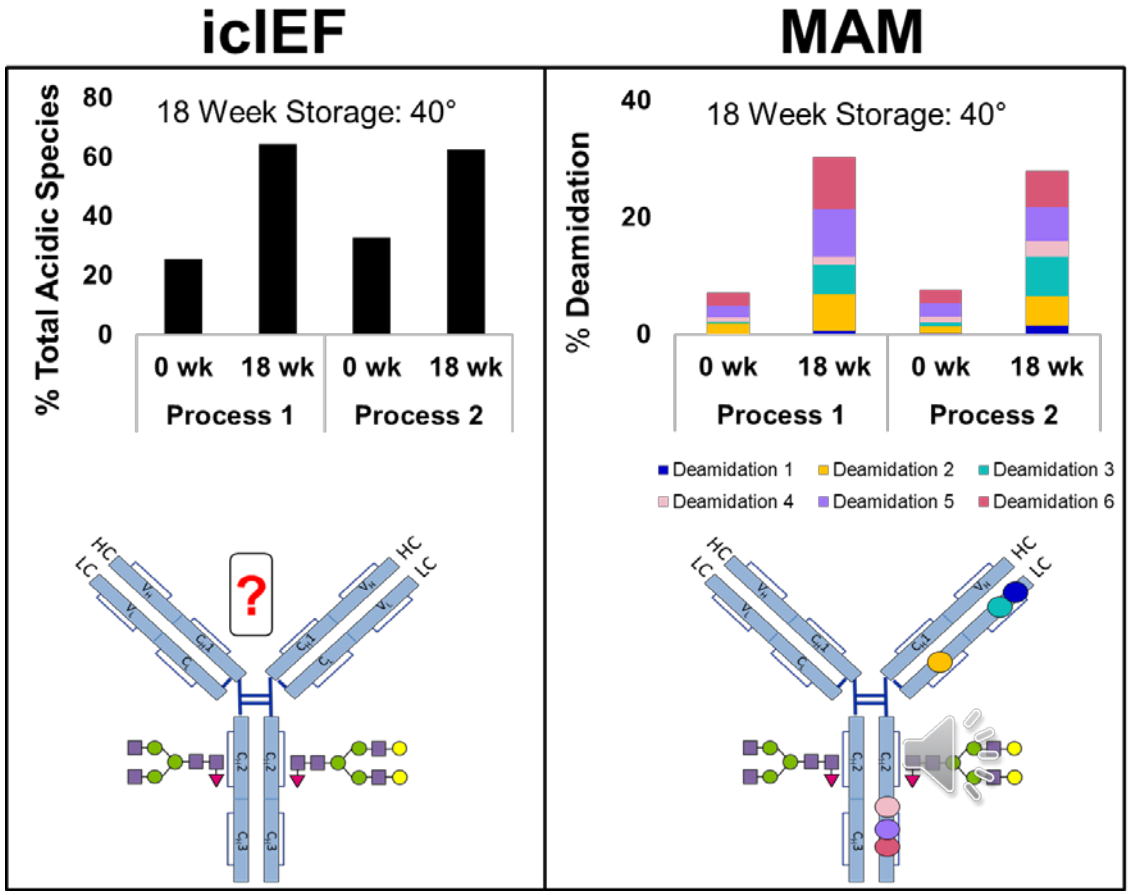
3 Case Studies

Use of MAM in Product Development

Case Study 1

- A therapeutic mAb produced by 2 different processes was subjected to thermal degradation at 40°C for 19 weeks
- Acidic charge variants determined by icIEF
- MAM was employed in parallel to monitor 6 previous-characterized Asn and Gln deamidation “hotpots”
- MAM provided a site-specific understanding of the thermal stability of the mAb produced by each process, which enables more precise and informed process changes
- NOTE: The absolute quantitation by each method is not expected to match due to MAM targeting the relative abundance of specific attributes, and icIEF providing quantitation of the total level of charge variants

Comparison of Thermal Stability by icIEF versus MAM for a mAb Manufactured by Two Different Processes

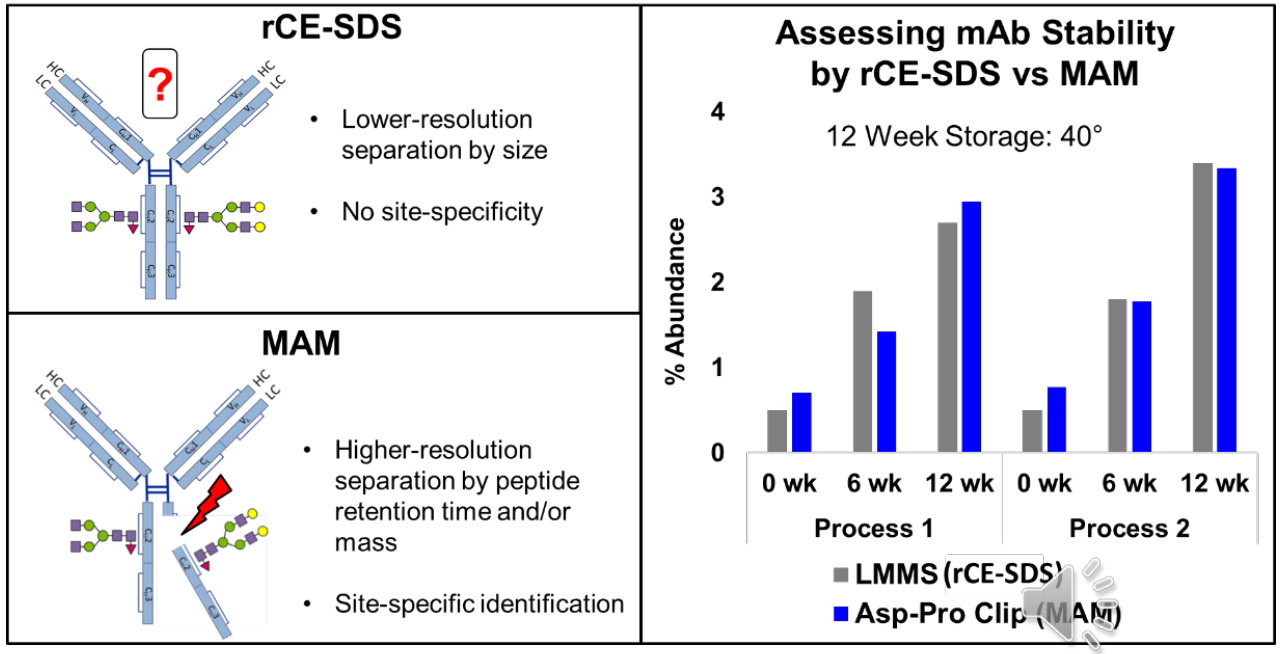


Use of MAM in Product Development

Case Study 2

- Reducing capillary gel electrophoresis (rCE-SDS) and MAM were used to assess the level of thermal stress-induced clipping of a mAb produced by two different processes
- The rCE-SDS assay monitored the composition of lower molecular mass species (LMMS) relative to the intact molecule
  - The individual species may separate but cannot be directly identified. Additionally, rCE-SDS may not have the resolution to separate all LMMS
- MAM not only quantitated the level of clipping comparably to rCE-SDS, but it also directly monitored the specific site responsible for the LMMS
- The relative abundance of the low molecular mass species (LMMS), as determined by rCE-SDS, is comparable to the relative abundance of a specific Asp-Pro clip, monitored by MAM

Comparison of Thermal Stability by rCE-SDS versus MAM for a mAb Manufactured by Two Different Processes

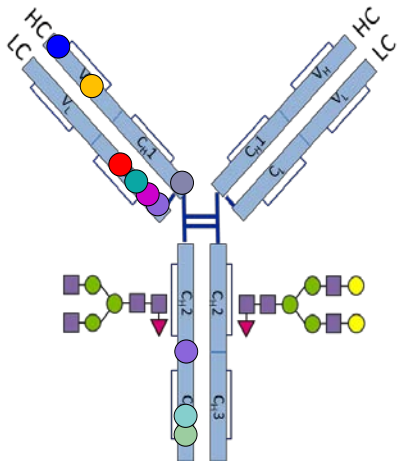


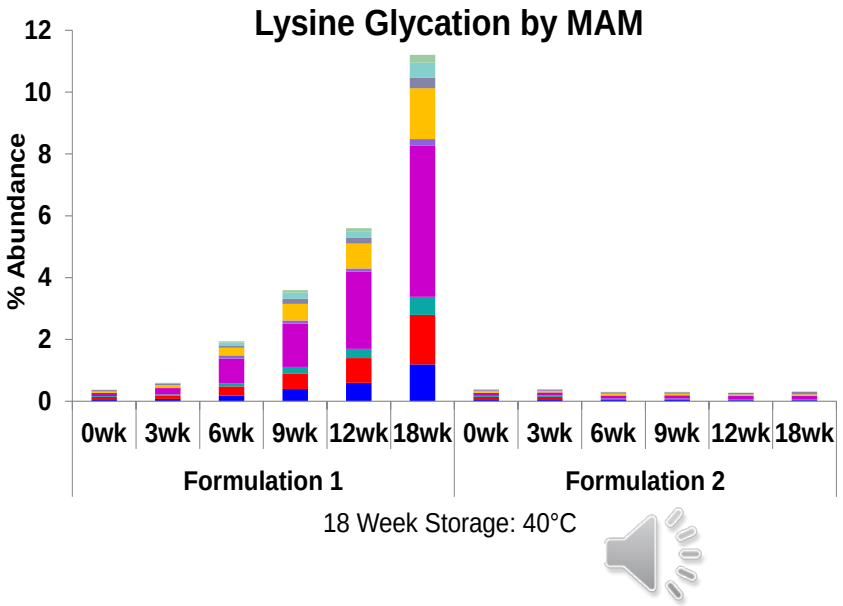
Use of MAM in Product Development

Case Study 3

- To study the effect of the formulation on non-enzymatic lysine glycation levels under thermal stress
- MAM analysis was used to elucidate the levels of lysine glycation at specific sites for two different formulations
- Though icIEF may be capable of detecting glycation as an acidic charge variant (data not shown), it can be difficult to separate and quantify
- The abundance of non-enzymatic lysine glycation is much higher using formulation 1

Assessing Non-Enzymatic Lysine Glycation Risk by MAM to Support Formulation Development





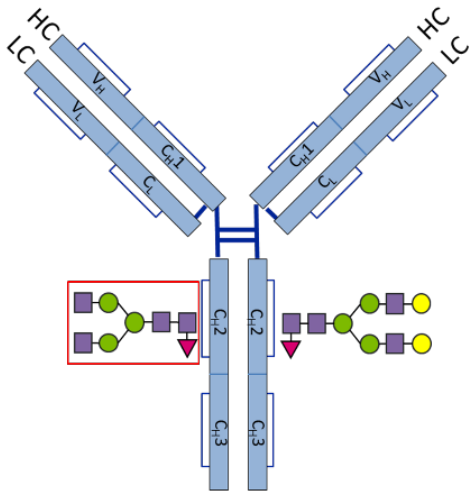
Use of MAM in Product Development

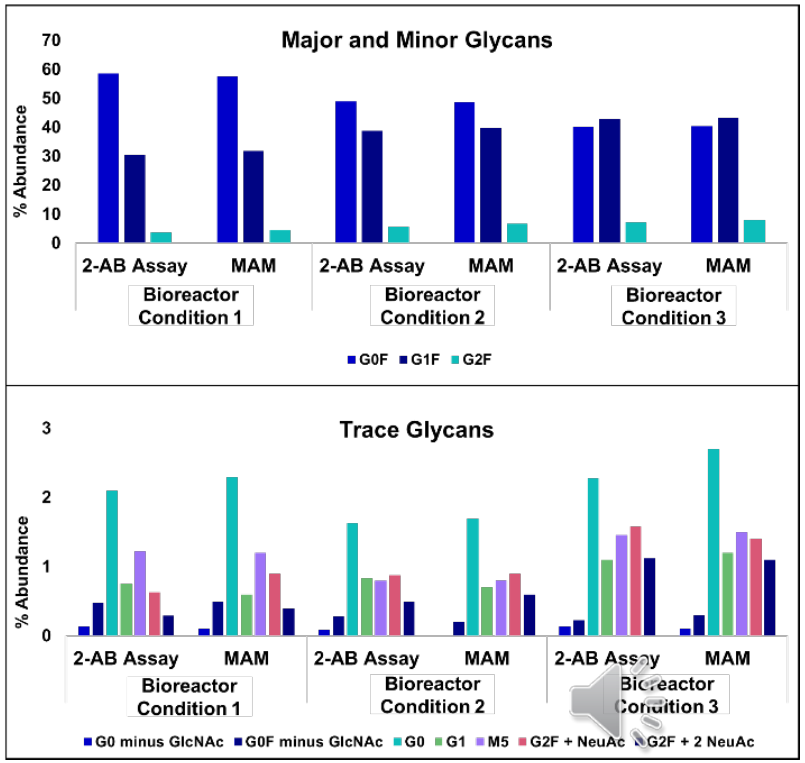
Case Study 4

- Upstream process developers sought to study the effects of bioreactor conditions on the presence and level of *N*-linked glycosylation
- This study demonstrates the ability of MAM to perform quantitation of *N*-glycosylation, with results comparable to the conventional 2-AB assay.
- Furthermore, MAM has several advantages over conventional assays. The conventional assay is agnostic to the presence of non-glycosylation, the presence of *O*-glycosylation, and the original location of *N*-glycosylation.

Comparison of N-Glycosylation Levels by HILIC Glycan Map versus MAM for a mAb from 3 Bioreactor Conditions

MAM enables unambiguous, label-free quantitation of glycans which may be used to inform process development





Summary and Next Steps

- USP Expert Panel has drafted new general chapter with best practices for MAM
  - *<1060> Mass Spectrometry Based Multi-Attribute Method for Therapeutic Proteins*
  - Expected to publish on September 1st, 2023, in Pharmacopeial Forum (PF): https://www.uspnf.com/pharmacopeial-forum
  - Will be open for 90 days for public comments

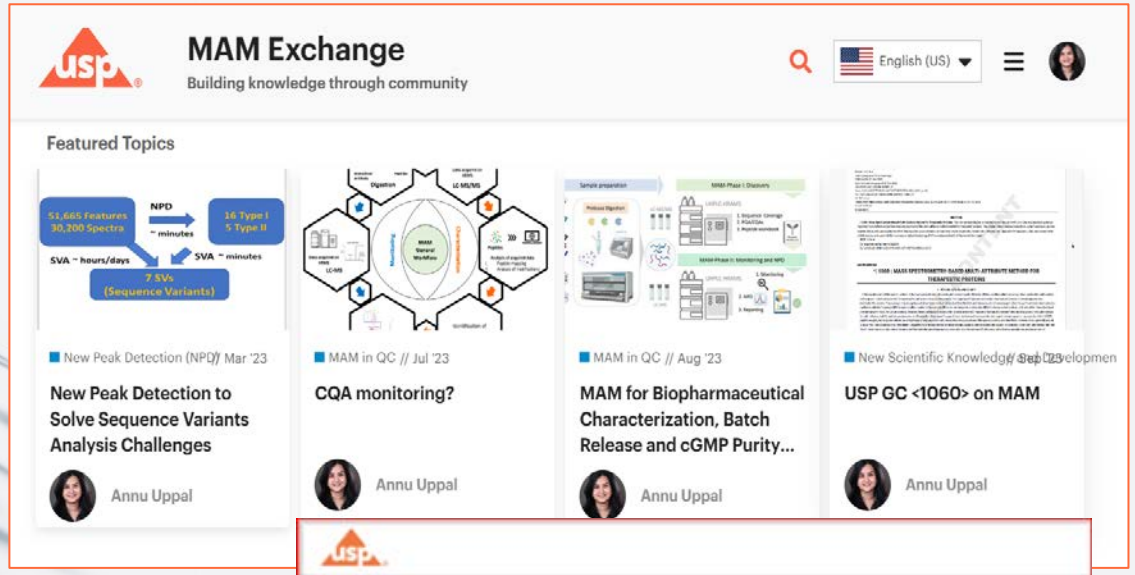
- Cooperative agreement with FDA under a BsUFA-funded research grant*
  - "Assessment of the performance of MAM vs conventional QC methods for evaluation of Product Quality Attributes of adalimumab and etanercept"

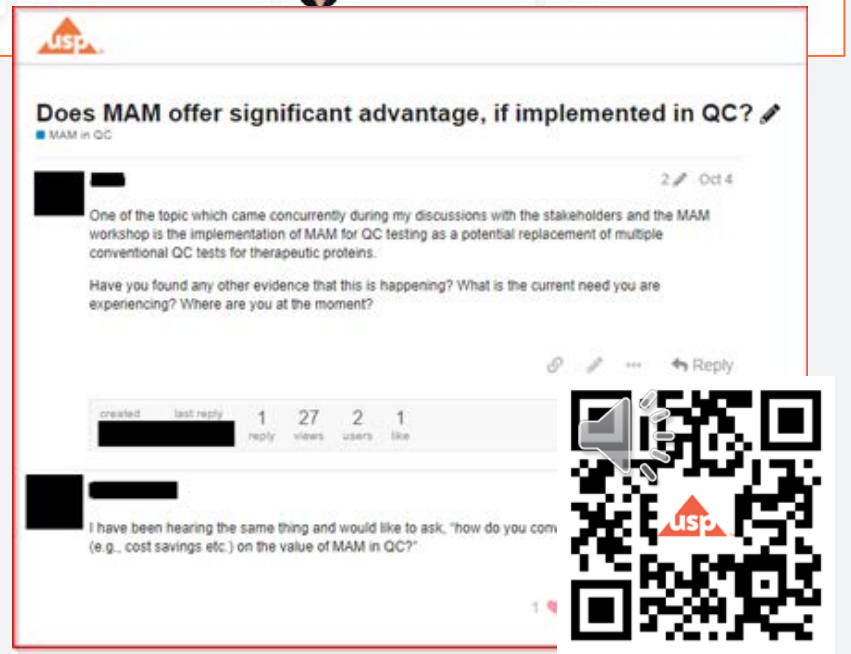
MAM Knowledge Hub

Knowledge Hub Online Community, WHY?

- Stakeholder engagement evolution
  - Transactional to Transformational
- Unleashing the power of online communities
  - Democratization and inclusion of knowledge
  - Engineered for asynchronous, hybrid work structures
- Increase and accelerate early scientific knowledge exchange in select topics
  - Community members shape, USP hosts

MAM Exchange Community





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Questions

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