Thermo Scientific HR Multi-Attribute Method Workflow for Critical Quality Attribute Monitoring and New Peak Detection

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ABSTRACT

Here we present Thermo Scientific™ HR Multi-Attribute Method (MAM) Workflow for biopharmaceutical product quality attribute (PQA) characterization, critical quality attribute (CQA) monitoring, and new peak detection (NPD). We introduce a system suitability test (SST) we developed to verify the LC and MS performance for peptide mapping. We demonstrate the importance of high resolution accurate mass (HRAM) in CQA quantitation and NPD and highlight unique features of Thermo Scientific™ BioPharma Finder™ Software and Thermo Scientific Chromeleon™ Chromatography Data System (CDS) for HR MAM. We describe the application of HR MAM to monitor selected CQAs of the NISTmAb reference standard under normal and stressed conditions. Finally, we showcase the capability of HR MAM for NPD in spiked and stressed samples.

INTRODUCTION

In accordance with Quality by Design (QbD) principles outlined by regulatory agencies, it is essential for the biopharmaceutical industry to identify, quantify, and monitor potential CQAs and impurities of protein therapeutics during process development and lot release.

In 2015, Rogers *et al*.[1] developed the MAM, taking advantage of the HRAM capabilities of Orbitrap™-based MS detection for simultaneous identification, quantitation, and monitoring of PQAs of two antibodies, MAb1 (IgG1) and anti-streptavidin IgG2. It was also demonstrated that MAM is well suited for NPD enabling new features ("impurities") to be identified when comparing to a reference [2]. It was proposed that MAM could replace several conventional methods used in quality control (QC) for lot release of drugs. Since MAM was introduced, it has gained popularity and acceptance in the biopharma industry, featuring as a hot topic in many recent conferences.

MATERIALS AND METHODS

System Suitability Test

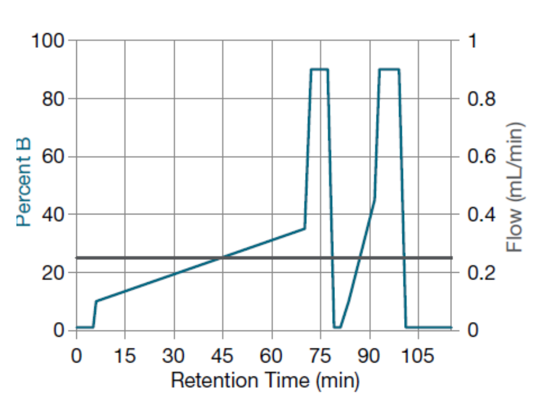
To verify the performance of LC and MS for HR MAM, a system suitability test (SST) was developed using Thermo Scientific™ Pierce™ BSA Protein Digest. The SST consists of 1 MS/MS run and 10 technical replicates of full scan MS runs. For each SST, 5 pmol of BSA digest was injected per run using a 35 min LC gradient (60 min total run time). The LC-MS methods, view template, processing method, and report template, as well as Chromeleon eWorkflow have been pre-built for easy execution of the SST and quick review of the Pass/Fail result.

Sample Preparation for NISTmAb Analysis

Trypsin digestion on NISTmAb (NIST, Gaithersburg, MD) was performed following the published protocol [3]. Briefly, NISTmAb standard was denatured, reduced, and alkylated. Subsequently, the sample was buffer exchanged and digested using Thermo Scientific™ Pierce™ trypsin in a 1:10 ratio. For each LC-MS analysis, 4 µL of the sample (~3 µg NISTmAb peptides) was loaded on-column. The thermally stressed sample was prepared by incubating NISTmAb in a ThermoMixer (Eppendorf) at 60°C for 120 hours, while oxidative stress was induced using 0.03% hydrogen peroxide at room temperature for 24 hours. To demonstrate the capability of MAM for NPD, Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture (PRTC) was spiked into the digested NISTmAb to yield 20 pmol NISTmAb to 0.5 pmol PRTC for each 4 µL sample injection.

LC and MS Methods for NISTmAb Analysis

Peptide separations were performed using a Thermo Scientific™ Accucore™ Vanquish™ C18+ UHPLC column (1.5 µm, 2.1 × 150 mm) using a Thermo Scientific™ Vanquish™ Horizon UHPLC system. The LC gradient used in this study is shown in Figure 1.



Time	% B
0	1
5	1
6	10
70	35
72	90
77	90
79	1
81	1
83.5	10
91.5	45
93	90
99	90
101	1
115	1

Figure 1. LC gradient for separation of NISTmAb peptides. The run consists of a 64 min linear gradient (6 min–70 min) and two washing steps. The total run time is 115 min.

The Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer was operated in standard pressure mode. Data were acquired using Chromeleon CDS software (Version 7.2.10). The data for peptide mapping were acquired with a Top5 data-dependent MS2 (ddMS2) method and processed in BioPharma Finder Software Version 3.1. Full scan data for CQA quantitation and NPD were acquired using a Full Scan MS only method and processed in Chromeleon CDS. A resolution setting of 140,000 (at *m/z* 200) was used to resolve overlapping peaks and to provide more accurate quantitation for deamidated peptides.

RESULTS

Acceptance Criteria for SST

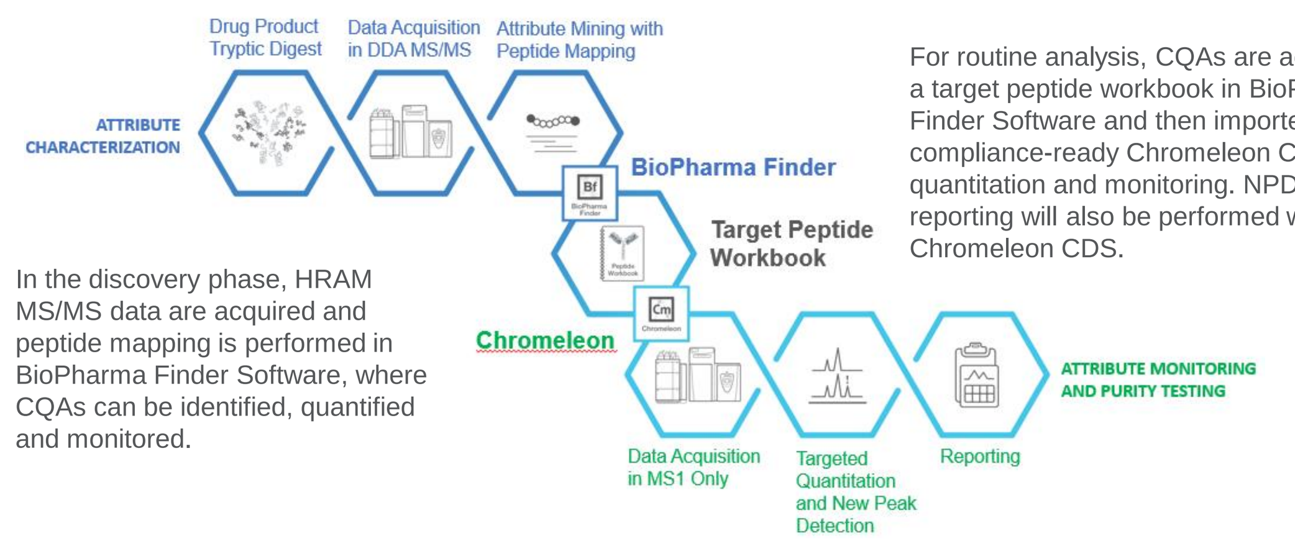
Nine BSA peptides covering the whole retention time (RT) range were selected for a general performance check, based on the data from BSA digests from seven different lots. The passing criteria for these nine peptides include %CV for peak area, RT, and peak width as well as absolute peak width and mass accuracy. The minimum peak areas of two BSA peptides were used to assess sensitivity. In addition, the %CV for the abundance of deamidation was calculated to check consistency in peak integration and quantitation. The degree of in-source fragmentation was also evaluated by measuring the ratio of integrated peak area of a gas phase fragment to its precursor.

Figure 2. Summary tables of SST in the prebuilt Chromeleon report showing the Pass/Fail results of peak area, retention time, peak width, and mass accuracy for nine BSA peptides (top) as well as the results of deamidation and in-source fragmentation measurements (bottom)

Peptides	Peak Area (Avg.)	%CV (Area)	Minimum Peak Area	Avg. RT (min)	%CV (RT)	Avg. FWHM (s)	%CV (FWHM)	Min. Mass Error (ppm)	Max. Mass Error (ppm)	Pass or Fail
1	1.07E+09	2.09%	1.02E+09	5.552	0.26%	1.732	2.84%	-1.90	-0.88	Pass
2	1.19E+09	2.79%	1.13E+09	6.939	0.26%	2.204	2.19%	-1.66	-0.34	Pass
3	3.38E+08	1.16%	3.33E+08	8.746	0.25%	2.258	2.28%	-0.26	0.69	Pass
4	1.43E+09	2.08%	1.37E+09	10.350	0.11%	2.754	3.76%	-1.43	-0.77	Pass
5	2.27E+09	1.80%	2.20E+09	13.434	0.17%	3.155	4.72%	-0.57	0.08	Pass
6	2.22E+09	2.63%	2.11E+09	15.558	0.11%	3.603	2.51%	-0.03	0.53	Pass
7	3.64E+08	1.69%	3.54E+08	16.465	0.08%	2.746	0.95%	-0.46	0.07	Pass
8	9.32E+08	1.83%	9.04E+08	20.225	0.05%	2.976	2.06%	-0.31	1.13	Pass
9	9.00E+08	1.12%	8.84E+08	23.733	0.11%	3.319	2.50%	-1.40	-0.43	Pass
		Pass	Pass		Pass	Pass	Pass	Pass	Pass	

Description	%Mod. (Avg.)	%CV (Mod.)	Pass or Fail
Deamidation	1.93	1.21%	Pass
Gas Phase y5	0.65	1.68%	Pass

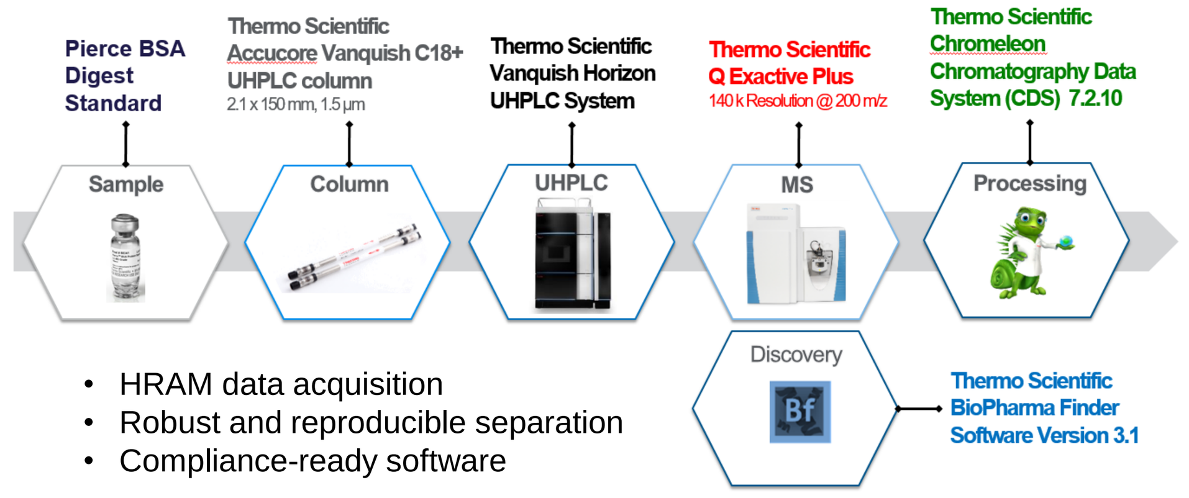
Figure 3. Illustration of the HR MAM workflow


Drug Product Tryptic Digest
Data Acquisition in DDA MS/MS
Attribute Mining with Peptide Mapping
ATTRIBUTE CHARACTERIZATION
BioPharma Finder
Target Peptide Workbook
Chromeleon
Data Acquisition in MS1 Only
Targeted Quantitation and New Peak Detection
Reporting
ATTRIBUTE MONITORING AND PURITY TESTING

In the discovery phase, HRAM MS/MS data are acquired and peptide mapping is performed in BioPharma Finder Software, where CQAs can be identified, quantified and monitored.

For routine analysis, CQAs are added to a target peptide workbook in BioPharma Finder Software and then imported into compliance-ready Chromeleon CDS for quantitation and monitoring. NPD and reporting will also be performed with Chromeleon CDS.

Figure 4. Components of HR MAM


Pierce BSA Digest Standard
Thermo Scientific Accucore Vanquish C18+ UHPLC column
2.1 x 150 mm, 1.5 µm
Thermo Scientific Vanquish Horizon UHPLC System
Thermo Scientific Q Exactive Plus
140 k Resolution @ 200 m/z
Thermo Scientific Chromeleon Chromatography Data System (CDS) 7.2.10
Sample
Column
UHPLC
MS
Processing
Discovery
Thermo Scientific BioPharma Finder Software Version 3.1

- HRAM data acquisition
- Robust and reproducible separation
- Compliance-ready software

Figure 5. Monitoring digestion efficiency and ratio of modifications in BioPharma Finder Software

Sample	PeptideMapQuality
Control	0.962904
Thermal	0.939886
Oxidative	0.967029

PeptideMapQuality is the ratio of perfectly digested peptides to all peptides. It represents a measure of the quality of the digestion. A value of 1 indicates that the peptides in the sample are neither under-digested nor over-digested.

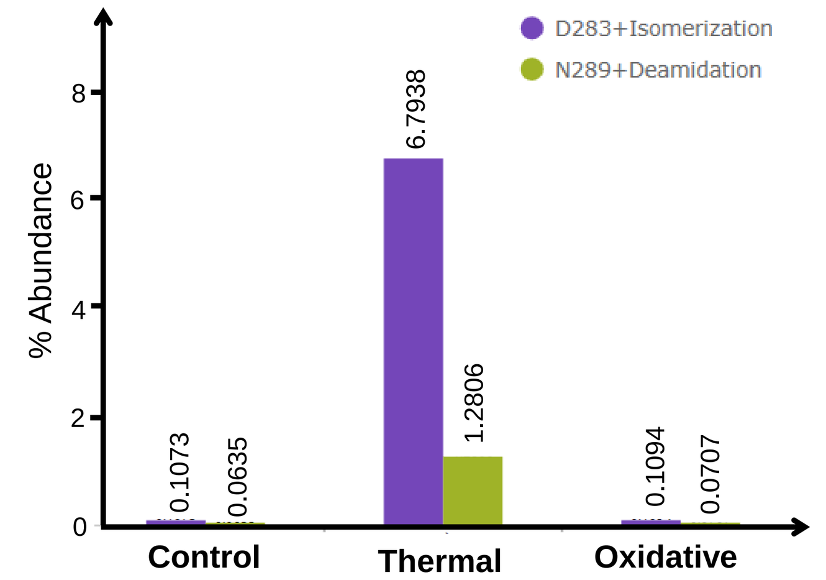
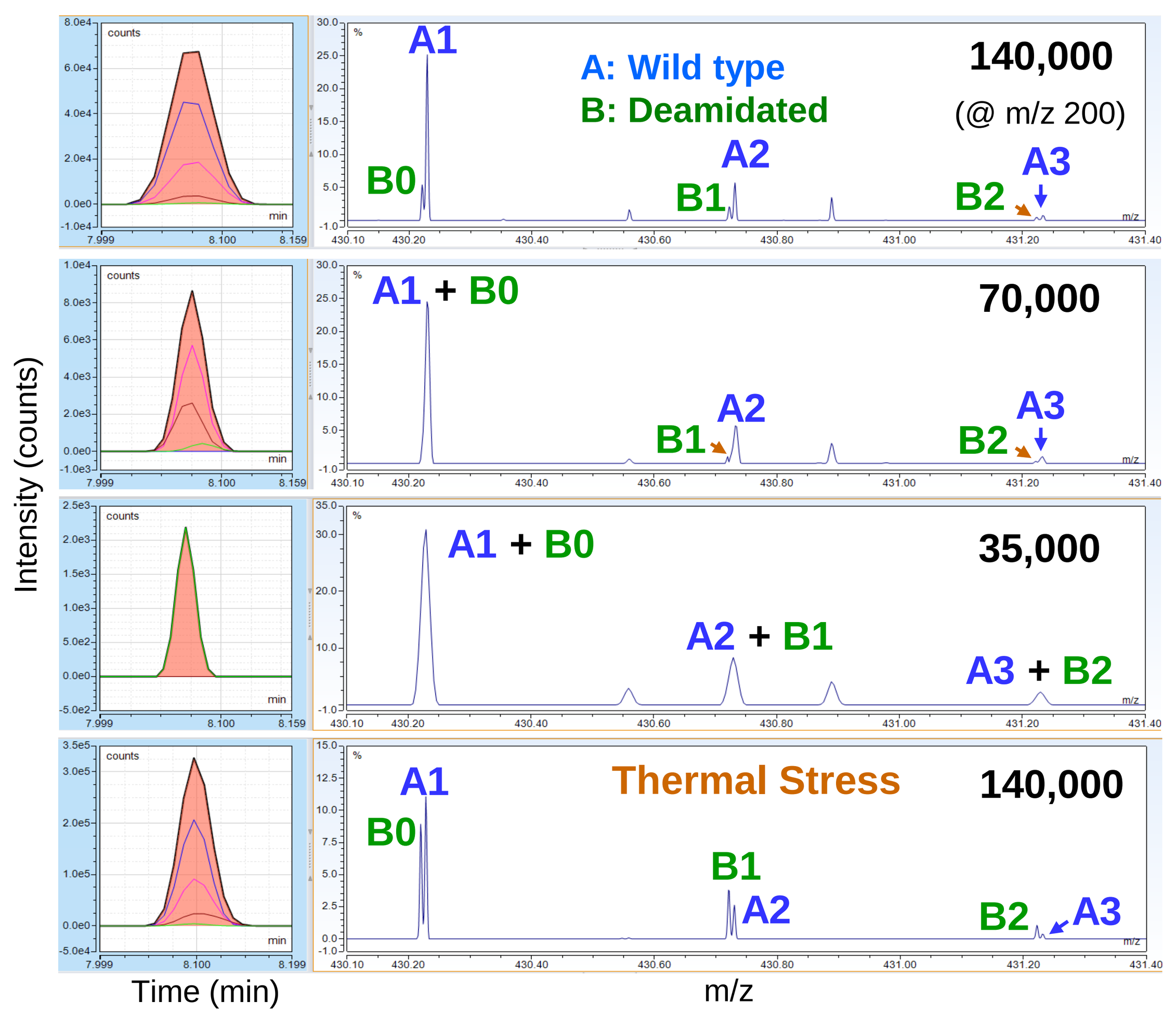


Figure 6. Use of Time Distance and Component Match for more robust and consistent peak integration in Chromeleon CDS


Retention Time: 1.350 min
Use this component as reference component
Interpretation
Absolute Time
Time Distance
Time Ratio
Component Match
Algorithm: Retention Time
The peak closest to the expected retention time (Nearest)
The 1st peak in the window (First)
The 2nd peak in the window
The 3rd peak in the window
The 4th peak in the window
+1.35 min 1st Peak
+1.35 min 2nd Peak
+1.35 min 3rd Peak
+1.35 min 4th Peak
Intensity (counts)
Time (min)

Unlike conventional targeted quantitation software, the extraction parameters for each peptide including RT and integration window can be set *a priori* in Chromeleon CDS. Shown above is an example of four partially overlapping peaks integrated using the Time Distance and Component Match features.

Figure 7. Importance of high resolution



HYNPSLK (component A) and HYN[Deamidation]PSLK (component B) cannot be chromatographically separated, nor can they be resolved in the mass spectra at resolution settings lower than 140,000 (at m/z 200). Therefore, component B would not be identified as a 'new peak' in the thermally stressed sample at resolution settings <140,000 (at m/z 200) due to peak overlapping. By comparison, the same two components can be nearly baseline resolved at resolution setting of 140,000 (at m/z 200), resulting in correct peak integration and quantitation.

Figure 8. Consistent and reproducible quantitation of selected NISTmAb CQAs

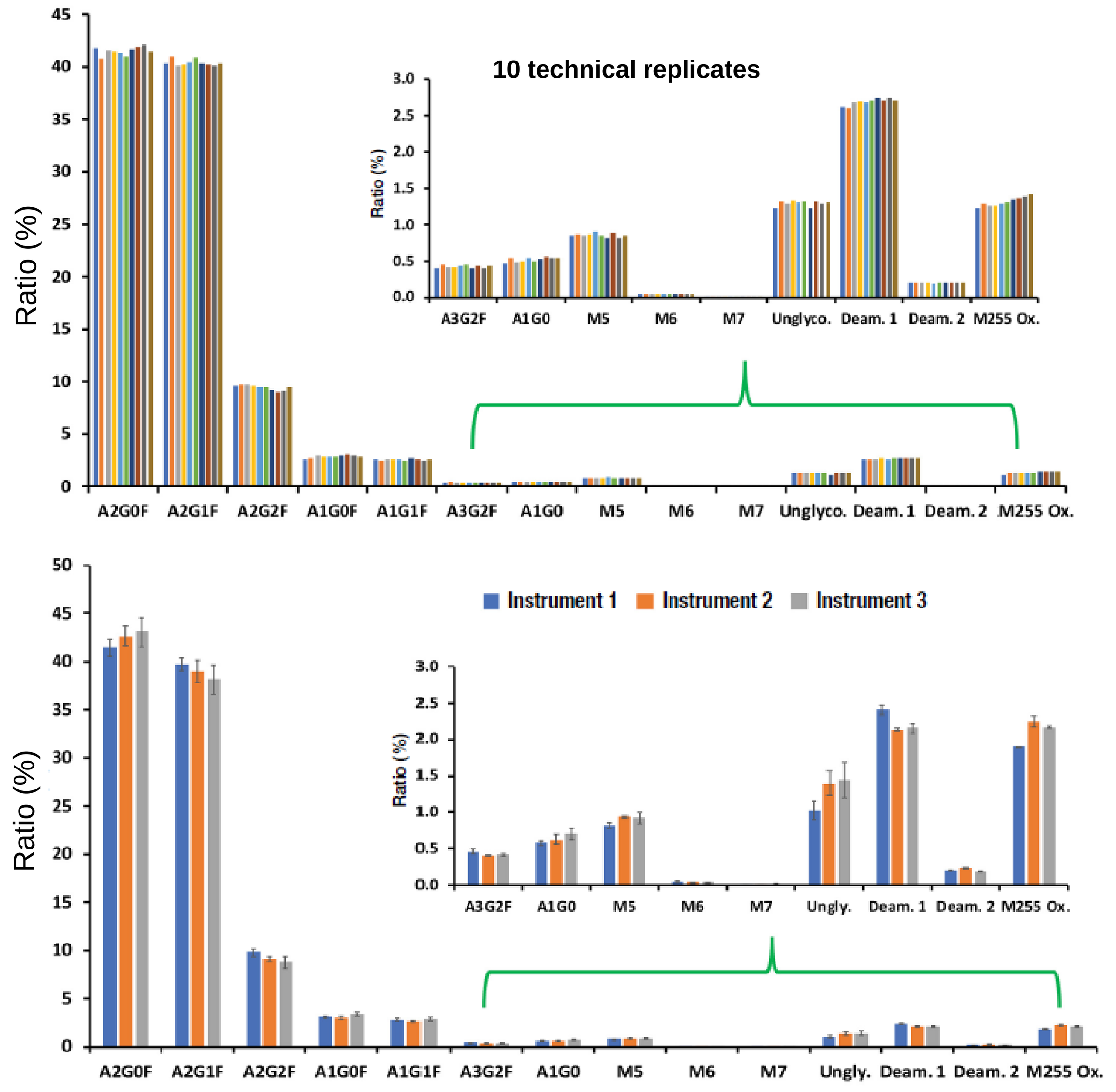
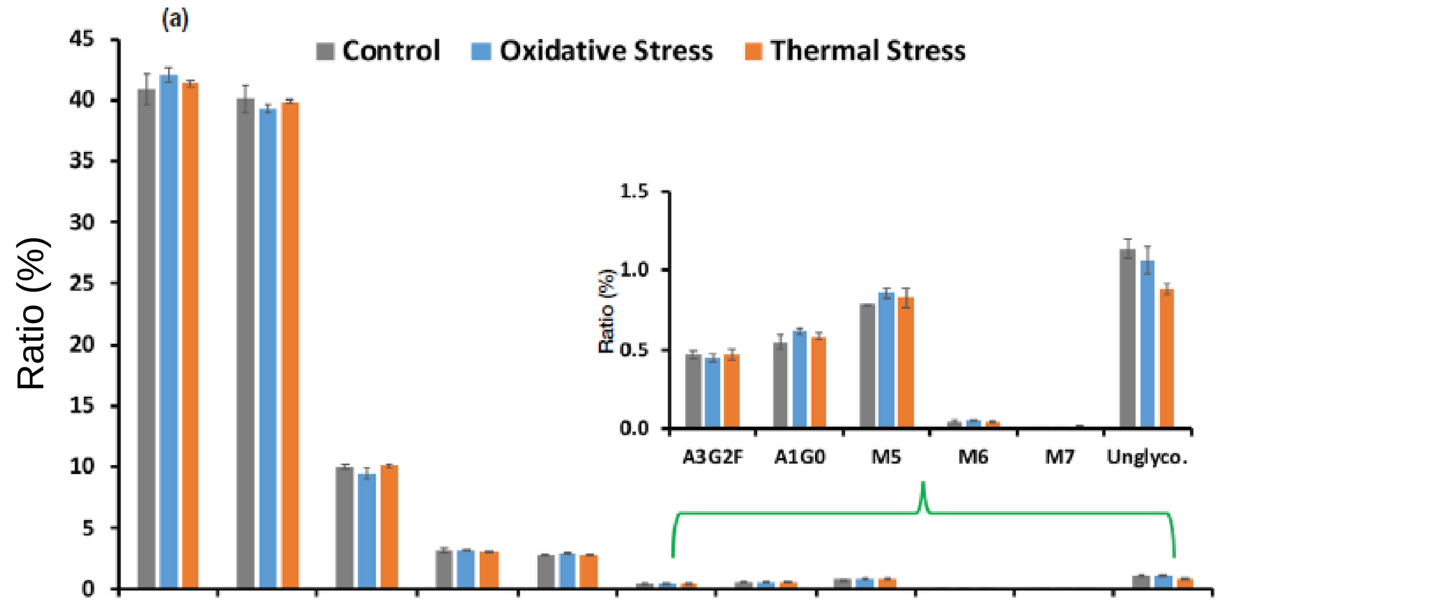
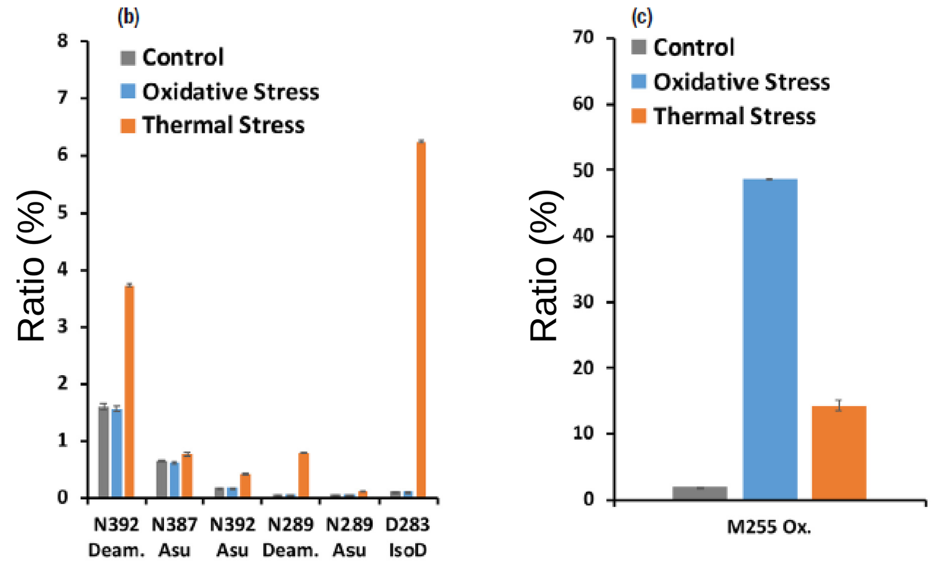


Figure 9. Results of stress studies

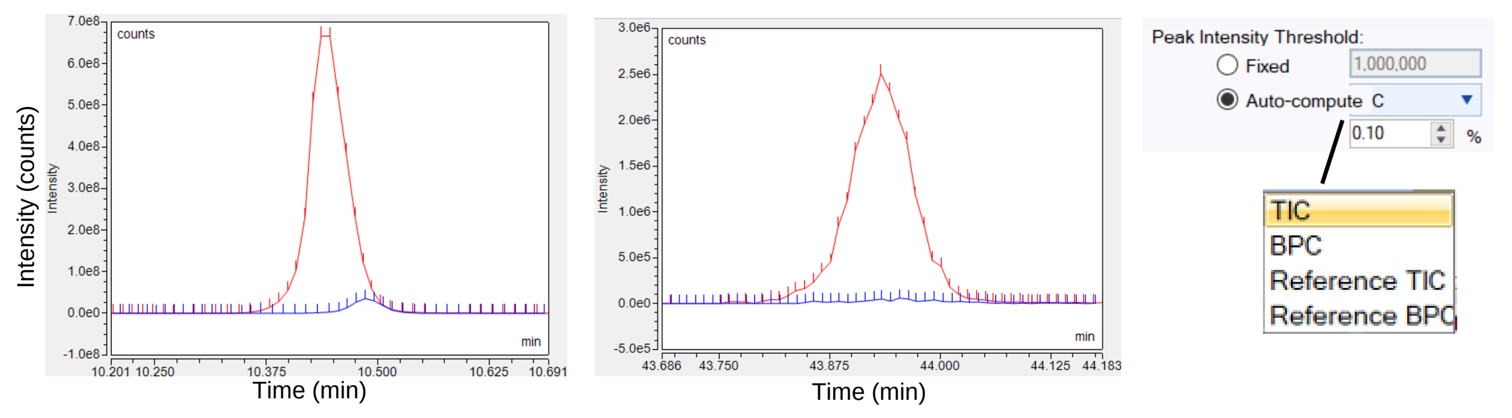




The stressed conditions employed in this work had a negligible effect on the glycosylation profile (a), lysine glycation (data not shown), and lysine clipping (~85% clipped, not shown), indicating that these modifications are neither temperature nor oxidant sensitive.

As expected, the most significant CQA variations for NISTmAb were deamidation and isomerization under thermal stress (b) and methionine oxidation with oxidative stress (c).

Figure 10. New peak detection in spiked and stressed NISTmAb samples



By correctly setting the peak intensity threshold and ratio, all 15 PRTC peptides can be identified without false positives in the spiked sample (data not shown). Shown here are two examples of "new peaks" detected in two stressed samples (red traces), where D315 isomerization (left) and M255 oxidation (middle) increased by >20-fold compared to the control sample (blue traces). In Chromeleon CDS version 7.2.10, peak intensity threshold can be set as a fixed value, or as the percentage of TIC or BPC intensity (right).

CONCLUSIONS

Thermo Scientific HR MAM workflow provides the robustness, flexibility, reproducibility, specificity, and sensitivity to not only identify PQAs and quantify multiple CQAs simultaneously, but also detect new features associated with changes induced by sample preparation, storage, and processing. The HRAM capability of the Q Exactive Plus mass spectrometer enables the resolution of species that would otherwise be overlapped, leading to accurate CQA quantitation and reliable NPD. Combined with the robust separation of the Vanquish Horizon UHPLC system and Accucore Vanquish C18+ column, this workflow produces reproducible results that can be confidently submitted for review by regulatory agencies.

BioPharma Finder software offers rapid peptide mapping, easy CQA selection, and accurate quantitation in a non-compliant environment. The seamless transition from BioPharma Finder software to Chromeleon software through a workbook, enables CQA quantitation and monitoring, as well as NPD, to be performed within a compliant GMP environment. This combination of software utilization provides flexibility in the different phases of drug development and release. It should be emphasized that Chromeleon software affords a comprehensive and fully realized GMP-compliant environment; from instrument configuration, calibration, and tuning through data acquisition, processing and reporting is fully audited with restricted user roles and signatory requirements.

REFERENCES

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2. Rogers, R. *et al.* A view on the importance of "multi-attribute method" for measuring purity of biopharmaceuticals and improving overall control strategy. *The AAPS Journal*, **2018,** *20*, 7.

3. Ren, D. *et al.* An improved trypsin digestion method minimizes digestion-induced modifications on proteins. *Analytical Biochemistry*, **2009**, *392*, 12.

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TRADEMARKS/LICENSING

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