

Monitoring Critical Quality Attributes with a Peptide-Mapping-Based Multi-Attribute Method

A user-friendly LC/MS workflow for quality control

Authors

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Abstract

Critical quality attributes (CQAs) play a vital role in ensuring the safety, efficacy, and consistency of biotherapeutics, including monoclonal antibodies (mAbs), making CQA monitoring essential for quality control (QC). Compared with conventional analytical approaches, the multi-attribute method (MAM) offers significant advantages by enabling integrated and comprehensive analysis of multiple CQAs within a single workflow. Although recent advances have accelerated the adoption of MAM in regulated QC environments, data analysis software suitable for MAM can be complex and may not fully meet regulatory requirements. Therefore, there remains a growing need for user-friendly workflows that support accurate, precise, and robust CQA monitoring while facilitating successful method transfer to QC laboratories.

In this application note, a stress study was performed using trastuzumab as a representative mAb. CQAs were identified and validated using an Agilent 6545XT AdvanceBio LC/Q-TOF (LC/MS/MS platform), and the validated method was subsequently transferred to an Agilent 6230C TOF LC/MS for routine CQA monitoring. Agilent MAM for OpenLab CDS streamlined data processing through an intuitive, web-based interface that supports efficient, regulatory-compliant analysis. Overall, the Agilent LC/MS/MS characterization workflow, LC/MS monitoring platform, and MAM for OpenLab CDS provide a streamlined, compliance-ready solution for implementing MAM and enabling routine CQA monitoring in biopharma QC laboratories.

Introduction

MAM for CQA monitoring

The rapid growth of the pharmaceutical industry has established biopharmaceuticals as a major segment of modern medicine, with mAbs representing one of the most important classes due to their broad and expanding therapeutic applications. As these complex molecules progress through development and manufacturing, monitoring CQAs is essential to ensure product safety, efficacy, and consistent performance. Conventional analytical strategies typically rely on multiple orthogonal methods to assess individual attributes, resulting in workflows that are often time consuming and labor intensive. In contrast, MAM provides a powerful alternative by enabling comprehensive characterization and monitoring of multiple CQAs within a single analytical platform. Although substantial progress has been made toward implementing MAM in regulated environments for QC applications, widespread adoption

remains challenging due to the inherent complexity of the methodology, data analysis requirements, and regulatory considerations. Therefore, there is a critical need to develop a robust, accurate, and precise MAM workflow for comprehensive CQA monitoring, supported by compliance-ready software solutions that streamline data processing and provide a user-friendly experience to facilitate routine deployment in QC laboratories.

Method development and transfer for MAM

As illustrated in Figure 1, MAM is initially developed using tandem mass spectrometry (MS/MS) to identify, characterize, and validate CQAs for mAb characterization during Phase I. Once the method has been established and validated, it can be transferred to QC laboratories for routine CQA monitoring in Phase II.¹ Since quantitative assessment of predefined CQAs does not require MS/MS information, routine QC analyses can be performed using MS-only acquisition, thereby simplifying operation, reducing data complexity, and enhancing the suitability of MAM for QC environments.

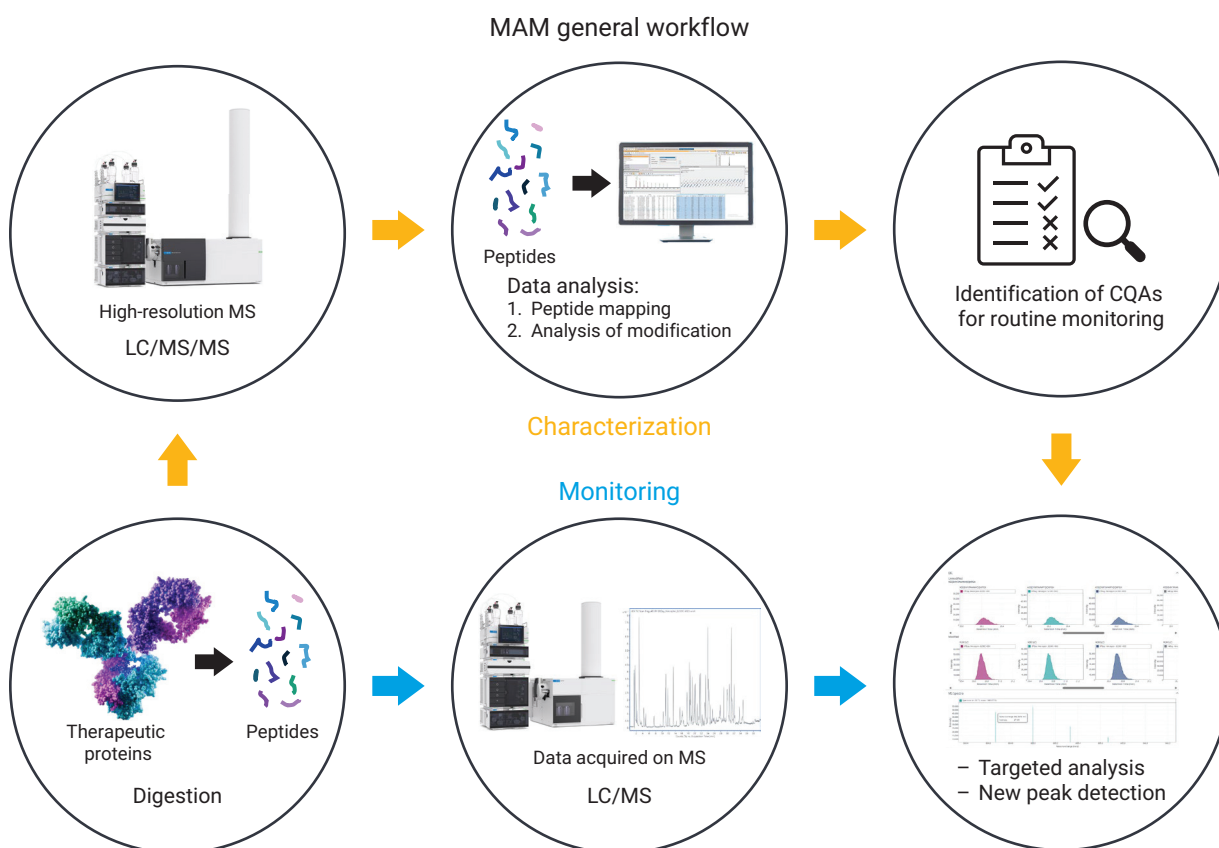


Figure 1. Overview of MAM general workflow, including CQA characterization in Phase I (yellow arrows) and monitoring in Phase II (blue arrows).

Experimental

Preparation of stressed samples

To generate samples with elevated levels of product-related modifications, trastuzumab was subjected to accelerated heat and agitation stress. The antibody was diluted to 5 mg/mL in 50 mM Tris-HCl buffer (pH 7.0) and incubated at 40 °C while agitating at 300 rpm under light-protected conditions. Aliquots were collected at 0, 1, 4, 7, 14, and 28 days to enable longitudinal monitoring of CQAs.

Sample preparation for MAM

Table 1. Materials for sample preparation.

Reagent and Material	Vendor	Part Number
Trastuzumab	Genentech	NDC 50242-134-68
Formic Acid (FA)	Sigma-Aldrich	5.33002
Acetic Acid	Sigma-Aldrich	5.33001
Purified Water, 18.2 MΩ·cm	In house	NA
Acetonitrile (ACN)	Agilent	5191-5101
1 M Tris-Hydrochloride Buffer, 7.5 ± 0.1	Corning	46-030-CM
8 M Guanidine Hydrochloride, pH 8.5	Sigma-Aldrich	G7294-100ML
DL-Dithiothreitol (DTT)	Sigma-Aldrich	D9779-1G
Iodoacetamide (IAM)	Sigma-Aldrich	I6125-5G
AdvanceBio Spin Columns, < 100 µL	Agilent	1980-1103
Trypsin Gold-Mass Spec Grade, 100 µg	Promega	V5280

Sample preparation consisted of the following steps.

1. Dilution

Dilute trastuzumab with water to 5 mg/mL. For example, add 4.55 µL of 22 mg/mL trastuzumab to 15.45 µL of water. Vortex gently to mix well.

2. Denature and reduction

- Mix 20 µL of 5 mg/mL sample and 60 µL of 8 M guanidine-HCl, pH 8.5, and add 5 µL of 300 mM DTT. Vortex gently to mix well.
- Incubate at 300 rpm, 37 °C for 30 minutes.

3. Alkylation

- Add 10 µL of 500 mM IAM to the sample. Vortex gently to mix well.
- Protect from light and incubate at 300 rpm and 25 °C for 30 minutes.

4. Buffer exchange²

- Snap or twist to remove the tip of Agilent AdvanceBio Spin columns, < 100 µL. Loosen or remove the cap.
- Place the 2 mL column wash tube in the centrifuge; spin at 1000 g for 2 minutes to remove the excess storage buffer. Ensure consistent column orientation in the centrifuge for each spin.
- Apply 400 µL of 50 mM Tris-HCl, pH 7.5, to the center of the column, taking care not to disturb the stationary phase. Place in the centrifuge, ensuring the same orientation that was previously used is used again. Spin at 1000 g for 2 minutes to equilibrate the column and discard the flow-through. Repeat the buffer application and spin two more times.
- Apply the sample (approximately 95 µL) to the center of the column, taking care not to disturb the stationary phase. Place in the centrifuge, ensuring the same orientation is used as was previously used. Spin at 1000 g for 2 minutes to recover the sample in a 1.7 mL tube.
- **Optional:** Add 50 mM Tris-HCl, pH 7.5, to bring the sample volume to 95 µL.

5. Digestion³

- Add 5 µL of 1 mg/mL trypsin in 50 mM acetic acid to the sample. Vortex gently to mix well. Confirm pH over 7.0.
- Incubate at 300 rpm and 37 °C for 2 hours.

6. Acidification

Add 10 µL of 10% FA to the sample. Confirm pH below 3.0. Transfer to a HPLC vial for injections.

Data acquisition and analysis for MAM

For CQA identification and characterization, LC/MS/MS analysis was performed using an Agilent biocompatible platform, consisting of an **Agilent 1290 Infinity III bio LC system** coupled to an **Agilent 6545XT AdvanceBio LC/Q-TOF system**. Data acquisition was conducted using Agilent OpenLab CDS software, version 3.0, and the resulting data were processed with **Agilent MassHunter BioConfirm software**, version 14.0, to identify CQAs.

LC/MS analysis was performed on an Agilent biocompatible platform comprising an Agilent 1290 Infinity III bio LC system coupled to an **Agilent 6230C TOF LC/MS**. Data were acquired using OpenLab CDS and analyzed with **Agilent MAM for OpenLab CDS** for routine CQA monitoring. Results for selected CQAs were exported to Microsoft Excel for trend analysis.

The LC conditions listed in Table 2 were applied to both LC/MS/MS and LC/MS analyses. MS parameters for LC/MS/MS and LC/MS analyses are provided in Tables 3 and 4, respectively. Instrument parameters may be further optimized as needed to accommodate specific analytical requirements.

Table 2. Instrument parameters for Agilent 1290 Infinity III bio LC.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping columns, 2.1 × 150 mm, 2.7 µm (p/n 653750-902)
Column Temperature	50 °C
Flow Rate	0.25 mL/min
Mobile Phase A	Water with 0.1% FA
Mobile Phase B	Acetonitrile with 0.1% FA
Gradient	Time (min) %B
	0 2
	5 2
	38 38
	47 95
	50 95
	51 2
	60 2

Table 3. Instrument parameters for MS/MS on Agilent 6545XT AdvanceBio LC/Q-TOF (auto MS/MS mode).

Parameter	Value	Parameter	Value
Polarity	Positive	MS/MS Range	100 to 3,000
Data Storage	Both	MS and MS/MS Acquisition Rate	3 spectra/s
Acquisition Mode	Auto MS/MS	Collision Energy	$3.6 \times (m/z)/100 - 4.8$
Gas Temperature	350 °C	Isolation Width	Medium ($m/z \sim 4$)
Drying Gas Flow	13 L/min	Precursors/Cycle	Top 5
Nebulizer	35 psi	Threshold for MS/MS	6,000 counts and 0.001%
Sheath Gas Temperature	375 °C	Precursor Charge	2+, 3+, > 3+
Sheath Gas Flow	12 L/min	Target	50,000 counts/spectrum
Capillary Voltage	3,750 V	Excluded After	3 spectra
Nozzle Voltage	0 V	Release After	0.2 min
Fragmentor	45 V	Reference Mass	322.0481, 922.0098
Skimmer	35 V		
MS Range	200 to 3,000		

Table 4. Instrument parameters for MS on Agilent 6230C TOF LC/MS (MS only mode).

Parameter	Value	Parameter	Value
Polarity	Positive	Fragmentor	45 V
Acquisition Mode	MS Only	Skimmer	35 V
Gas Temperature	250 °C	MS Acquisition Rate	3 spectra/s
Drying Gas Flow	13 L/min	Octopole RF Voltage	750
Nebulizer	35 psi	Centroid Absolute MS Threshold	200
Sheath Gas Temperature	300 °C	Centroid Relative MS Threshold	0.01
Sheath Gas Flow	12 L/min	Profile MS Threshold	200
Capillary Voltage	3,000 V	Reference Mass	322.0481, 922.0098
Nozzle Voltage	0 V		
MS Range	200 to 3,000		

Results and discussion

CQA identification and method validation

It is critical to ensure the method is capable of successful and reliable CQA monitoring; therefore, the relevant CQAs must first be identified and validated. The auto MS/MS acquisition mode on the 6545XT AdvanceBio LC/Q-TOF system provides comprehensive peptide fragmentation, enabling confident peptide identification and sequence confirmation. This capability also allows potential CQA-related modifications to be accurately detected, verified, and localized, establishing a robust foundation for subsequent MAM-based monitoring in QC applications.

As shown in Figure 2, the sequence coverage map achieved 89.29% coverage of the combined heavy- and light-chain sequences. Notably, this experiment did not aim to achieve full sequence coverage, because complete coverage was obtained for all critical regions, including the complementarity-determining regions, ensuring confident sequence verification and enabling accurate identification and characterization of CQA-related modifications.

Figure 3 presents a comprehensive fragmentation map of a peptide containing a CQA. The extensive MS/MS fragmentation enabled confident assignment of the deamidation modification to asparagine 6 (N6), unambiguously differentiating it from other possible deamidation sites within the peptide sequence. Such site-specific characterization is essential for accurate CQA identification and provides a strong foundation for robust MAM method development and validation.

01Day_Herceptin-r002.sirsl

A: Herceptin Heavy Chain

1	<u>EVQLVESGGGLVQPGGSLRLS</u> <u>CAASGFNIKDTYIHWVRQAPGKGLEWVAR</u>	50
51	<u>IYPTNGYTRYADSVK</u> <u>GRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG</u>	100
101	<u>GDGFYAMDYWGQGT</u> <u>LVTVSSASTKGPSVFPLAPSSK</u> <u>STSGGTAALGCLVK</u>	150
151	<u>DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT</u>	200
201	<u>YICNVNHKPSNTK</u> <u>VDKKVEPK</u> <u>SCDKTHTCPPCPAPELLGGPSVFLFPPKP</u>	250
251	<u>KDTLMISRTPEVTCVVVDVSHEDPEVK</u> <u>FNWYVDGVEVHNAK</u> <u>TKPREEQYN</u>	300
301	<u>STYRVVSVLTVLHQDWLNGKEYK</u> <u>CKVSNKALPAPIEK</u> <u>TISKAKGQPREPO</u>	350
351	<u>VYTLPPSR</u> <u>EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK</u> <u>TTPPV</u>	400
401	<u>LDSDGSFFLYSKLTVDK</u> <u>SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG</u>	449

B: Herceptin Light Chain

1	<u>DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGK</u> <u>APKLLIYS</u>	50
51	<u>ASFLYSGVPSR</u> <u>FSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQ</u>	100
101	<u>GTKVEIK</u> <u>RTVAAPSVFIFPPSDEQLKSGTASVVCLLNFFYP</u> <u>REAQVQWKV</u>	150
151	<u>DNALQSGNSQESVTEQDSK</u> <u>DSTYLSSTLTLSKADYEK</u> <u>HKVYACEVTHQG</u>	200
201	<u>LSSPVTKS</u> <u>FNRRGEC</u>	214

Figure 2. A representative sequence-coverage map for trastuzumab, demonstrating peptide coverage across the heavy and light chains obtained by LC/MS/MS analysis.

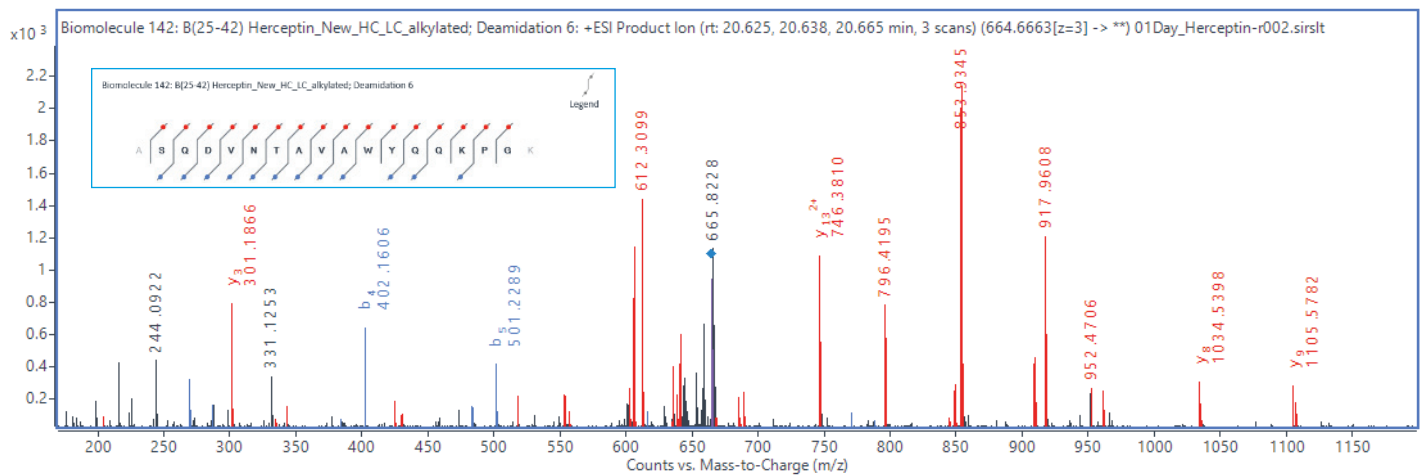


Figure 3. Representative MS/MS fragmentation map of the trastuzumab light-chain peptide (residues 25 to 42), showing site-specific localization of the deamidation modification.

CQA verification and analysis

CQAs were established by evaluating quantitative trends across the stress time course, while MS/MS data were used to identify and confirm peptides containing CQA-related modifications. The resulting MAM workflow was developed and validated for robustness, accuracy, and reproducibility, allowing subsequent CQA quantification to be performed using MS-only acquisition. After CQAs were validated, MAM for OpenLab CDS was employed for routine monitoring of the defined CQAs in trastuzumab. Table 5 summarizes the CQAs that were identified and validated through MS/MS characterization.

Table 5. Summary of monitored trastuzumab CQAs for QC.

Peptide Name	CQA Position	Modification	Sequence
HC:51-59	N55 [HC]	Deamidation	IYPTNGYTR
HC:77-87	N84 [HC]	Deamidation	NTAYLQMNSLR
HC:99-124	D102 [HC]	Isomerization	WGGDGFYAMDYWGQGLTVT-VSSASTK
HC:252-258	D252 [HC]	Isomerization	DTLMISR
HC:252-258	M255 [HC]	Oxidation (M)	DTLMISR
HC:278-291	N289 [HC]	Deamidation	FNWYVDGVEVHNAK
HC:305-320	N318 [HC]	Deamidation	VVSVLTVLHQDWLNGK
HC:364-373	N364 [HC]	Deamidation	NQVSLTCLVK
LC:25-42	N30 [LC]	Deamidation	ASQDVNTAVAWYQQKPGK
LC:150-169	N158 [LC]	Deamidation	VDNALQSGNSQESVTEQDSK

CQA monitoring in MAM for OpenLab CDS

CQAs settings in MAM for OpenLab CDS consist of Data Processing, which defines the parameters used for peptide identification and quantification, and Targets, which specifies the peptides containing CQAs of interest. As shown in Figure 4, LC/MS characteristics of validated CQA peptides (including retention time, predominant charge state, and monoisotopic mass) were imported as target attributes for analysis. These parameters enabled automated extraction and quantification of the corresponding extracted ion chromatogram (EIC) peak areas using the streamlined, web-based MAM for OpenLab CDS. This targeted approach facilitated efficient and reliable MS-based quantification of predefined CQAs while simplifying data analysis for routine QC applications.

MAM for OpenLab CDS offered a user-friendly interface for CQA monitoring. The software enabled straightforward definition and grouping of modified peptides with their corresponding unmodified forms, facilitating efficient method setup and data processing. CQA quantification was performed using the EIC peak areas of the relevant peptides, and the relative abundance of each modification was calculated according to the following equation:

%Modification =
$$\frac{\text{Area of modified peptide}}{\text{Area of unmodified peptide} + \text{Area of modified peptide}} \times 100\%$$

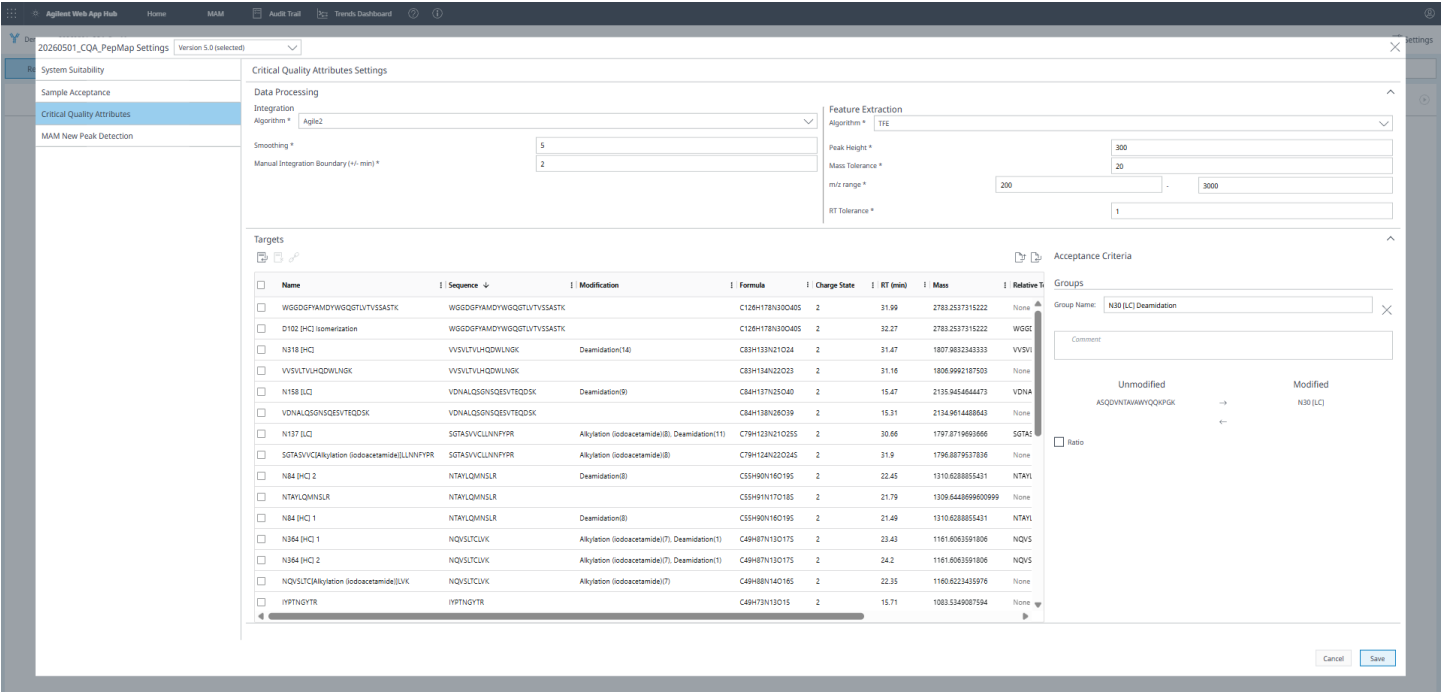


Figure 4. User interface of Agilent MAM for OpenLab CDS for configuring parameters used in CQA monitoring, including data processing settings and target peptide definitions.

CQA monitoring results are presented in Figure 5. For each monitored CQA, the corresponding peptide EICs and integrated peak areas were displayed to facilitate quantitative analysis. In addition, the MS spectrum from the apex scan of the selected peak provided confirmation of the target peptide at the specified charge state. Quantitative results for all monitored CQAs were summarized in a tabulated format and could be exported directly for further analysis.

It is noteworthy that MAM for OpenLab CDS supports complex quantification strategies for multiple CQAs occurring within the same peptide. For instance, peptide DTLMISR in trastuzumab heavy-chain residues 252 to 258 contains two hotspots for CQAs: aspartic acid (D) 252, which is susceptible to isomerization, and methionine (M) 255, which is prone

to oxidation. To enable independent quantification of these attributes, two separate target groups were defined using three related peptide species: the unmodified peptide, the isomerized peptide, and the oxidized peptide.

Figure 6 depicts software settings together with the resulting data, which demonstrate the successful monitoring of both CQAs. All peptide species were correctly identified and integrated, and the relative abundance of each modification was calculated independently based on the D252 isomerization and M255 oxidation target groups. Moreover, the software allowed quantitative results to be automatically compared against predefined acceptance criteria or threshold values, facilitating efficient assessment of product quality and compliance with established specifications.

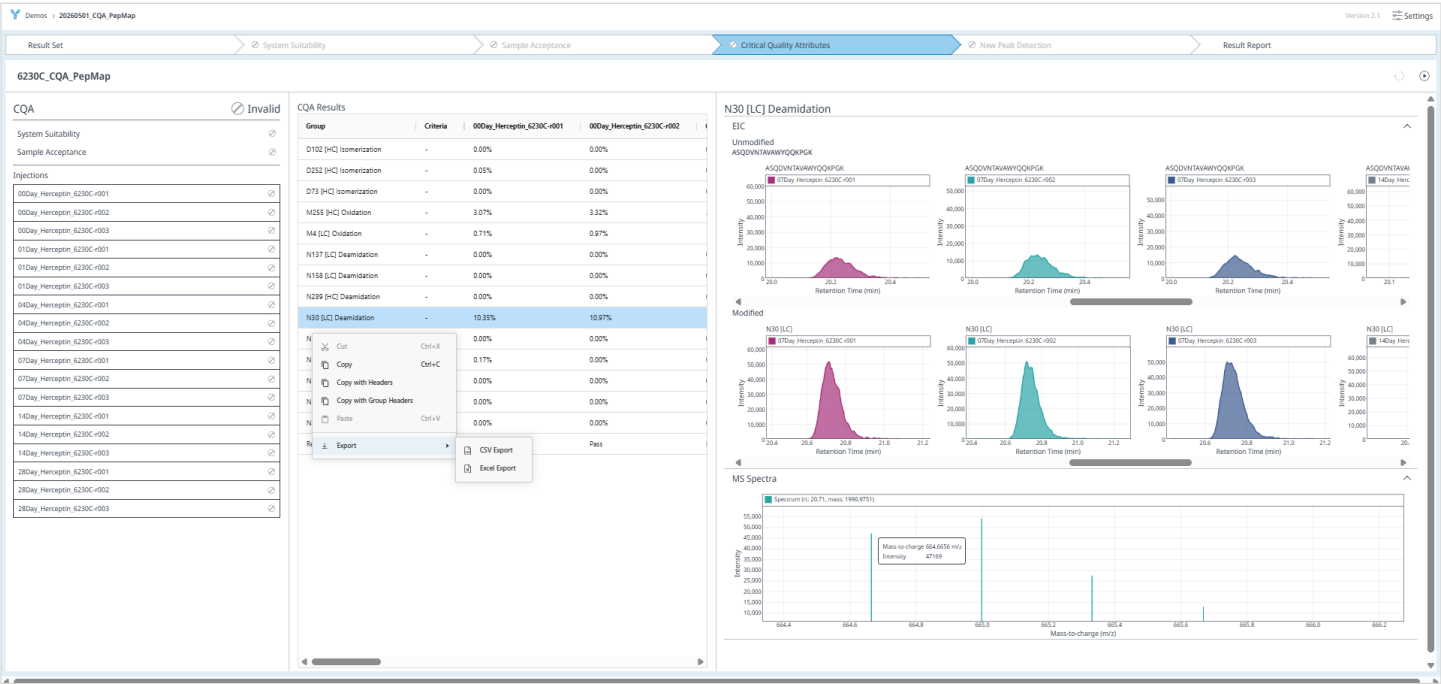


Figure 5. Results interface in Agilent MAM for OpenLab CDS for CQA monitoring, including EICs with integrated peaks, apex-scan mass spectra, and quantitative results.

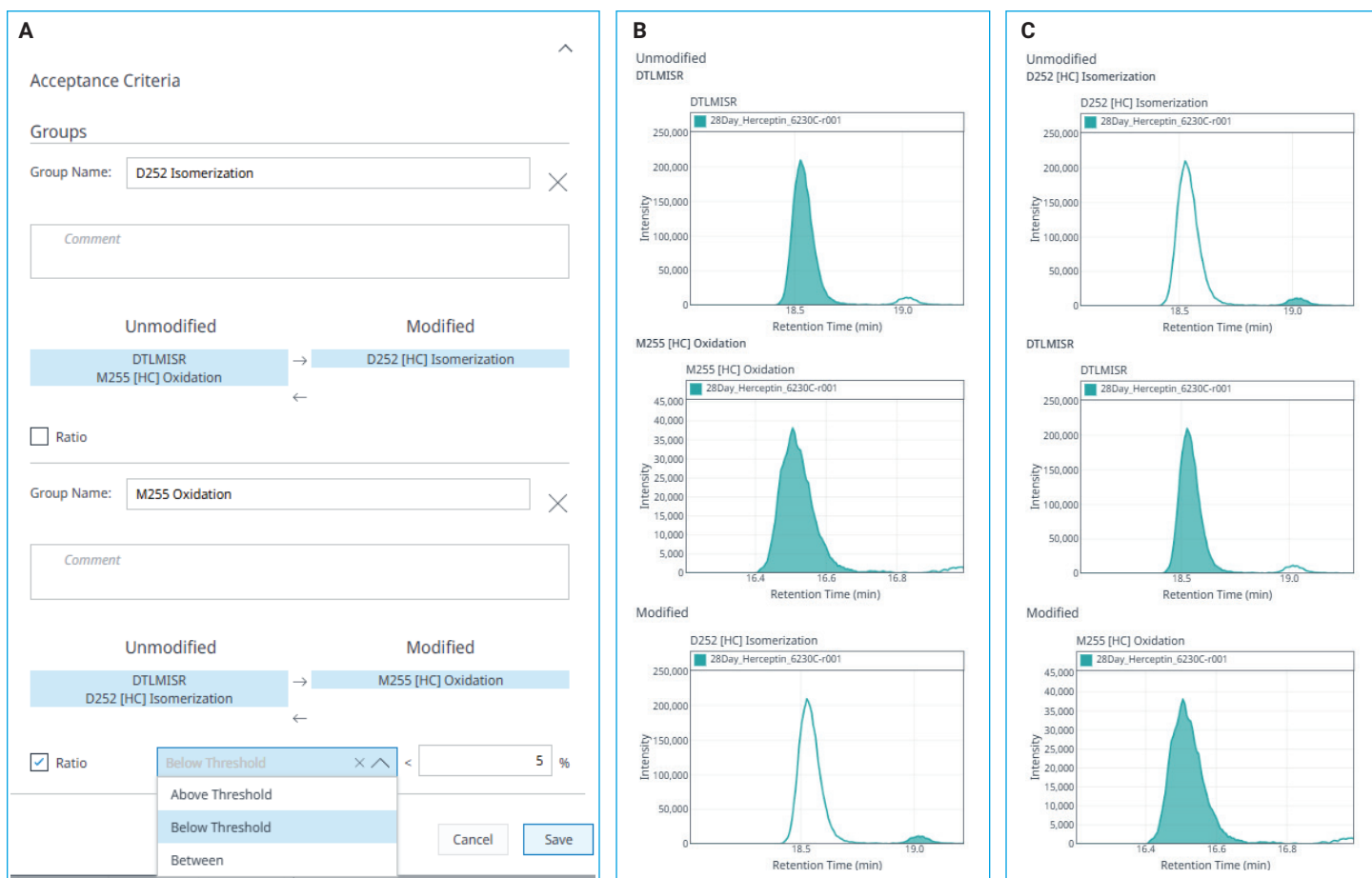


Figure 6. Setting and results of the monitored CQAs on peptide DTLMISR in Agilent MAM for OpenLab CDS: (A) Setting for D252 isomerization and M255 oxidation in separated groups; (B) EIC results of peptides for D252 isomerization; (C) EIC results of peptides for M255 oxidation.

Trend analysis for CQA monitoring

Quantification of the monitored CQAs exhibited acceptable reproducibility across three replicates and spanned several orders of magnitude in relative abundance. These results demonstrated the robustness and reliability of the MAM workflow for routine CQA monitoring. The quantitative results for the monitored CQAs are listed in Table 6.

Table 6. Results of selected CQAs for monitoring.

CQA	0	1	4	7	14	28
N55 [HC] Deamidation	0.10	0.48	2.30	16.03	46.93	71.89
N84 [HC] Deamidation	0.00	0.00	3.19	5.18	11.67	21.81
D102 [HC] Isomerization	0.00	13.24	15.37	20.29	28.16	33.62
D252 [HC] Isomerization	0.03	0.04	0.60	0.99	2.20	3.96
M255 [HC] Oxidation	3.31	3.12	3.81	4.81	7.71	14.08
N289 [HC] Deamidation	0.00	0.00	0.00	0.00	2.48	5.14
N318 [HC] Deamidation	0.00	0.00	0.00	0.00	0.00	0.16
N364 [HC] Deamidation	0.15	0.36	0.56	0.58	1.36	2.88
N30 [LC] Deamidation	10.69	27.88	65.14	79.38	98.20	100.00
N158 [LC] Deamidation	0.00	0.00	0.00	0.32	1.99	3.93

Figure 7 presents a bar chart of individual hotspots for selected CQAs. All monitored CQAs, including deamidation, oxidation, and isomerization, showed progressive increases over time, consistent with the expected degradation behavior of stressed trastuzumab samples. Notably, N55 [HC] deamidation, D102 [HC] isomerization, and N30 [LC] deamidation presented more rapid increases than the other monitored CQAs, indicating that these residues are particularly susceptible to stress-induced modification. These results demonstrated the ability of the MAM workflow to sensitively track the formation and progression of individual CQA hotspots, providing valuable insights into product stability and degradation pathways.

In addition to monitoring individual CQA hotspots, related CQAs can be grouped to provide an overall assessment of specific modification types. As shown in Figure 8, selected deamidation sites were combined to generate an aggregate deamidation profile for trastuzumab across multiple stress-study time points. The grouped analysis exhibited a trend consistent with that observed for the individual deamidation hotspots, showing a gradual increase in overall deamidation over time. This capability enabled both site-specific and global monitoring of CQAs, providing a comprehensive view of product quality and facilitating routine implementation of MAM in QC environments.

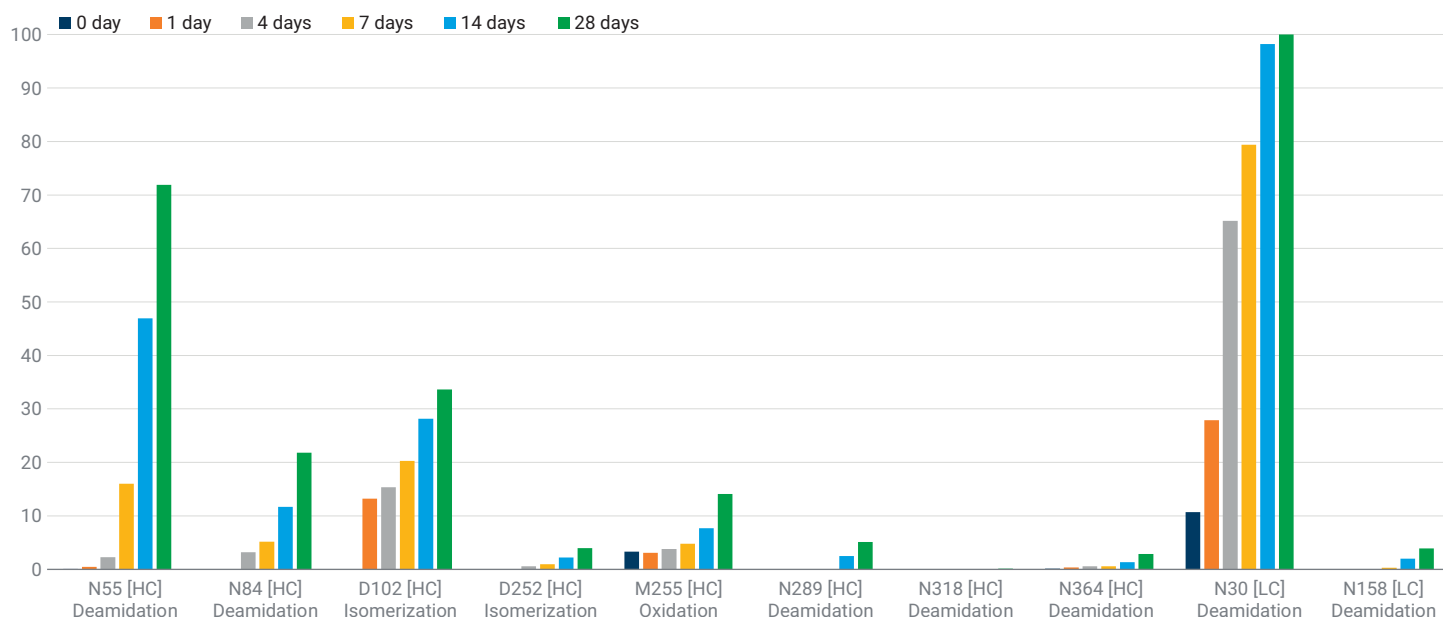


Figure 7. Trend analysis of individual CQA hotspots for trastuzumab monitored across multiple sampling time points during the stress study.

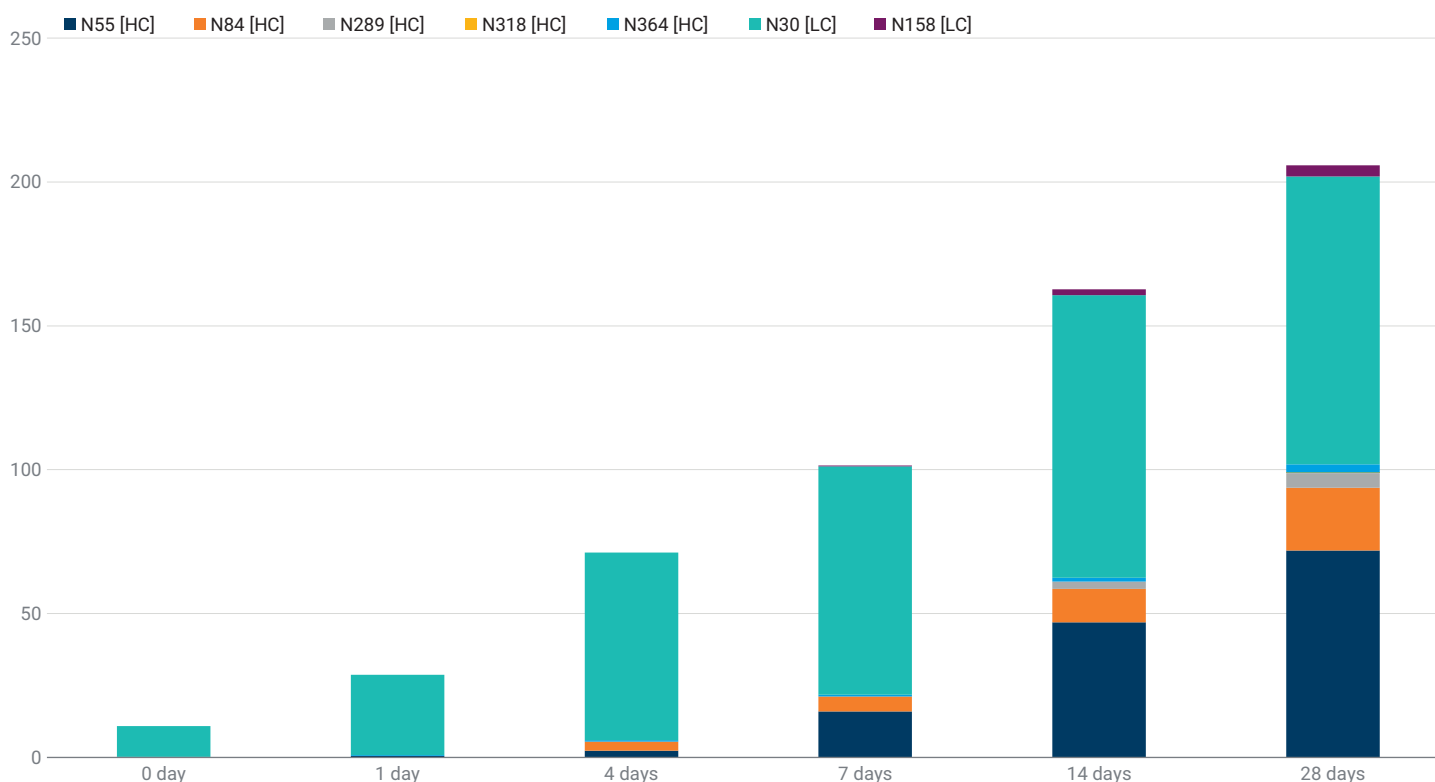


Figure 8. Trend analysis of overall deamidation for trastuzumab across multiple stress-study time points. Quantitative results from selected deamidation sites were combined to enable global monitoring of deamidation and assessment of its progression over time.

Conclusion

Trastuzumab was subjected to heat and agitation stress to induce product-related modifications, and the resulting CQAs were identified and validated using LC/MS/MS on the Agilent 6545XT AdvanceBio LC/Q-TOF system. Following method development and validation, the MAM was successfully transferred to an LC/MS platform for routine QC monitoring using the Agilent 6230C TOF LC/MS system.

Agilent MAM for OpenLab CDS enabled streamlined and efficient analysis for CQA monitoring through a user-friendly, web-based interface. The software supported data processing, automated quantification, and flexible CQA reporting under regulatory compliance guidelines, providing a practical solution for implementing MAM in biopharma QC environments.

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