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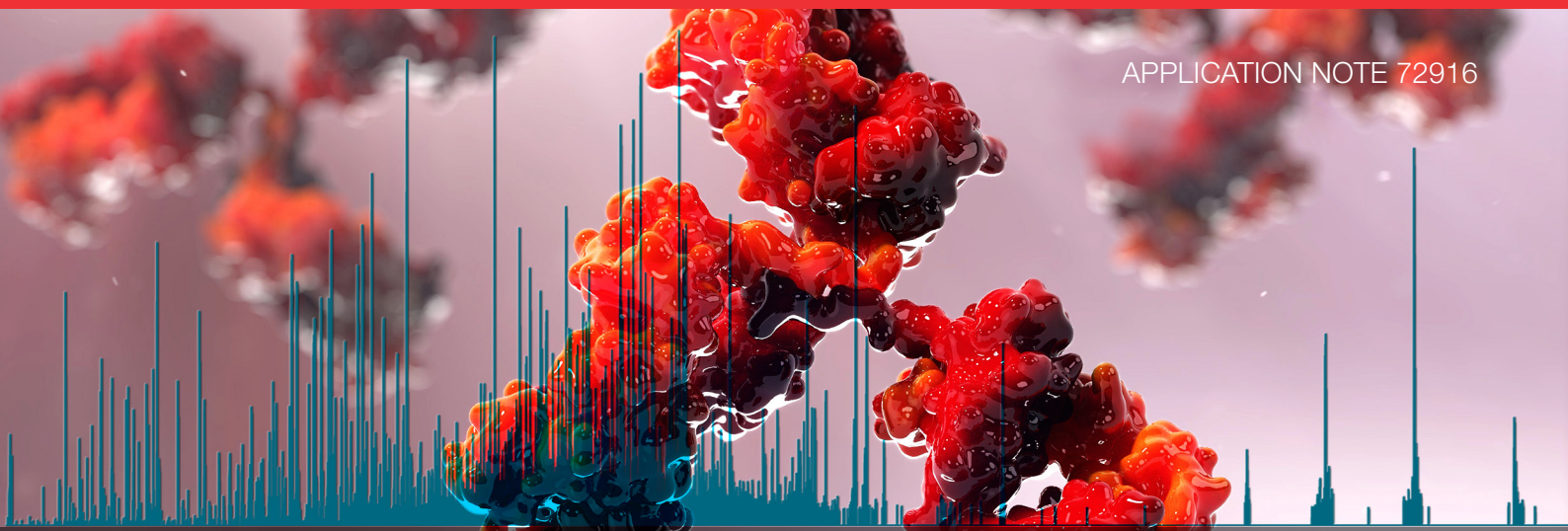

APPLICATION NOTE 72916

A high-resolution accurate mass multi-attribute method for critical quality attribute monitoring and new peak detection

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Keywords

Multi-attribute method, MAM, peptide mapping, Q Exactive Plus, Orbitrap, mass spectrometry, MS, LC/MS, product quality attributes, PQAs, critical quality attributes, CQAs, high resolution, accurate mass, HRAM, Vanquish, Accucore, monoclonal antibody, mAb, NIST, BioPharma Finder, Chromeleon Chromatography Data System, Part 11, compliance, quality control, QC, development

Goal

The aim of this work is to develop a high-resolution accurate mass (HRAM) multi-attribute method (MAM) for the analysis of monoclonal antibody (mAb) critical quality attributes (CQAs). In this application note, we will describe the optimization and application of the Thermo Scientific™ HR Multi-Attribute Method as a complete workflow to monitor CQAs of the NISTmAb standard, including glycosylation, deamidation, isomerization, succinimide formation, oxidation, C-terminal lysine truncation, N-terminal pyroglutamate, and glycation, under normal and stressed conditions. In addition, we will demonstrate the capability of the HR MAM workflow for new peak detection (NPD) using spiked and stressed samples. The importance of HRAM in CQA quantitation and NPD will be discussed.

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.[1,2] Traditionally, a variety of separation techniques such as hydrophilic-interaction liquid chromatography (HILIC), size-exclusion chromatography (SEC), cation-exchange chromatography

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(CEX), capillary electrophoresis (CE), and reversed-phase high performance liquid chromatography (RP-HPLC) are used in conjunction with ultraviolet (UV) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and enzyme-linked immunosorbent assay (ELISA) to comprehensively measure these attributes and assess purity. 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 product quality attributes (PQAs) of two antibodies, Mab1 (IgG1) and anti-streptavidin IgG2. It was also demonstrated that MAM is well suited for NPD, enabling new peaks ("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. In this application note, we describe the HR MAM workflow developed using the Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer and Thermo Scientific™ Vanquish™ Horizon UHPLC system combined with Thermo Scientific™ Chromeleon™ 7.2.10 Chromatography Data System (CDS) and Thermo Scientific™ BioPharma Finder™ 3.1 software for user intuitive data processing and reporting (Figure 1).

Experimental

Methods and materials

Equipment

- Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (P/N 0726030)
- Thermo Scientific Vanquish Horizon UHPLC system (P/N 5400.0105) consisting of:
  - Thermo Scientific™ Vanquish™ System Base (P/N VF-S01-A-02)
  - Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A-02)
  - Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02)
  - Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
  - MS Connection Kit Vanquish (P/N 6720.0405)
- Fisherbrand™ accuSpin™ Micro 17 Microcentrifuge (P/N 13-100-675)
- Eppendorf™ ThermoMixer® C (P/N 5382000023)
- Fisherbrand™ accumet™ AB150 pH Benchtop Meters (P/N 13-636-AB150)

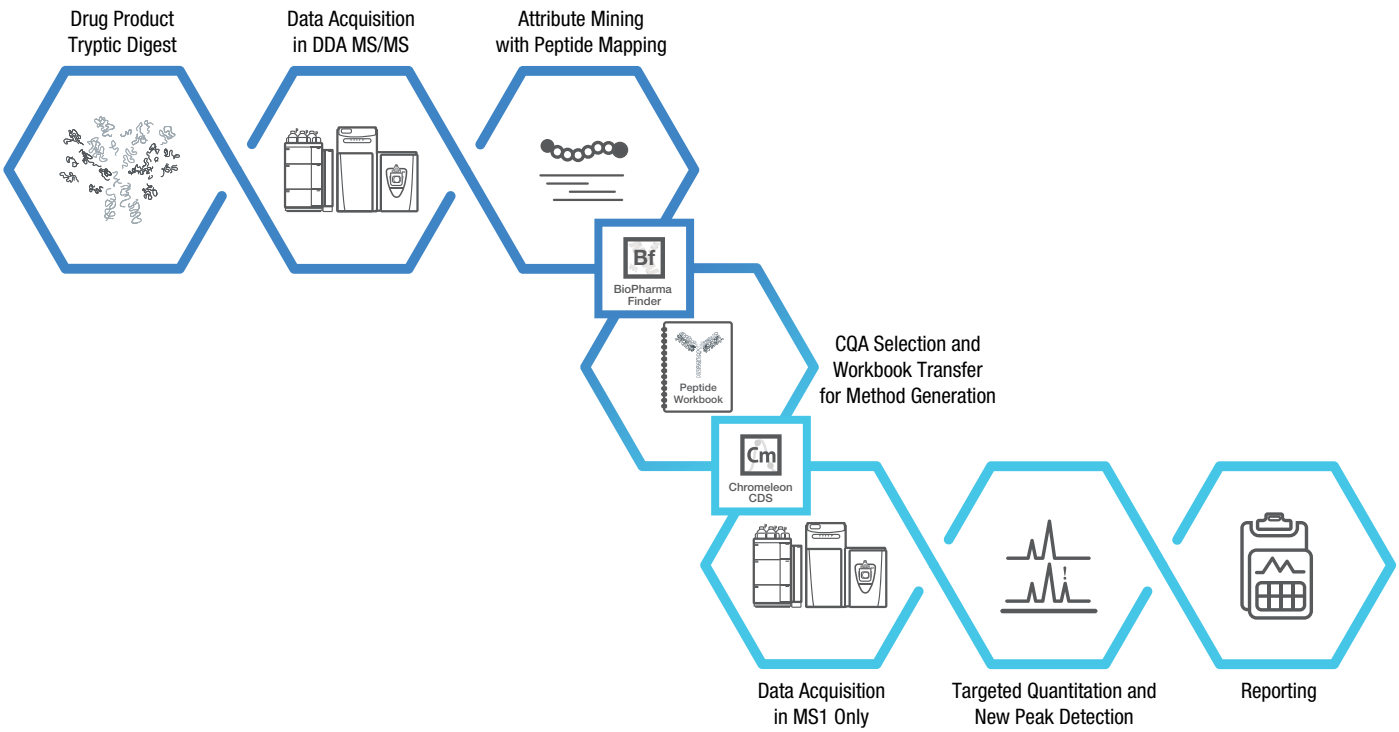


Figure 1. Illustration of the Thermo Scientific HR MAM workflow. A peptide mapping experiment followed by analysis in BioPharma Finder software comprises the discovery phase to identify the PQAs. A list of CQAs is created in a BioPharma Finder workbook, which is then exported to Chromeleon CDS for routine GMP-compliant monitoring of these targets, detection of new features, and reporting. DDA = data dependent acquisition

Software

- Thermo Scientific BioPharma Finder 3.1 software Peptide Mapping Only (OPTON-30888)
- Chromeleon CDS software with the following components:
  - Chromeleon Enterprise Client (P/N 7200.0300)
  - Biopharma QC Package (P/N 7200.0044)
  - Thermo Scientific Instrument Control (P/N 7200.1000)
  - License Key New (P/N 7050.0104A)

Liquid chromatography / mass spectrometry consumables

- Thermo Scientific™ Accucore™ Vanquish™ C18+ UHPLC column, 1.5 µm, 2.1 × 150 mm (P/N 27101-152130)
- Fisher Scientific™ 0.1% Formic Acid in Water, Optima™ LC-MS grade (P/N LS118-212)
- Fisher Scientific™ 0.1% Formic Acid in Acetonitrile, Optima™ LC-MS grade (P/N LS120-212)
- Thermo Scientific™ Water, UHPLC-MS grade (P/N W8-1)
- Thermo Scientific™ Acetonitrile, UHPLC-MS grade (P/N A956-1)
- Thermo Scientific™ Pierce™ LTQ Velos ESI Positive Ion Calibration solution, 10 mL (P/N 88323)

Sample digestion consumables

- NISTmAb Humanized IgG1κ Monoclonal Antibody Lot 14HB-D-001 (NIST, RM 8671)
- Thermo Scientific™ Pierce™ Trypsin Protease MS grade (P/N 90058)
- Thermo Scientific™ Pierce™ Peptide Retention Time Calibration (PRTC) Mixture 200 µL (P/N 88321)
- Thermo Scientific™ Pierce™ Formic Acid, LC-MS grade (P/N 28905)
- Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 (P/N 15567027)
- 8.0 M Guanidine Hydrochloride Solution (Sigma, P/N G7294-100ML)
- Bio-Spin® P-6 Gel Columns, Tris Buffer (Bio-Rad, P/N 732-6227)
- Sodium Iodoacetate (IAC) BioUltra >98% purity (Sigma, P/N I-9148)
- DL-Dithiothreitol (DTT) BioXtra ≥99% purity (Sigma, P/N D-5545)
- Sodium Hydroxide Concentrate (Sigma, P/N 43617-1L)
- Eppendorf™ Protein LoBind Microcentrifuge Tube 0.5 mL (P/N 022431064)
- Eppendorf™ Protein LoBind Microcentrifuge Tube 1.5 mL (P/N 022431081)

Preparatory steps

Prior to sample preparation, the BioSpin-6 columns were inverted vigorously several times and allowed to equilibrate at room temperature for 1 hr. The Eppendorf ThermoMixer was set to 37 °C (or 60 °C for the thermally stressed sample) and allowed sufficient time to adjust to this temperature. The Q Exactive Plus mass spectrometer was calibrated using the positive ion CalMix (P/N 88323).

Solution preparation[4]

The following five solutions were prepared in advance to facilitate sample digestion:

Solution I: 7.0 M Guanidine HCl, 100 mM Tris @ pH 8.3

87.5 mL of 8 M Guanidine HCl solution and 10.0 mL of 1 M Tris-HCl pH 7.5 were added to a 100 mL volumetric flask. After mixing thoroughly by inversion, the pH was measured using a pH meter and adjusted to pH 8.3 with sodium hydroxide concentrate. LC-MS grade water was then added to reach a final volume of 100 mL.

Solution II: 500 mM DTT (with 7.0 M Guanidine HCl, 100 mM Tris @ pH 8.3)

To 50.0 mg freshly weighed DTT, 649 µL of Solution I was added to give a final concentration of 500 mM in a 1.5 mL microcentrifuge tube. The tube was vortexed to mix well.

Solution III: 500 mM IAC (with 7.0 M Guadidine HCl, 100 mM Tris @ pH 8.3)

To 50.0 mg of freshly weighed IAC, 481 µL of Solution I was added, to give a final concentration of 500 mM IAC in a 1.5 mL microcentrifuge tube. The tube was vortexed and stored in the absence of light.

Solution IV: 50 mM DTT (with 7.0 M Guanidine HCl, 100 mM Tris @ pH 8.3)
100 µL of Solution II were diluted with 900 µL of Solution I in a 1.5 mL microcentrifuge tube to yield a final concentration of 50 mM DTT.

Solution V: 50 mM Tris pH 7.9
10 mL of 1 M Tris-HCl pH 7.5 was added to a 200 mL volumetric flask and the final volume was adjusted to 200 mL using LC-MS grade water. The flask was mixed by inversion. The pH was measured by a pH meter.

Solution VI: 10% Formic acid
1 mL of formic acid was taken out of an ampule and was mixed with 9 mL of LC-MS grade water, followed by vortexing.

Reduction

The commercially available NISTmAb standard is provided in 800 µL volumes at a specified concentration of 10.004 ± 0.08 mg/mL stored with 12.5 mM L-histidine and 12.5 mM L-histidine HCl (pH 6.0). Subsequent to thawing the vial was inverted gently five times and aliquot into eighty 10 µL portions.

The concentration of each aliquot used in this study was adjusted to 1 mg/mL by adding 90 µL of Solution I (7 M Guanidine HCl, 100 mM Tris, pH 8.3). To this solution, 2.0 µL of Solution II (500 mM DTT) were added and mixed by pipette yielding a final concentration of approximately 10 mM DTT. The reaction was carried out for 30 minutes at room temperature.

Alkylation

To the reduced sample above, 4.0 µL of Solution III (500 mM IAC) was added and mixed by pipette to give a final concentration of 20 mM IAC. The solution was stored in the absence of light for 20 minutes at room temperature.

Buffer exchange

Alkylation was arrested by further addition of 4.0 µL of Solution IV (50 mM DTT), resulting in a total volume of 110 µL (11 mM DTT).

The tip of the BioSpin-6 column(s) was broken off and the column was placed in a 2 mL Eppendorf microcentrifuge tube. After removing the cap, the column was centrifuged for 2 minutes at 1000 × g. The liquid collected in the Eppendorf tube was disposed of and 500 µL of Solution V (50 mM Tris at pH 7.9) was added carefully to the top of the BioSpin-6 column. Centrifugation was repeated at 1000 × g for 2 minutes. This step was repeated three more times (four times in total) to completely buffer exchange the BioSpin-6 column. At each step, the collected liquid was discarded.

Following buffer exchange into 50 mM Tris at pH 7.9, the spin column was placed in a new 1.5 mL microcentrifuge tube and 110 µL of the alkylated sample was added carefully to the center of the column bed. Next, the column was centrifuged for 4 minutes at 1000 × g. The collected sample was ready for trypsin digestion.

Digestion

100 µL of LC-MS grade water was added to a vial of 100 µg Pierce Trypsin to give a final concentration of 1 mg/mL. The solution was mixed by gentle vortexing. This solution was added in a 1:10 ratio to the NISTmAb sample above; for example, 10 µL of 1 mg/mL Trypsin to 100 µL of sample solution. The resulting solution was placed in an Eppendorf ThermoMixer and heated at 37 °C for 30 minutes. Digestion was halted by adding 10% formic acid (1:10 ratio in volume with the digestion solution) and the pH verified to be acidic (approximately pH 1.5). The final sample solution was transferred to an autosampler vial and placed in the sample rack held at 5 °C.

Heat stressed sample

10 µL of 10 mg/mL NISTmAb was placed in an Eppendorf ThermoMixer at 60 °C for 120 hours. Afterwards, it was reduced, alkylated, and digested precisely as described above.

Oxidized sample

10 µL of 0.03% hydrogen peroxide were added to 10 µL of 10 mg/mL NISTmAb. The oxidation took place at room temperature for 24 hours. Afterwards the concentration of NISTmAb was brought to 1 µg/µL by adding 80 µL of Solution I (7 M Guanidine HCl, 100 mM Tris, pH 8.3). The sample was then reduced, alkylated, and digested precisely as described above.

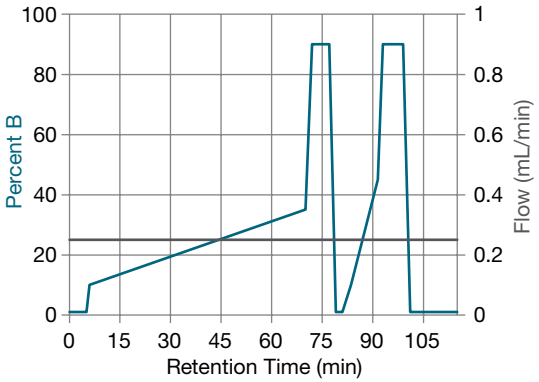
PRTC spiked sample for NPD

To demonstrate the capability of the HR MAM workflow for NDP, 2 µL of PRTC stock solution (5 pmol/µL, 200 µL) were spiked into 78 µL of the digested NISTmAb, resulting in a final concentration of PRTC at 0.125 pmol/µL. This yielded 20 pmol of the NISTmAb to 0.5 pmol of the PRTC for a 4 µL sample injection.

Liquid chromatography

Peptide separations were performed with an Accucore Vanquish C18+ UHPLC column (1.5 µm, 2.1 × 150 mm) using a Vanquish Horizon UHPLC system. The autosampler was held at 5 °C while the column was maintained at 50 °C with the column oven Thermostatting Mode set to Still Air.

The digest of NISTmAb was loaded onto the column, with 4 µL injected per analysis, using a draw and dispense speed of 0.5 µL/s and a sample loop of 25 µL. The binary solvent system consisted of 0.1% formic acid in water (A1) and 0.1% formic acid in acetonitrile (B1) at a fixed flow rate of 0.250 mL/min. The LC gradient used in this study is shown in Figure 2.



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 2. 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.

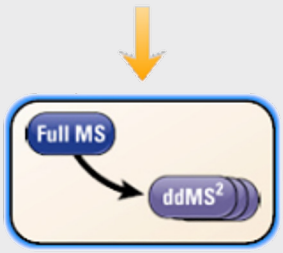
Mass spectrometry

The Q Exactive Plus mass spectrometer was operated in positive ion mode with the source and MS method settings shown in Table 1. Instrument calibration was performed using the positive ion CalMix within the Exactive Series Tune software. Data were acquired using Chromeleon CDS software 7.2.10. The data for peptide mapping were acquired with a Top5 data-dependent MS2 (ddMS2) method and processed in BioPharma Finder software. Full scan data for CQA quantitation and NPD were acquired using a Full Scan MS only method and processed in Chromeleon software. All experiments were performed in the standard pressure mode. Further details of method parameters and settings are provided in Table 1. 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, as described below.

Table 1. Mass spectrometry tune and method settings

MS source setting	Value
Sheath gas	35 arb
Aux gas	10 arb
Sweep gas	0 arb
Spray voltage	3.5 kV
S-lens RF level	50
Aux gas temp.	250 °C
Capillary temp.	250 °C

Properties of Full MS - SIM	Value
General	
Runtime	0 to 72 min
Polarity	Positive
Full MS - SIM	
Resolution	140,000
AGC target	3e6
Maximum IT	200 ms
Scan range	300 to 1800 *m/z*



Properties of Full MS/ dd-MS² (Top5)	Value
General	
Runtime	0 to 72 min
Polarity	Positive
Default charge state	2
Inclusion	–
Exclusion	–
Tags	–
Full MS	
Resolution	140,000
AGC target	3e6
Maximum IT	100 ms
Scan range	300 to 1800 *m/z*
dd-MS² / dd-SIM	
Resolution	17,500
AGC target	1e5
Maximum IT	250 ms
Loop count	5
TopN	5
Isolation window	1.2 *m/z*
Fixed first mass	–
(N)CE / stepped (N)CE	nce: 27
dd Settings	
Minimum AGC target	2.00e3
Intensity threshold	8.0e3
Apex trigger	–
Charge exclusion	Unassigned, 1, >8
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	8.0 s

Peptide mapping and CQA selection in BioPharma Finder software

The raw data for peptide mapping were exported from Chromeleon CDS and mapped against the NISTmAb sequence in BioPharma Finder 3.1 software. The BioPharma Finder software settings used in this study are listed in Table 2. It is recommended to perform peptide mapping on the MS/MS data of both control and stressed samples to determine which PQAs are critical for quantitation and monitoring, as some of the modifications (e.g. isomerization and a deamidated form of VVSVLTVLHQDWLNGK) may be absent, or present at very low levels, under normal conditions and hence not identified in peptide mapping.

The modifications of NISTmAb have been well documented in recent publications.[3] The CQAs selected for quantitation in this study were glycosylation, deamidation, isomerization, succinimide formation, oxidation, C-terminal lysine truncation, N-terminal pyroglutamate, and glycation. For each CQA, the four isotopes of the identified charge states were selected in the BioPharma Finder Process and Review table and saved to Target Peptide Workbook. The CQAs in the workbook were then exported to a BioPharma Finder workbook file (.wbpf) and imported into the MS Component Table of the Chromeleon processing method. Any new CQAs can be added to the BioPharma Finder workbook and hence the Chromeleon processing method at any time. This allows for flexibility during the method development and optimization phase with post-acquisition data mining. Once a standardized method has been established within Chromeleon software, it can be locked and is therefore immune to further modifications for application in a GMP environment.

Table 2. BioPharma Finder 3.1 search settings

Database Parameters	
Protease	Trypsin (C-term KR)
Specificity	High
Fixed Modification	Carboxymethylation (C)
Variable Modifications	Deamidation (N) Deamidation (Q) Glycation (K) NH_3 loss (NQ) Oxidation (MW) Lys (C-term) Gln→Pyro-Glu (N-term) N, O Glycans (CHO)
Component Detection Parameters	
Task To Perform	Find All Ions in the Run
Absolute MS Signal Threshold (MS Noise Level * S/N Threshold)	Automatic determination by software
MS Noise Level	Automatic determination by software
S/N Threshold	Automatic determination by software
Typical Chromatographic Peak Width (min)	0.30
Maximum Chromatographic Peak Width (min)	Automatic determination by software
Maximum RT Shift	Automatic determination by software
Identification Parameters	
Search by Full MS Only	No
Use MS/MS	Use All MS/MS
Maximum Peptide Mass	7000
Mass Accuracy (ppm)	8
Minimum Confidence	0.8
Maximum Number of Modifications for a Peptide	1
Enable Mass Search for Unspecified Modifications	Unchecked
Glycosylation	CHO
Search for Amino Acid Substitutions	None
Perform Disulfide Bond Search	No
Enable HDX	Unchecked

Building the processing method in Chromeleon software

The Chromeleon MAM processing method was created based on the MS Quantitative template. The basic settings for the MAM processing method are shown in Table 3. The CQAs in the BioPharma Finder workbook were imported into the MS Component Table of the processing method. The peak integration parameters for each CQA can be optimized in the Chromeleon software to ensure consistent and confident peak integration, and therefore quantitation. Chromeleon software offers an array of options and controls for peak integration. For example, ICIS and Genesis algorithms are available for integrating the extracted ion chromatogram (XIC) peaks. In addition, a smoothing algorithm (e.g. Gaussian) can be used to improve integration of low abundant species. Several retention time (RT) parameters can be adjusted to achieve accurate and consistent integration of a CQA. As an example, Figure 3 illustrates using the retention "Time Distance" to integrate an isoaspartic acid (isoAsp) variant from its wild type form. The component, FNWYVDGVEVHNAK, was set as reference, with the RT of its isoAsp form set to -0.715 min from the reference. Further adjustment of the Window and Component Match parameters ensure correct integration of the modified form. This approach can be very useful to separately integrate multiple forms in a single XIC.

Table 3. Chromeleon CDS processing settings

MS Detection	Value
Extracted Ion Chromatogram	<MS Default Detection Settings>
Detection Algorithm	ICIS Default Values
MS Settings	**Value**
MS No. of Decimal Points	5
Mass Tolerance	Manually Define Mass Tolerance, 8 ppm
Integration	Inhibit Integration for TIC Channel
Smoothing	None
MS Spectra Bunching	No baseline correction, 1 scan
Composite Scoring	**Value**
Pass Score if at Least	2 criteria passed
Fail Score if Less Than	1 criterion passed
Isotopic Dot Product	≥0.900
Mass Accuracy	≤5.00 ppm
Peak Apex Alignment	≤0.50 min

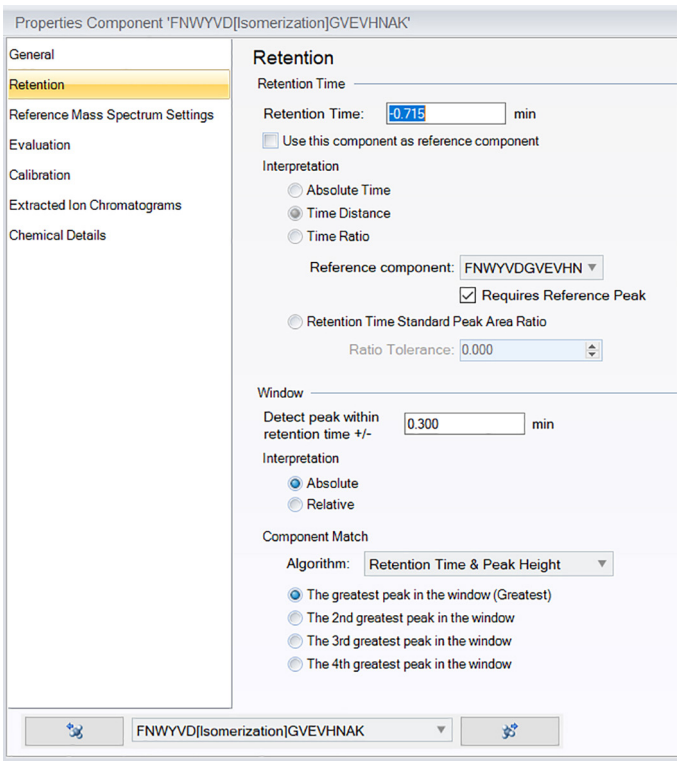


Figure 3. Unlike conventional targeted quantitation software, the extraction parameters for each peptide including RT and integration window can be set *a priori* in Chromeleon software.

Figure 4 illustrates how a combination of Time Distance (+1.35 min) and Component Match parameters (Algorithm = Retention Time, The 1st-4th peak in the window settings checked for Figure 4a-4d, respectively) can be used to properly integrate four partially overlapping deamidation peaks. It should be noted that the four peaks can be also integrated separately using the Retention Time & Peak Height (or Peak Area) algorithm, demonstrating the versatility of peak integration in Chromeleon software.

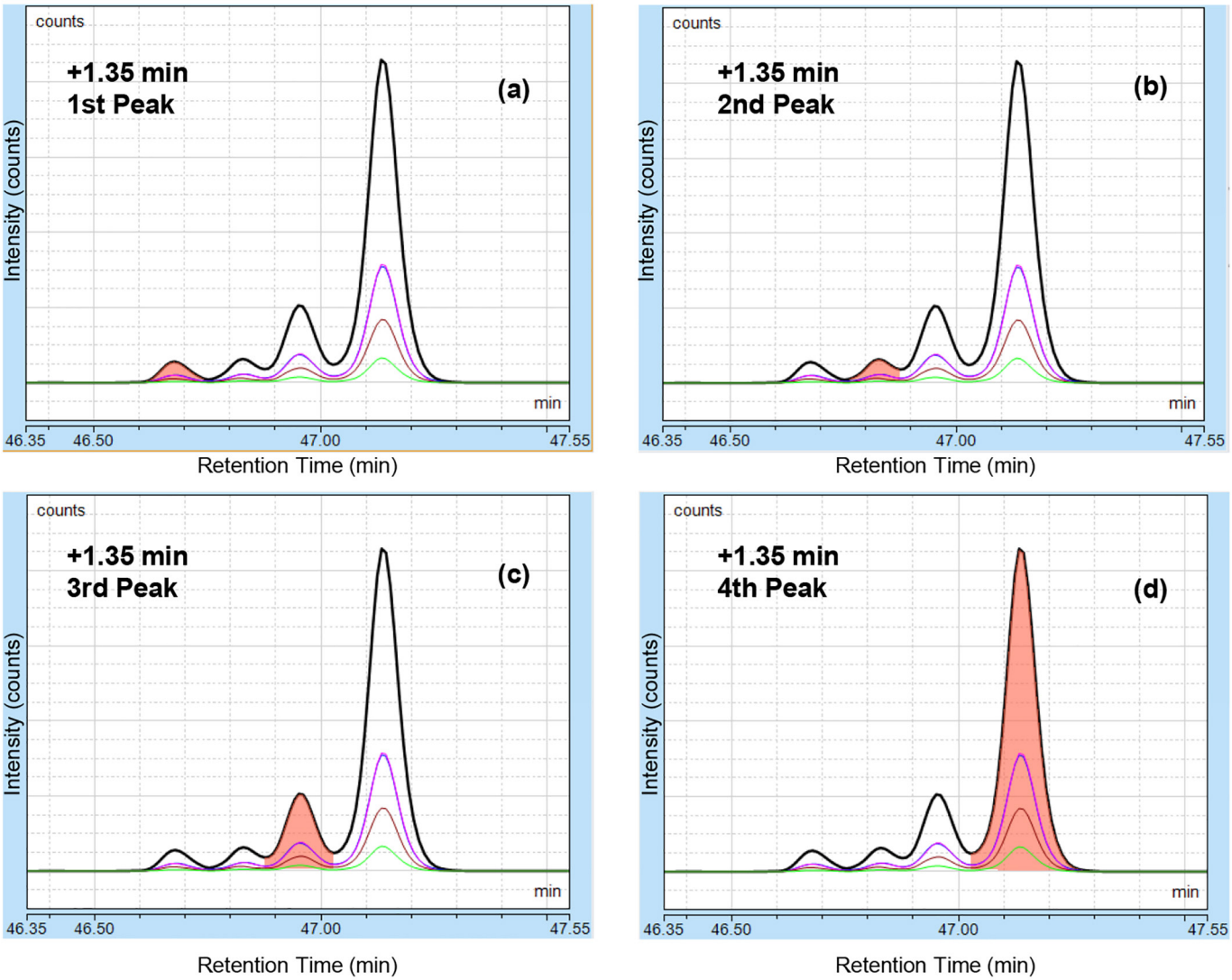


Figure 4. Integration of four partially overlapping peaks using a Time Distance of +1.35 min and Component Match = The 1st-4th peak in the window for (a)-(d), respectively, using the Retention Time algorithm, following initial integration with the ICIS algorithm and with multiplet resolution of 1 and 7 points Gaussian smoothing

Another useful feature of the Chromeleon processing method is the component isotopic distribution display. This provides confidence in the integration and can help to confirm detection of a peptide component. Figure 5 shows that when Component B was not integrated due to interference from a very abundant Component A (Figure 5a), the experimental isotopic distribution (dark bars in Figure 5b) deviated from the theoretical distribution (light bars in Figure 5b). With proper integration of Component B (Figure 5c), an agreement was found between the experimental and theoretical isotopic distributions (Figure 5d).

Non-targeted MS processing for NPD in Chromeleon software

To demonstrate the NPD process in Chromeleon software, the NISTmAb digest spiked with Pierce PRTC was compared to a reference sample (unspiked NISTmAb digest). The NPD settings used for this study are given in Table 4. The Peak Intensity Threshold (in Frame Settings) and Ratio (in Filter Settings) need to be optimized for different experiments. In Chromeleon CDS Version 7.2.10, one can set a fixed value or a percentage of total ion chromatogram (TIC) or base peak chromatogram (BPC) of the current or reference injection for the peak intensity threshold. Since PRTC was absent in the reference, a large ratio (≥1000) was used to filter the result of the NISTmAb sample spiked with PRTC. For the stressed samples, a ratio of 20 was used to identify significant changes compared to the control sample. Lowering the Peak Intensity Threshold and Ratio may increase the number of frames (potential new features) but result in more false positive identifications.

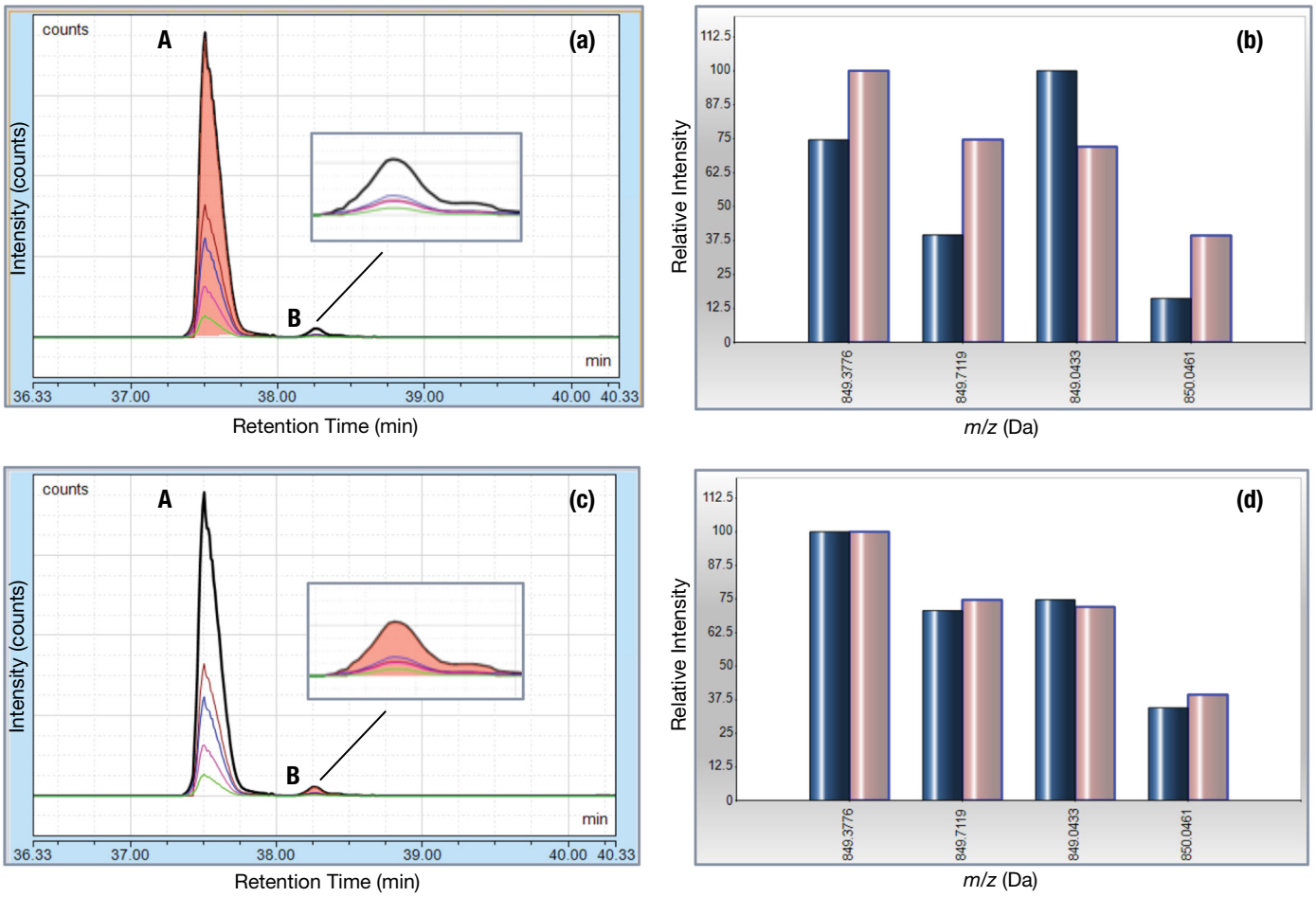


Figure 5. Comparison of experimental (dark bars) and theoretical (light bars) isotopic distributions of Component B (b) and (d) with incorrect (a) and correct (c) peak integration

Table 4. Chromeleon CDS new peak detection settings for the data of NISTmAb digest spiked with PRTC

Frame Settings	Value
m/z Min	300
m/z Max	1800
m/z Width	10 ppm
Retention Time Start	0 min
Retention Time Stop	72 min
Frame Time Width	0.5 min
Maximum Number of Frames	16,000
Peak Intensity Threshold – Fixed	1E+06
Minimum Intensity Threshold	FTMS + p ESI ms [300.00–1800.00]

MORE	Value
Alignment Bypass	False
Alignment Min Intensity	1000
Correlation Bin Width	1
Max RT Shift	0.5 min
Tile Size	300
Filter Settings	**Value**
PR Element	0
PR Size	>1
Charge	2 for the PRTC spiked sample 2–5 for the stressed samples
Ratio	≥1000 for the PRTC spiked sample >20 for the stressed samples

Results and discussion

A TIC for a NISTmAb digest (~3 µg), not spiked with PRTC, generates an intensity of 1-3E+9 (Figure 6). The small particle size (1.5 µm) of the Accucore column used in this study produced highly resolved peaks. The sequence coverages for NISTmAb LC and HC were consistently >96%. The regions that were not mapped contain few amino acid residues (3–5) or multiple lysine residues. In either case, the corresponding tryptic peptides are too short to be detected in this study.

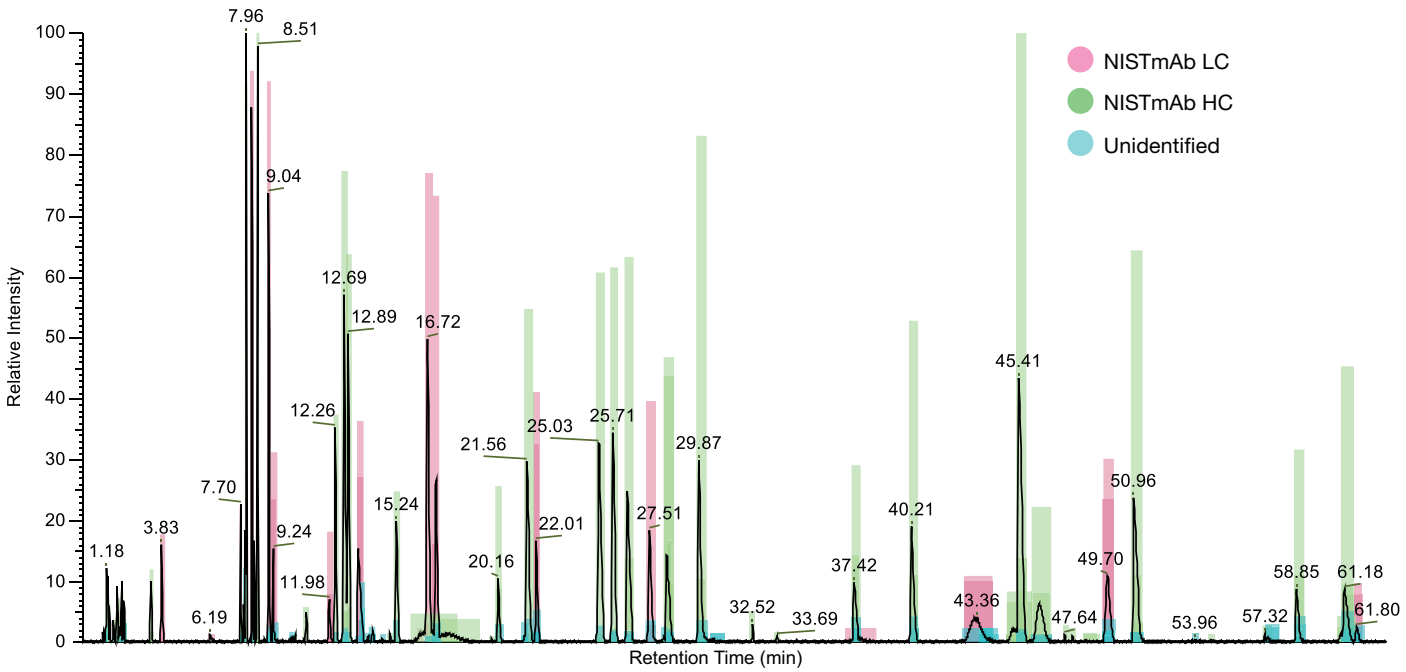


Figure 6. Total ion chromatogram (TIC) of control NISTmAb digest. Peptides of the NISTmAb light chain (LC) and heavy chain (HC) as identified from peptide mapping using BioPharma Finder 3.1 software, are highlighted in pink and light green, respectively.

Importance of high-resolution accurate mass

The HRAM capability of Orbitrap mass spectrometers promotes resolution of overlapping peaks, ensures precise peak integration, and enables accurate CQA quantitation using MS full scan data. At a resolution setting of <140,000, ions with similar *m/z*, for example, the monoisotopic peak of a deamidated peptide (B0 in Figure 7) and the C13 peak of the wild type form (A1 in Figure 7), may overlap or even merge into a single peak. This negatively influences mass accuracy and results in incorrect peak integration. At a resolution setting of 70,000 (Figure 7b), the B0 peak of deamidated HYNPSLK completely overlaps with the A1 peak of the wild type form, resulting in the absence of this isotope in the peak integration window. At even lower resolution setting of 35,000 (Figure 7c), all three isotopes of the deamidated peptide (B0-B2) overlap with the neighboring isotopes of the wild type form (A1-A3). Only the very low abundant fourth isotope of the deamidated form was integrated (Figure 7c, left panel). An Orbitrap resolution setting of 140,000, however, can resolve the isotopes of the low abundance deamidated peptide from the abundant native form (Figure 7a). Therefore, the first four isotopes of the deamidated peptide generated suitable XICs, using an 8 ppm mass tolerance, enabling accurate quantitation of this CQA. Figure 7d shows the data acquired at resolution setting 140,000 for the NISTmAb digest prepared under the thermally stressed condition. Due to

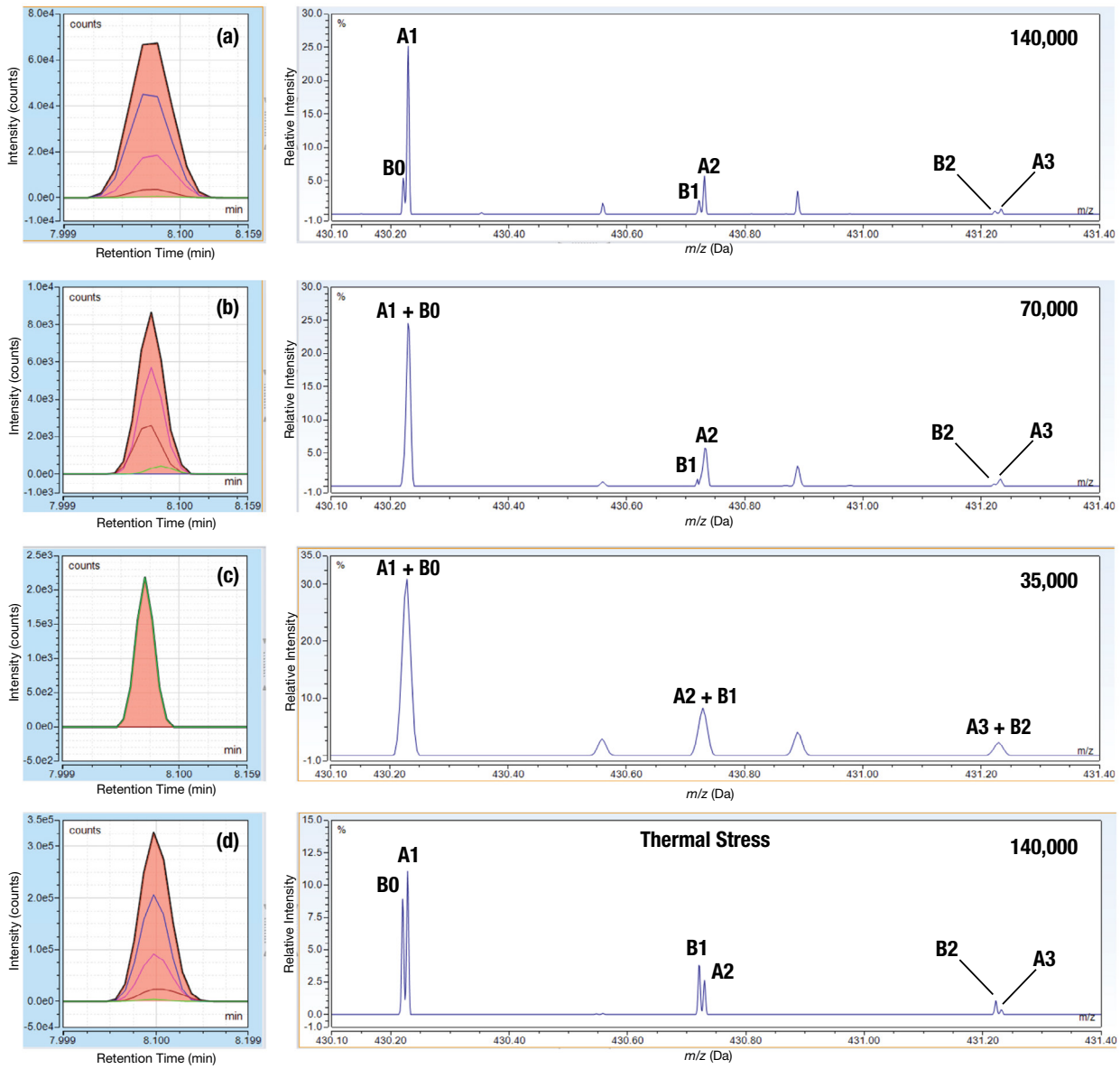


Figure 7. Left panels: XICs of HYNPSLK (component A) and HYN[Deamidation]PSLK (component B) in the control (a-c) sample at Orbitrap resolution settings of 140,000, 70,000, and 35,000 (at *m/z* 200) and in the thermally stressed (d) sample at a resolution setting of 140,000 (at *m/z* 200). Right panels: mass spectra at the apex RTs. A1–A3 represent the second through fourth isotopes of the wild type form while B0–B2 correspond to the first through third isotopes of the deamidated form.

significant increase in the level of deamidation, it is more obvious to see the isotopes of the deamidated forms (B0-B2) that are resolved from the neighboring isotopes (A1-A3) of the wild type form. This is an excellent example that highlights the importance of high resolution in CQA quantitation. There were also other examples in the NISTmAb digest where the isotopes of two peptides have very close *m/z.* In such cases, inaccurate quantitation was obtained for the data acquired at a resolution setting of <140,000 because the overlapped isotopes could not be resolved and extracted properly.

High mass accuracy also serves to improve peak extraction and integration. Shown in Figure 8 are XICs of the deamidated "PENNYK" peptide using 5 ppm and 10 ppm mass tolerance settings, respectively. In the case of 10 ppm extraction (Figure 8a), the wild type form of this peptide dominated the XIC, which led to an incorrect peak integration when the Component Match was set to Greatest. By comparison, the wild type form was not evident in the XIC of the deamidated form when a 5 ppm extraction was used (Figure 8b). It should be noted that Peak A in the 10 ppm XIC (Figure 8a) can be correctly integrated in the presence of abundant Peak B by using the Component Match = Nearest or by setting a narrow RT window, again demonstrating the versatility of peak integration in Chromeleon software.

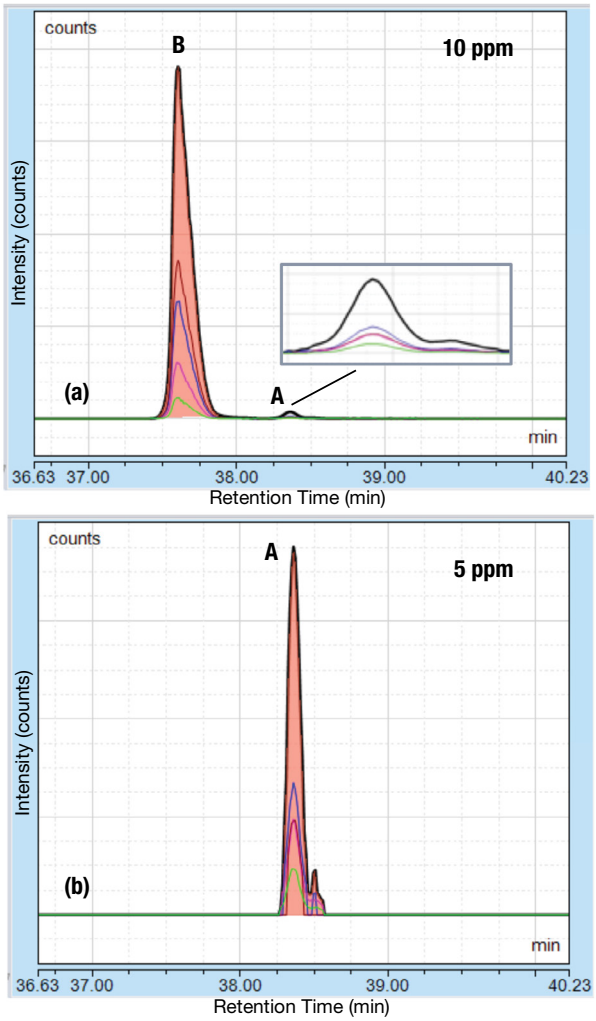


Figure 8. XICs of deamidated GFYPSDIAVEWESNGQPENNYK ("PENNYK" peptide) with 10 ppm (a) and 5 ppm (b) mass tolerance. Component Match = Greatest. A: Deamidated form, B: Wild type form. While the deamidated form (Peak A) was correctly integrated in the 5 ppm XIC (b), the wrong peak (Peak B, wild type form) was integrated in the 10 ppm XIC (a).

Technical and biological replicate reproducibility

The ratios of selected NISTmAb CQAs, including glycosylation at EEQYNSTYR, deamidation at GFYPSDIAVEWESNGQPENNYK and FNWYVDGVEVHNAK, and oxidation at DTLMISR, were measured under different conditions to assess the effects of digestion, sample storage, and instrumentation on the quantitative results (Figure 9). The reproducibility of ratio measurement across ten technical replicates of the same NISTmAb digest was excellent (Figure 9a), as was the reproducibility across the three biological replicates prepared by the same individual (data not shown). There was also good consistency between % glycosylation measurements under different conditions, i.e. the same NISTmAb analyzed at different times (Figure 9b), and on three different Q Exactive Plus mass spectrometers with two different Vanquish Horizon and Accucore columns (Figure 9c), plus between two different digests prepared by two individuals (Figure 9d). Higher variations were seen for deamidation and oxidation in the sample stored in a freezer for extended time (Figure 9b). This result is expected since the levels of these two modifications may increase with the increasing time of storage. Overall, these results demonstrate that the HR MAM workflow can generate consistent results under various conditions.

It should be noted that very low abundance CQAs such as glycopeptides M6 (0.04%–0.05%) and M7 (0.01%–0.02%) can be consistently detected and quantified. However, care needs to be taken to check the integration of low abundant peaks from injection to injection. Generally, once the signal counts fall below 5E+4, peak integration and quantitation become challenging. However, this signal threshold is expected to be dependent on the physiochemical properties, ionizability, and detectability of each peptide.

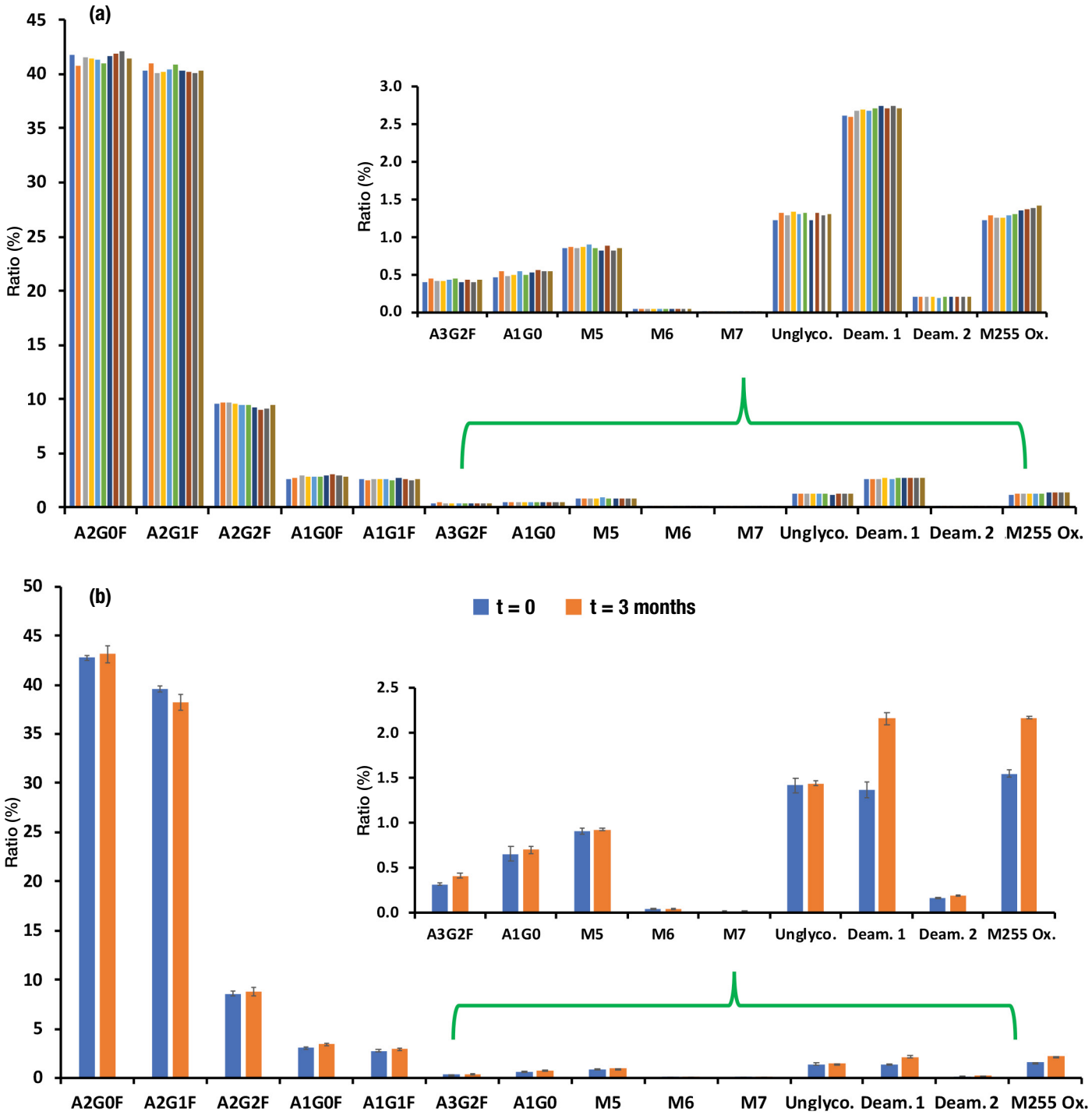


Figure 9-1. Ratios of selected CQAs of NISTmAb, including common glycan forms of EEQYNSTYR, deamidation of GFYPSDIAVEWESNGQPENNYK ("Deam. 1") and FNWYVDGVEVHNAK ("Deam. 2"), and Met oxidation of DTLMISR ("M255 Ox."). (a) Ratios for 10 consecutive injections of the same NISTmAb digest on the same instrument. (b) Average ratios for the same NISTmAb digest measured at different times (sample kept at -80 °C in between) on the same instrument. The error bars show the standard deviations of the ratios measured from 3 to 6 technical replicates. Note that the ratios of "Deam. 1" and "Deam. 2" for GFYPSDIAVEWESNGQPENNYK and FNWYVDGVEVHNAK, respectively, are the sums of deamidation, succinimide, and isomerization (for "Deam. 2").

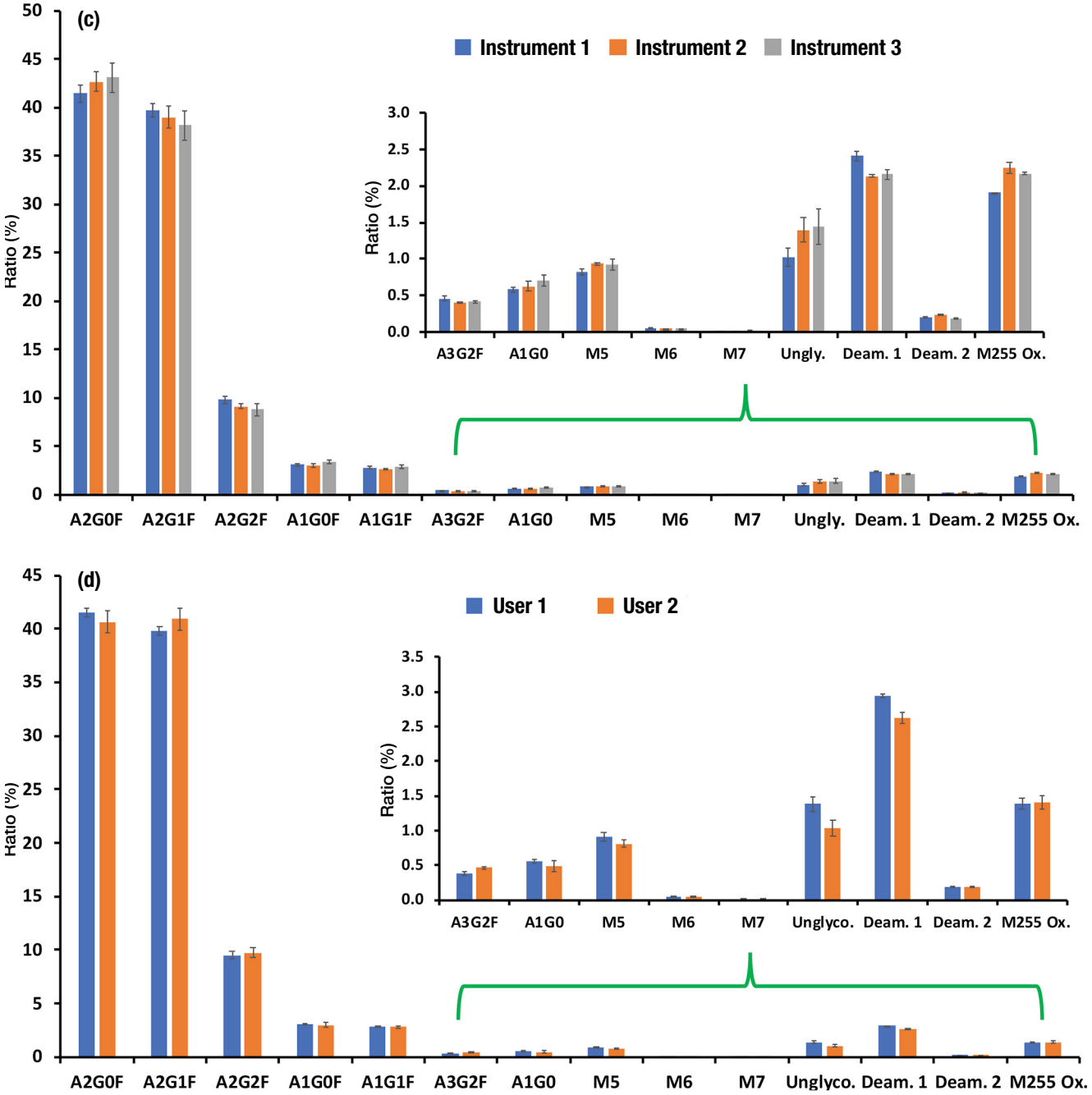


Figure 9-2. Ratios of selected CQAs of NISTmAb, including common glycan forms of EEQYNSTYR, deamidation of GFYPSDIAVEWESNGQPENNYK ("Deam. 1") and FNWYVDGVEVHNAK ("Deam. 2"), and Met oxidation of DTLMISR ("M255 Ox."). (c) Average ratios for the same NISTmAb digest analyzed on three different Q Exactive Plus mass spectrometers. (d) Average ratios for NISTmAb digests prepared by two individuals measured at different times on the same instrument. The insets show the expanded view of the low abundant forms. The error bars show the standard deviations of the ratios measured from 3 to 6 technical replicates. Note that the ratios of "Deam. 1" and "Deam. 2" for GFYPSDIAVEWESNGQPENNYK and FNWYVDGVEVHNAK, respectively, are the sums of deamidation, succinimide, and isomerization (for "Deam. 2").

Some low-level host cell proteins (HCPs) in the NISTmAb digest were identified in our previous experiments. As an example, peptide TFTTQETITNAETAK from glucose 6-phosphate isomerase was monitored in this work. This peptide was reproducibly detected and quantified with a monoisotopic ion intensity of less than 2E+4 (Figure 10). Previous analyses of NISTmAb have quantified this protein at approximately 10–15 ppm relative to the drug substance. Despite the significant dynamic range, the HR MAM workflow was able to quantify this peptide easily and consistently.

Thermal and oxidative stress studies

To investigate the thermal and oxidative effects on NISTmAb degradation, one aliquot (10 µL) of NISTmAb was incubated at 60 °C for 5 days, while another aliquot was treated with 0.03% hydrogen peroxide for 24 hours prior to trypsin digestion. The results of the control and two stressed samples for selected modifications were compared in Figure 11. The stressed conditions employed in this work had a negligible effect on the glycosylation profile (Figure 11a), lysine glycation (data not shown), and lysine clipping (~85% clipped, not shown), indicating that these modifications are neither temperature nor oxidant sensitive. By comparison, there was a slight decrease in abundance of the non-glycosylated peptide EEQYNSTYR under both stress conditions. The abundance of N-terminal pyroglutamate increased from ~99.2% to ~100% under thermal stress but remained nearly unchanged under the oxidatively stressed condition (data not shown).

The most significant CQA variations for NISTmAb were deamidation and isomerization under thermal stress and methionine oxidation with oxidative stress. The dynamic range between normal and stressed conditions, in terms of relative abundance, spanned multiple orders of magnitude. For asparagine residue N289, deamidation increased more than 10-fold from ~0.06% to 0.78% (13-fold), while other positions, such as N392, had a more modest increase from ~1.6% to 3.7% (2.3-fold). Exposure to thermal stress also had a significant effect on the isomerization of the aspartic acid. The ratio of isoAsp (D283) of FNWYVDGVEVHNAK increased by >60-fold (from ~0.08% to ~5.2) (Figure 11b). Similarly, D315 isomerization of VVSVLTVLHQDWLNGK was consistently present at ~0.1% ratio in the thermally stressed sample, but barely detected in the control sample (Figure 12). Two deamidation forms (at N318 and Q314) of this peptide followed a similar pattern (data not shown). By contrast, the abundance of succinimide formation at Asn was not greatly affected by thermal stress (e.g. N392 Asu and N289 Asu in Figure 11b).

While oxidative stress did not appear to affect the level of deamidation in DTLMISR (blue bars, Figure 11b), the abundance of M255 oxidation did increase significantly (from ~2% to ~14%) under thermal stress (Figure 11c), indicating increased reaction kinetics of oxidation at higher temperatures. The oxidatively stressed condition increased oxidation at most monitored sites for the NISTmAb, including a 15- to 25-fold increase in abundance for M255 and M4 oxidation in DTLMISR (Figure 11c) and DIQMTQSPSTLSASVGDR (data not shown), respectively.

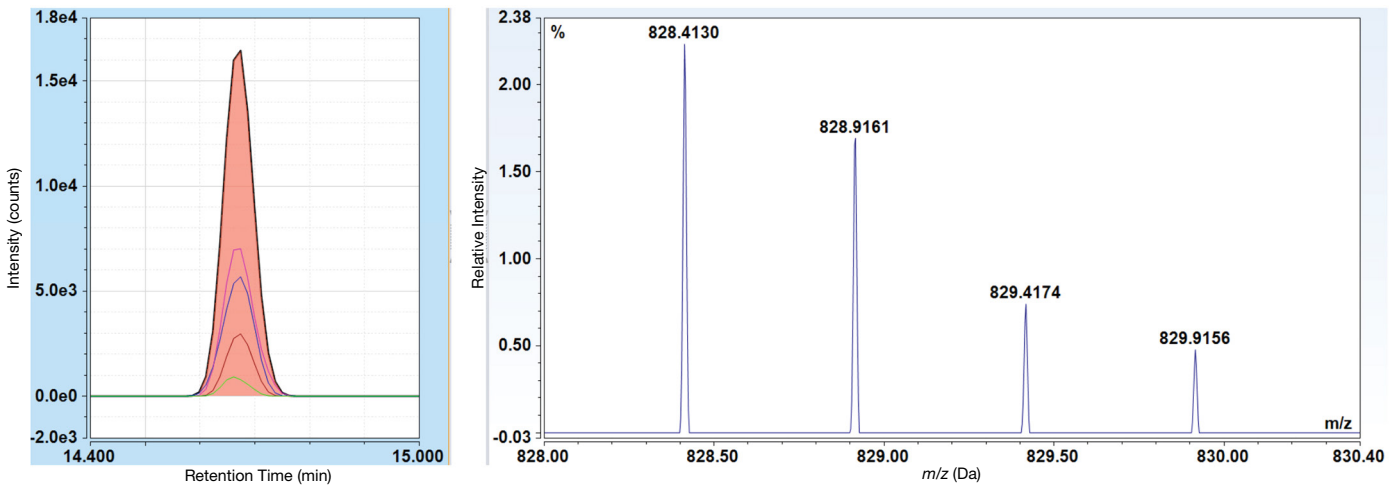


Figure 10. XIC (left panel) and mass spectrum (right panel) of peptide TFTTQETITNAETAK from glucose 6 phosphate isomerase, a low-level host cell protein present in NISTmAb at approximately 10–15 ppm relative to the drug substance. Despite the significant dynamic range, the HR MAM workflow was able to quantify this peptide easily and consistently.

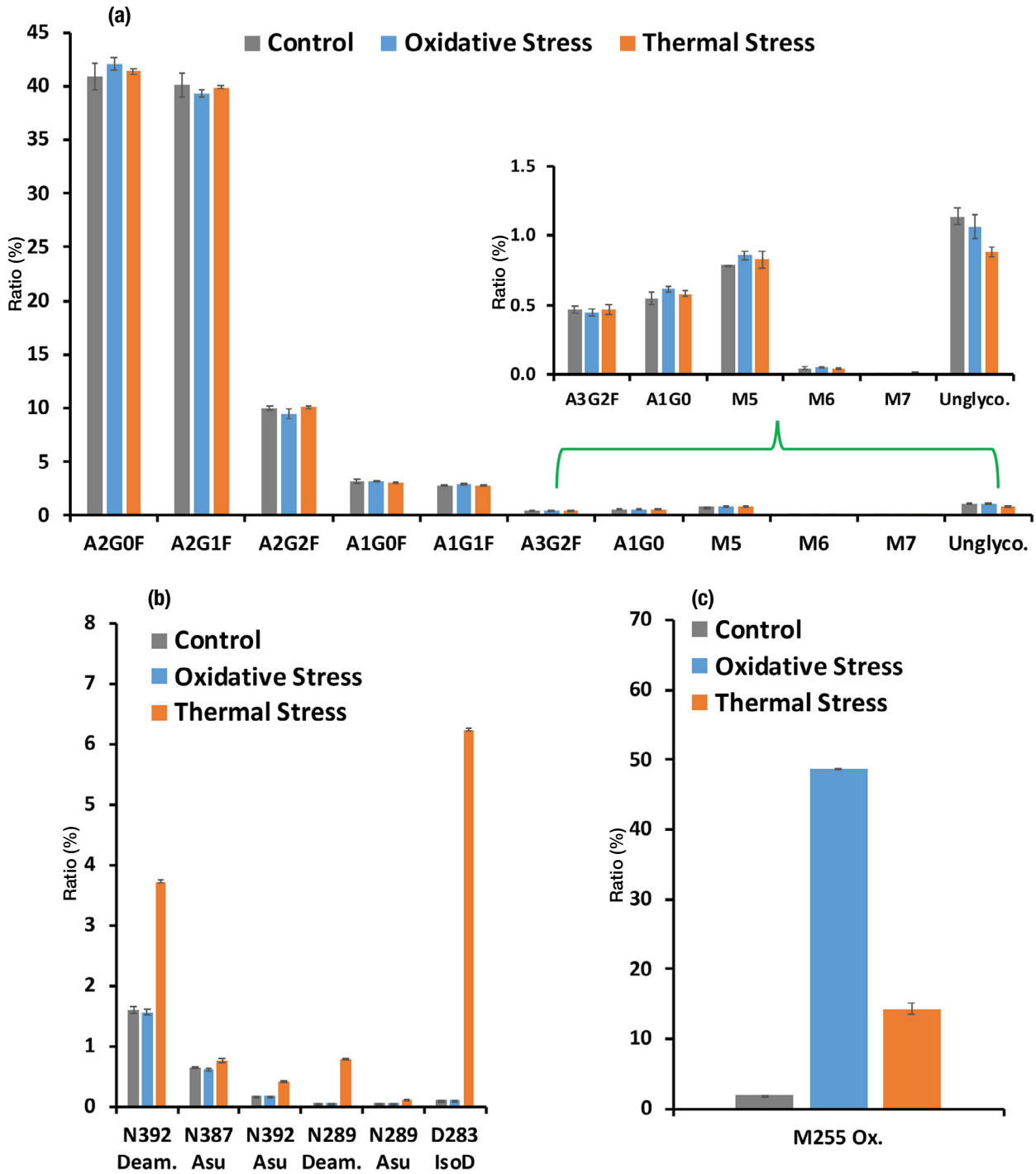


Figure 11. Quantitative results of modifications in EEQYNSTYR (a), GFYPSDIAVEWESNGQPENNYK and FNWYVDGVEVHNAK (b), and DTLMISR (c) in the control (gray), oxidative stress (blue), and thermal stress (orange) samples (error bars represent standard deviation measured from three technical replicates)

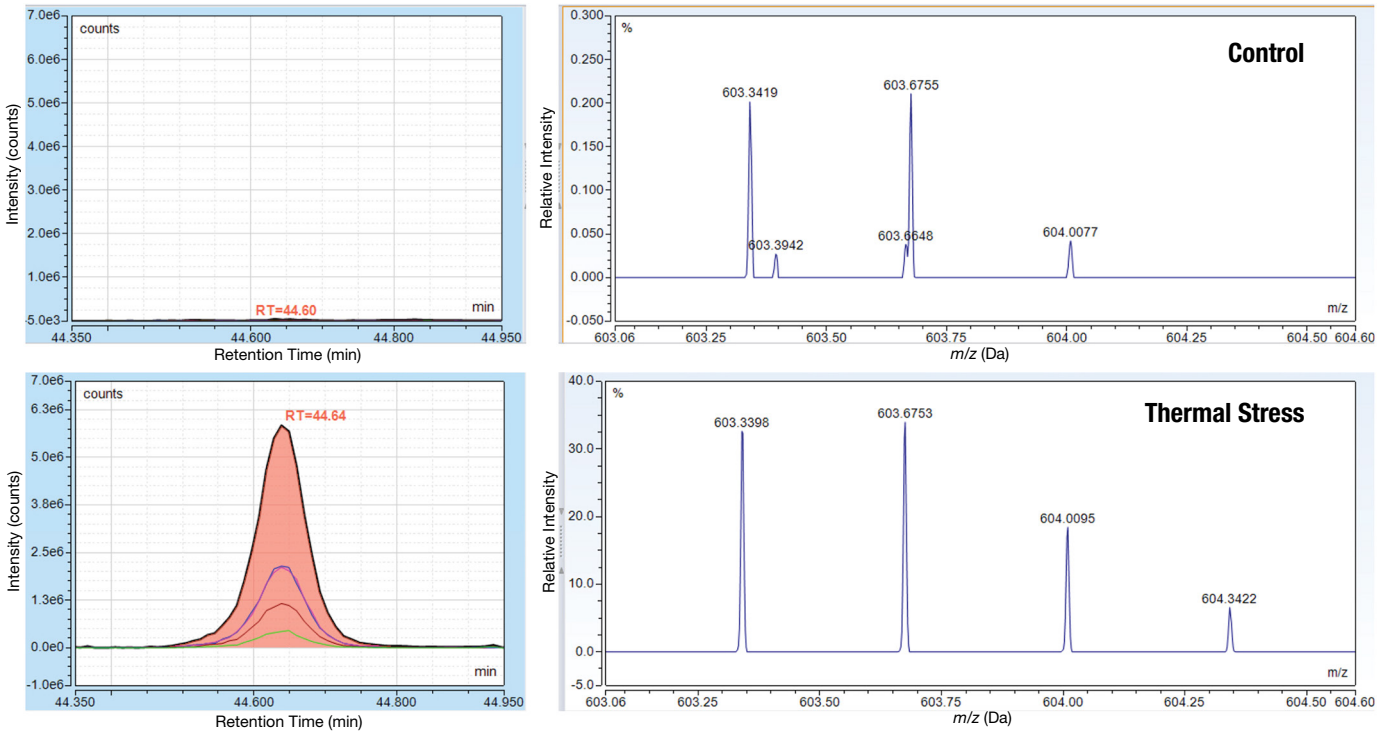


Figure 12. D315 isomerization of VVSVLTVLHQDWLNGK in the control (top panels) and thermally stressed (bottom panels) NISTmAb digests. XICs (left panels) are shown at the same scale. Mass spectra (right panels) as recorded at apex RT. D315 isomerization was negligible in the control sample (top panels). By contrast, its abundance was significantly increased under a thermally stressed condition (bottom panels).

New Peak Detection (NPD)

In addition to detection and quantitation of known quality attributes, an essential component of the HR MAM workflow is detection of new features, including potential unknowns and impurities. The settings used for NPD are given in Table 4. Generally, the maximum number of frames (potential new features) can be set to the maximum value of 16,000 with minimal impact on computing duty cycle. The frame width in mass accuracy (ppm) can be set to 10 ppm (±5 ppm) given the observed Orbitrap mass accuracy, while the width in RT (min) depends upon the chromatographic performance. The most critical parameter is the Peak Intensity Threshold. This value must be carefully chosen based on not only the intensity of the total ion or base peak chromatogram but also on the question to be answered. Setting a very low threshold may greatly increase the chance of finding false positives. With careful testing and benchmarking, this threshold value could be used for purity test and lot release.

The detected features were filtered with the settings shown in Table 4. Briefly, only features with an identified monoisotopic mass (PR Element = 0) and at least one additional isotope (PR Size > 1) for charge state +2 were considered. Finally, those features not showing significant change relative to the reference injection were filtered out. A ratio of 1000 was used to filter the result of the NISTmAb + PRTC data as PRTC peptides were absent in the reference data (control). For stressed samples, a minimum ratio of 20 was applied to the result to identify the features with a significant fold change from the reference.

Among the control samples, both replicate digests and replicate injections (using the first injection from the first digest as the reference) revealed no significant changes, as expected. To act as a positive control for NPD, the PRTC was spiked into NISTmAb digest at a concentration of 500 fmol/µL. All 15 PRTC peptides were detected using the non-targeted MS processing feature in Chromeleon software (Table 5). Figure 13 shows a peak (HVLTSIGEK) detected in the NISTmAb + PRTC sample but not in the control sample. The ratio of all PRTC peptides in the spiked vs. control samples were >10,000, except SAAGAFGPELSR, whose abundance was ~1000 times higher in the spiked sample than in the control (Table 5). The second isotope of a low abundant +5 peak with an *m/z* nearly identical to SAAGAFGPELSR was present in the control sample, making the measured ratio lower than it should be. This indicates that caution must be taken to set the threshold for NPD, as some ratios may be under-represented due to interfering peaks. This is particularly concerning with low resolution, low mass

accuracy instruments. However, the HRAM feature of the Thermo Scientific HR MAM workflow helps minimize the occurrence of any interferences, thus improving the confidence in NPD. It should be noted that some impurities already present in PRTC were also detected as new peaks.

Table 5. All 15 PRTC peptides identified as new peaks in NISTmAb spiked with PRTC by comparing to the reference data (control). Results were filtered using the settings shown in Table 4.

m/z (Da)	RT (min)	Ratio	Charge	PRTC Peptides
493.7678	8.20	99999.9	2	SSAAPPPPPR
613.3159	8.43	99999.9	2	GISNEGQNASIK
496.2857	9.16	99999.9	2	HVLTSIGEK
422.7358	11.09	99999.9	2	IGDYAGIK
451.2831	11.26	148197.2	2	DIPVPKPK
695.8317	13.70	10780.1	2	TASEFDSAIAQDK
586.8009	16.26	1002.6	2	SAAGAFGPELSR
773.8978	19.66	99999.9	2	ELGQSGVDTYLQTK
558.3266	26.46	99999.9	2	GLILVGGYGTR
745.3932	27.54	99999.9	2	SFANQPLEVVYSK
801.4116	27.69	41221.1	2	GILFVGSGVSGGEEGAR
498.8022	31.54	27315.4	2	LTILEELR
573.3033	35.99	99999.9	2	NGFILDGFPR
680.3743	39.65	99999.9	2	ELASGLSFPVGFK
787.4203	42.94	99999.9	2	LSSEAPALFQFDLK

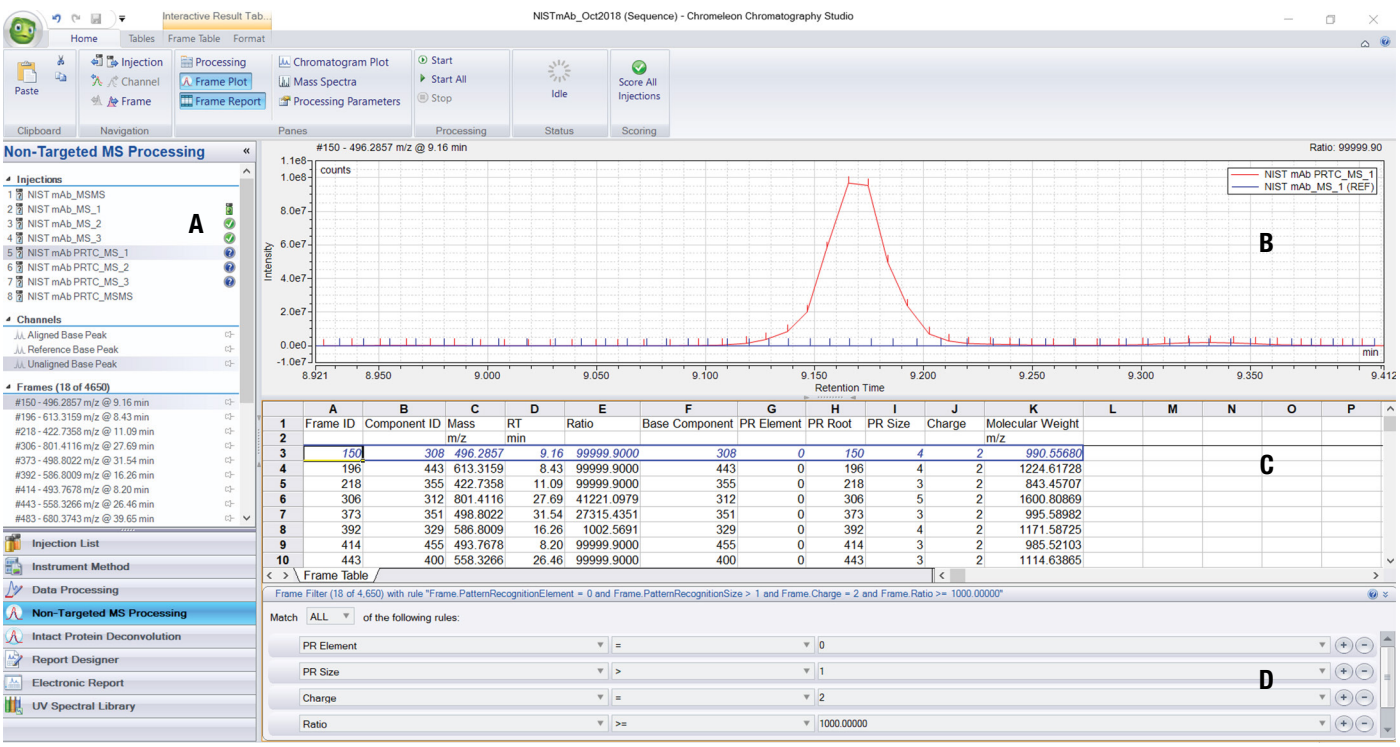


Figure 13. An example of NPD (HVLTSIGEK in PRTC) reported by Chromeleon software. Region A: Injections, Channels, and Frames information; Region B: XICs of the selected component in the PRTC spiked and reference samples; Region C: new peaks detected; Region D: filtering rules. The blue and red traces in Region B represent the XICs of HVLTSIGEK in the reference (NISTmAb control) and spike (NISTmAb + PRTC) samples, respectively.

The NPD strategy described above can be applied to the stressed NISTmAb samples to detect “new” or dramatically changing peptides. Most of these correspond to deamidation and isomerization in the thermally stressed sample and oxidation in the oxidatively stressed sample. Shown in Figure 14 are two examples of “new peaks” detected in two stressed samples, where D315 isomerization and M255 oxidation increased by >20-fold compared to the control sample.

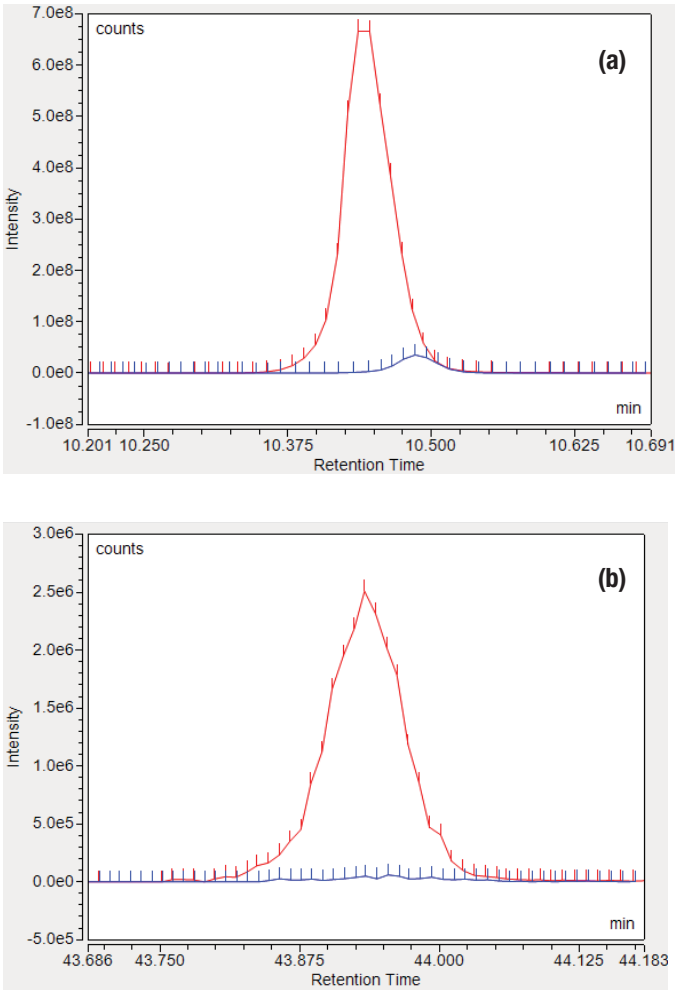


Figure 14. Examples of new peaks detected in the thermal (D315 isomerization in VVSVLTVLHQDWLNGK (a), and oxidatively (M255 oxidation in DTLMISR) (b) stressed samples by comparing to the control sample. The blue and red traces represent the XICs of this peptide in the control and stressed samples, respectively.

Conclusion

The Thermo Scientific HR MAM workflow described in this application note provides the robustness, flexibility, 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 ability of the Q Exactive Plus mass spectrometer makes it possible to resolve species that would otherwise be overlapped, leading to accurate CQA quantitation and reliable NPD. This, combined with the robust separation offered by the Vanquish Horizon UHPLC system and Accucore Vanquish C18+ column, 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.

As the MAM gains in popularity and recognition within the biopharmaceutical industry and with the regulatory agencies, the workflow described herein can serve as useful guidance for those who are using, or wish to use, this technology in different phases of drug development and QC.

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