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A User-Friendly, Regulatory-Compliant Multi-Attribute Method Workflow for Monitoring Critical Quality Attributes and Enabling Method Transfer

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Introduction

MAM for CQA Monitoring

The rapid growth of the pharmaceutical industry has positioned biotherapeutics as a leading sector, with monoclonal antibodies (mAbs) representing a major class due to their broad therapeutic applications. As these complex molecules advance through development and manufacturing, monitoring critical quality attributes (CQAs) is essential to ensure safety, efficacy, and performance. Conventional analytical approaches often rely on multiple orthogonal methods, which can be time-consuming and labor-intensive. In contrast, multi-attribute method (MAM) offers a powerful alternative for comprehensive CQA characterization and monitoring. Despite recent efforts in deploying MAM in the regulatory environment for quality control (QC) purposes, it remains challenging to address issues that are caused by the complexity of the method. It is important to develop a MAM workflow to monitor CQAs comprehensively with accuracy, precision, and robustness, with compliant software streamlined with user-friendly modules.

Method Development and Transfer for MAM

MAM is developed with MSMS to determine and validate CQAs for mAb characterization (Phase I), then it can be transferred to QC for monitoring (Phase II).[1] Because CQA monitoring does not need MSMS data for quantitation, so the data can be acquired with MS only mode after MAM is transferred.

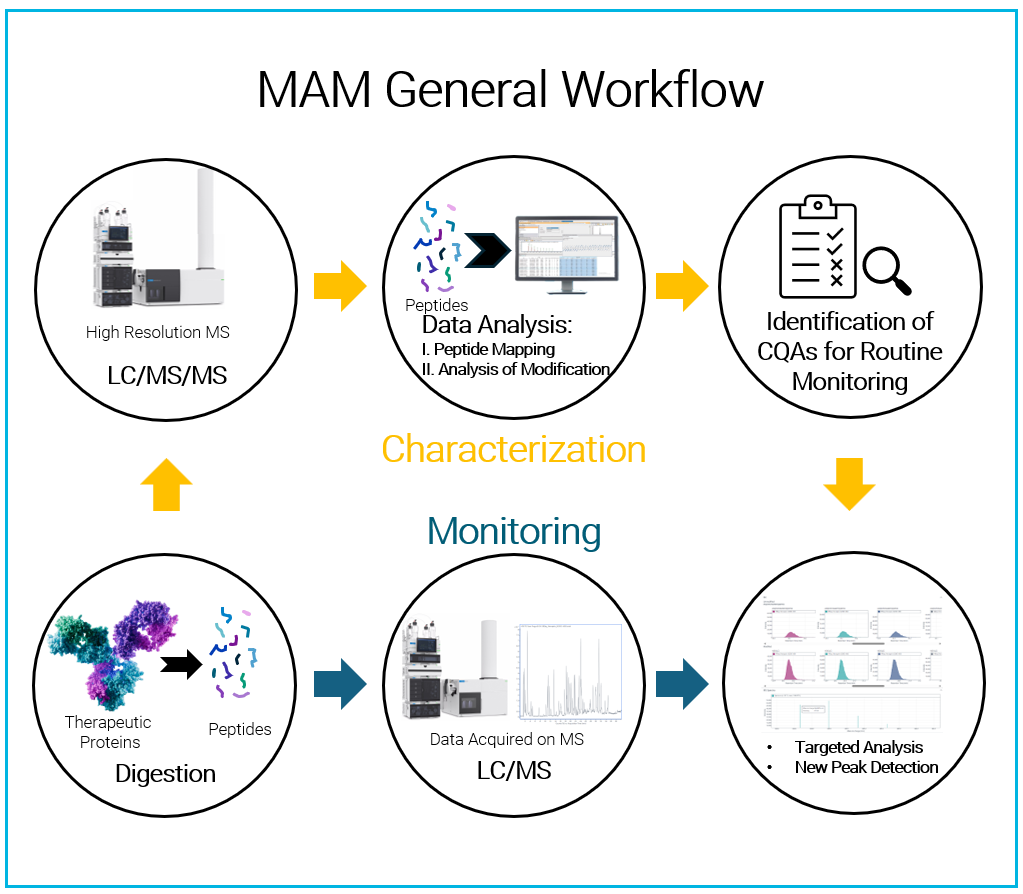

Figure 1. MAM workflow, including characterization (orange arrows) and monitoring (blue arrows).

Experimental

Preparation of Stressed Samples

Herceptin (Trastuzumab) was stressed with heat and agitation to stimulate the molecule for increasing modification, and the mAb samples were collected at multiple time points to monitor CQAs: Herceptin was diluted to 5 mg/mL with 50 mM Tris-HCl (pH 7.0), and stressed at 40 C and 300 RPM under light-protected conditions; samples were collected at 0, 1, 4, 7, 14, and 28 days for CQA monitoring.

Sample Preparation for MAM

Herceptin samples were denatured by guanidine and reduced by dithiothreitol, followed by iodoacetamide alkylation; then buffer exchanged to 50 mM Tris-HCl (pH 7.0) using Agilent AdvanceBio spin columns, and digested by trypsin, and finally quenched by formic acid. The sample preparation conditions were optimized to minimize artificial modifications.

Data Acquisition and Analysis for MAM

LC-MS/MS analysis was conducted on the Agilent Bio-inert system: an Agilent 1290 Infinity III BioLC and an Agilent 6545XT AdvanceBio LC/Q-TOF system; data was acquired by Agilent pre-release software OpenLab CDS 3.0, and results were analyzed by pre-release software Agilent BioConfirm 14.1 to confirm CQAs.

LC-MS analysis was performed on the Agilent Bio-inert system: an Agilent 1290 Infinity III BioLC and a pre-release Agilent 6230C LC/TOF system; data was acquired by Agilent pre-release software OpenLab CDS 3.0, and the results were analyzed to monitor CQAs by pre-release software Agilent MAM Analysis Workflow 1.0. Results of selected CQAs were exported for trend analysis in Microsoft Excel.

Table 1. Instrument parameters for LC-MS analysis

Agilent 1290 Infinity III BioLC		
Column	Agilent AdvanceBio Peptide Map column, 2.1 x 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

6545XT AdvanceBio LC/Q-TOF (Auto MS/MS) and 6230C TOF (MS Only)			
Polarity	Positive	MS1 Range	200 to 3,000
Gas Temperature	350 °C	Data Storage	Both
Sheath Gas Temperature	375 °C	MS1 Acquisition Rate	3 spectra/sec
Drying Gas Flow	13 L/min	Sheath Gas Flow	12 L/min
Nebulizer	35 psi	Fragmentor	45 V
Skimmer	35 V	Nozzle Voltage	0 V
Capillary Voltage	3,750 V	Reference Mass	322.0481, 922.0098
MS/MS Range	100 to 3,000	Precursor Charge	2+, 3+, > 3+
Isolation Width	Medium (m/z ~ 4)	MSMS Acquisition Rate	3 spectra/sec
Precursors/ Cycle	Top 5	Collision Energy	3.6*(m/z)/100-4.8
Release after	0.2 min	Excluded after	3 spectra
Threshold for MS/MS	6,000 counts and 0.001%	Target	50,000 counts/spectrum

Results and Discussion

CQA Identification and Method Validation

It is critical to ensure the method is capable of successful CQA monitoring, so it is required to identify the CQAs and validate the method. The Auto MS/MS mode in Agilent 6545XT provides sufficient fragmentation on identified peptides, so that the peptides can be verified, and possible modifications in CQAs will be verified and localized.

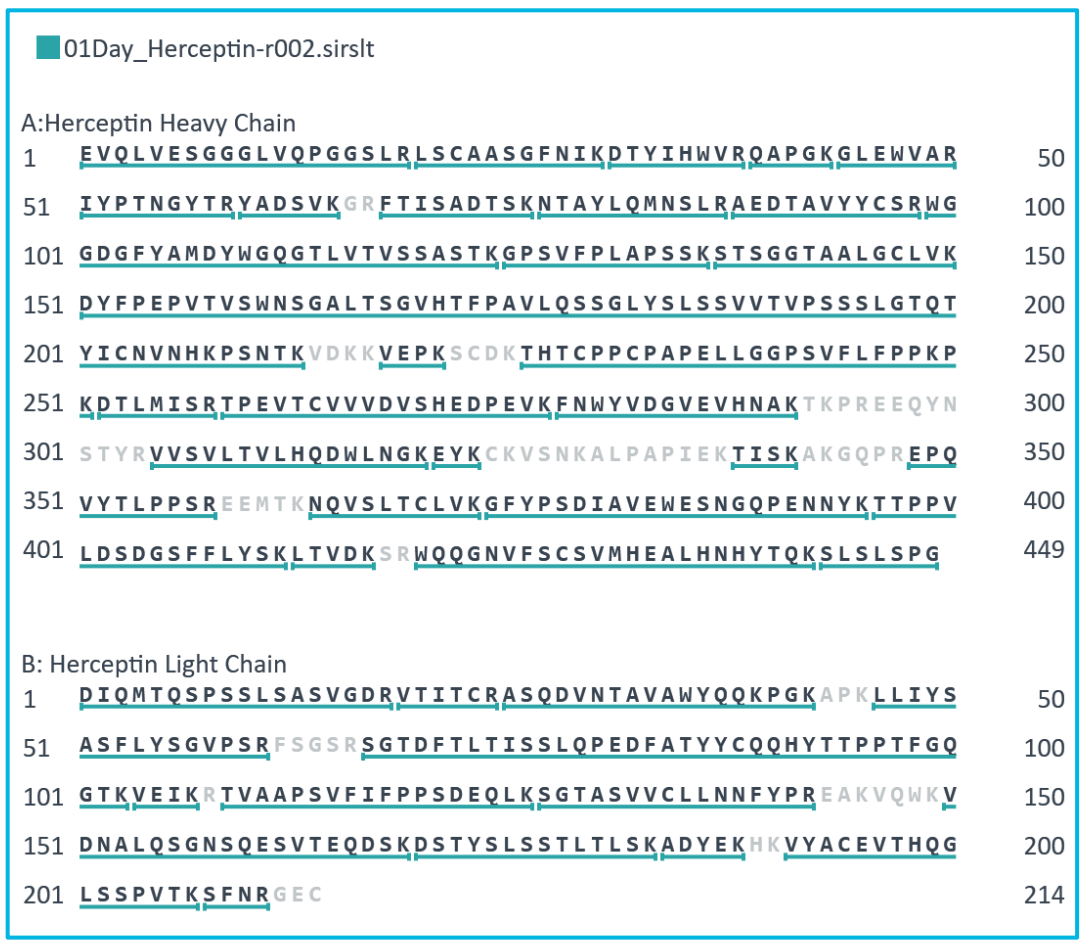

Figure 2. A representative sequence coverage map for Herceptin.

As shown in Figure 2, the sequence coverage map presented 89.29% confirmation for combined heavy and light chains. All critical sequences including complementarity determining regions (CDRs) were fully covered.

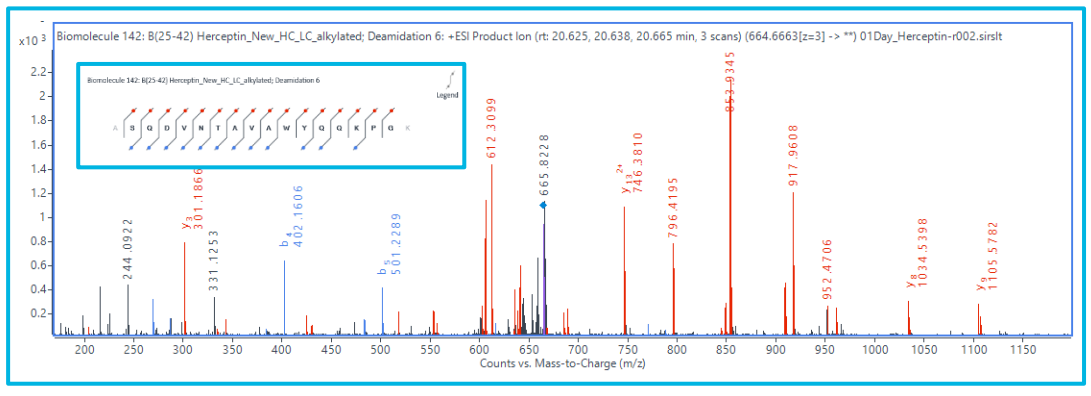

Figure 3. A typical MS/MS fragment coverage for peptide (Herceptin light chain 25-42) with confirmed location of the deamidation modification.

A comprehensive fragmentation map was displayed in Figure 3 for an identified peptide containing CQA. The fragment ions were able to locate the deamidation modification at Asparagine 6, distinguishing from other possible deamidation sites in the peptide.

CQA Verification and Analysis

CQAs were defined by comparing the quantitative trend over time, and with help of MS/MS, peptides with possible modifications of CQAs were identified. The MAM workflow was developed and validated for robustness, so that MS-only data could be used in quantification for CQA monitoring.

After the method was validated, MAM Analysis Workflow was used for monitoring the defined CQAs of Herceptin. Table 2 summarized the CQAs validated by MS/MS.

Table 2. Summary of Monitored Herceptin 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	WGGDGFYAMDYWGQGTLVTVSSASTK
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 Analysis Workflow 1.0

As shown in Figure 4, LCMS attributes of validated CQAs (including retention time, most abundant charge state, and corresponding monoisotopic mass) were used to quantify extracted ion chromatogram (EIC) peak area for MS quantification in streamlined, web-based MAM Analysis Workflow 1.0.

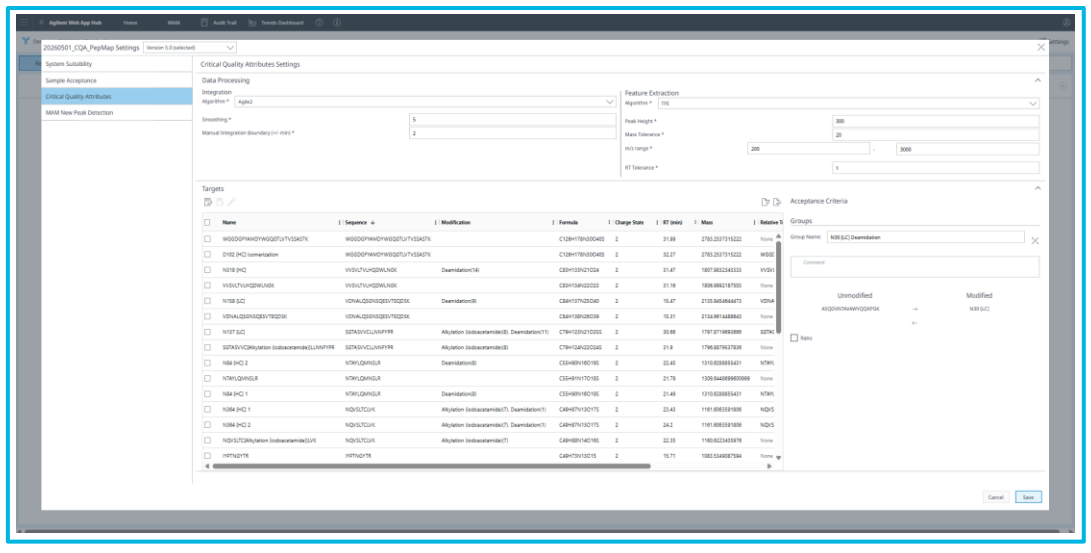

Figure 4. The interface of parameter settings for CQA monitoring in MAM Analysis Workflow 1.0.

MAM Analysis Workflow 1.0 offered a user-friendly interface to monitor CQAs with regulatory-compliance. The quantitation was based on the EIC peak area of peptides containing CQAs and calculated with equation:

$$\%\text{Modification} = \frac{\text{Area of Modified Peptide}}{\text{Area of Unmodified Peptide} + \text{Area of Modified Peptide}} \times 100\%$$

Results and Discussion

CQA monitoring results were presented in Figure 5: related peptides of respective CQAs showed EICs and integrated peak area; the MS spectrum of apex scan was available to show the peptide of interest at defined charge state for selected peak.

The results of CQA monitoring were displayed in the table, and the results could be exported for further analysis.

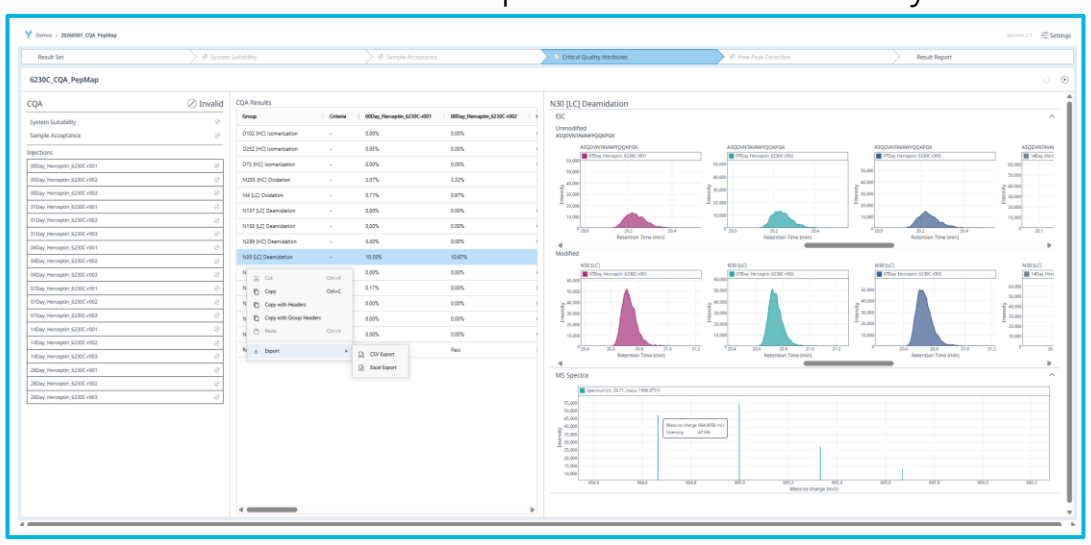

Figure 5. The interface of results for CQA monitoring in MAM Analysis Workflow 1.0.

Trend Analysis for CQA Monitoring

The CQA quantification showed acceptable reproducibility across several orders of magnitude on three replicates, and the results for CQA monitoring were listed in Table 3.

Table 3. Results of Selected CQA 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

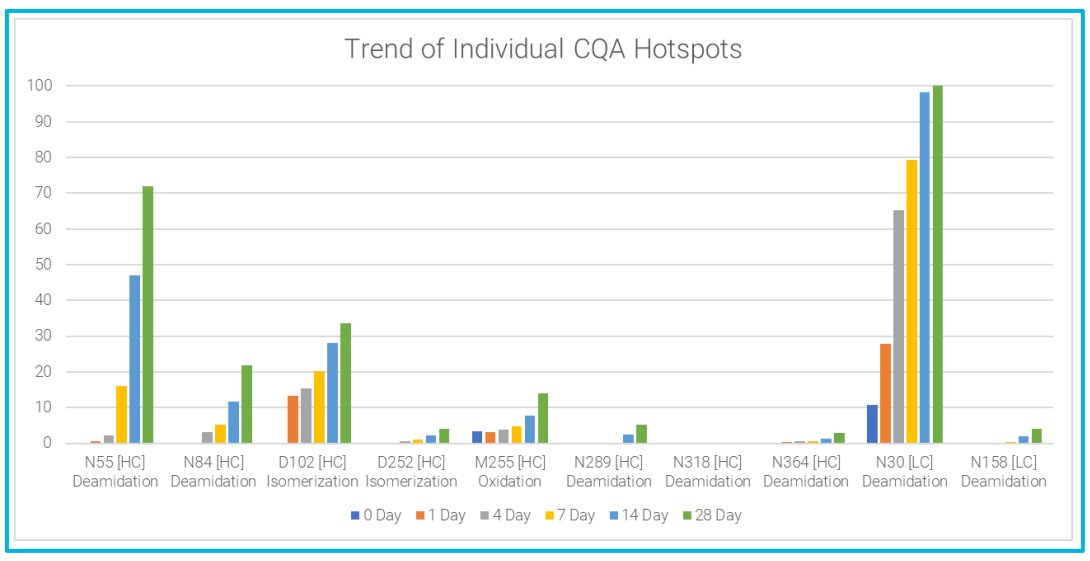

Figure 6. Trend analysis of individual CQA hotspots for Herceptin across multiple timepoints.

Figure 6 depicted a bar chart of individual hotspots for selected CQAs. All CQA hotspots increased with time, including deamidation, oxidation, and isomerization. This observation agreed with the expected trend of stressed Herceptin samples. N55 [HC] deamidation, D102 [HC] isomerization, and N30 [LC] deamidation presented a relatively rapid growth in comparison to other CQAs, indicating susceptible sites of Herceptin molecules.

Besides monitoring individual hotspots, selected CQAs could be grouped to overview the combined quantitation of related CQAs. As shown in Figure 7, an example of overall deamidation same type of trend of deamidation for Herceptin was displayed with various timepoints. This empowers the site-specific CQA monitoring for QC.

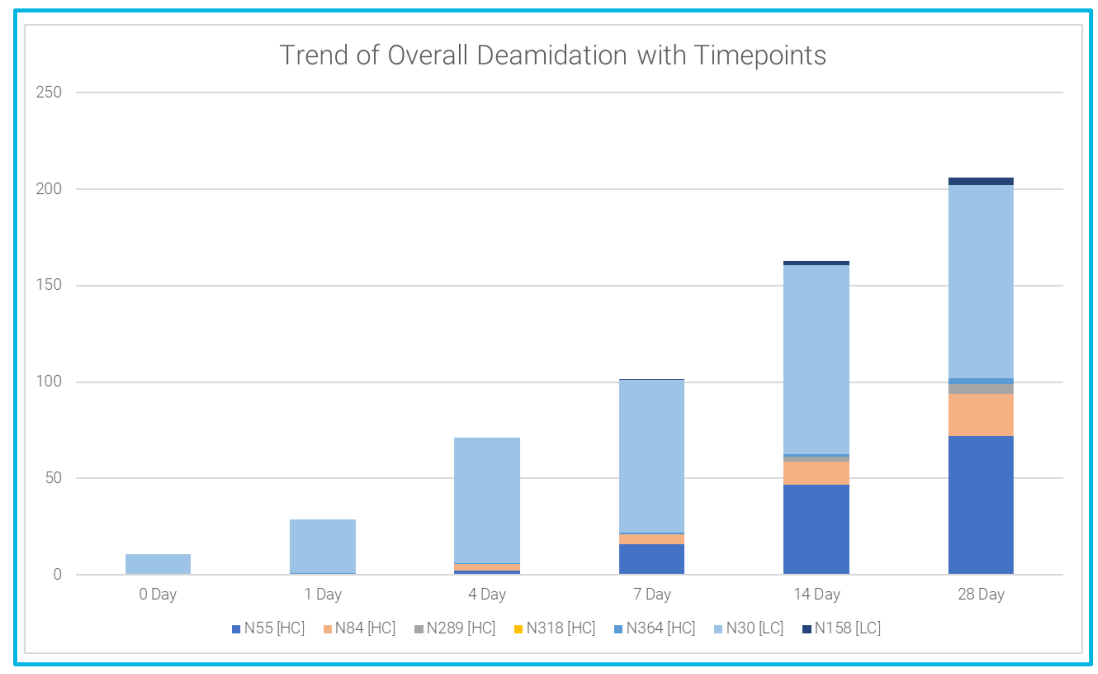

Figure 7. Trend analysis of overall deamidation for Herceptin at various timepoints.

Conclusions

Herceptin was stressed under heat and agitation conditions, and the CQAs were validated by LC-MS/MS on Agilent 6545XT Q-TOF; the validated MAM was transferred to LC-MS for QC monitoring on pre-release Agilent 6230C TOF.

Pre-release software Agilent MAM Analysis Workflow 1.0 demonstrated streamlined analysis for CQA monitoring, and offered a user-friendly and regulatory-compliant solution for MAM under QC environment.

References

[1]United States Pharmacopeial Convention, USP General Chapter <1060> Analytical Data—Interpretation and Treatment (Rockville, MD: USP, 2024), 43–R1.

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