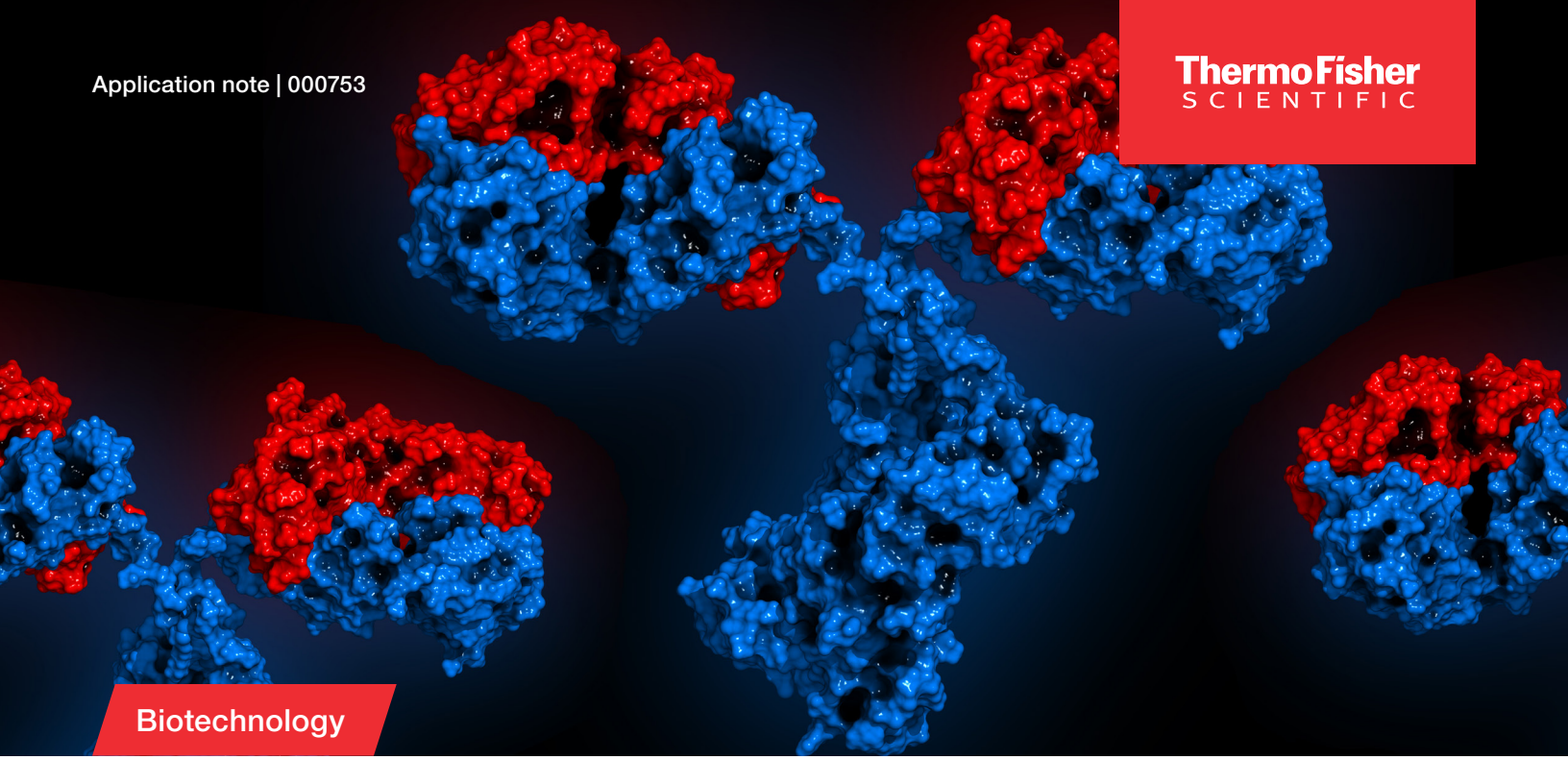
Application note | 000753



Biotechnology

Development of a robust multi-attribute method suitable for implementation in a QC environment

Authors

Silvia Millán-Martín[1], Sara Carillo[1], Craig Jakes[1], Hao Yang[2], Melissa Sato[2], Royston Quintyn[3], Catharina Crone[4], Julia Kraegenbring[4], Kai Scheffler[5], Min Du[6], Jonathan Bones[1]

[1]National Institute for Bioprocessing Research and Training (NIBRT), Ireland

Thermo Fisher Scientific, [2]San Jose, CA, USA; [3]Somerset, NJ, USA; [4]Bremen, Germany; [5]Germering, Germany; [6]Boston, MA, USA

Keywords

Multi-attribute method (MAM), monoclonal antibody (mAb), quality control (QC), liquid chromatography—high-resolution accurate-mass mass spectrometry (LC-HRAM-MS), critical quality attribute (CQA), new peak detection (NPD), Thermo Scientific MAM 2.0 workflow, Orbitrap Exploris MX mass detector, BioPharma Finder software, Chromeleon CDS

Application benefits

- The robust LC-HRAM-MS based multi-attribute method (MAM) developed on the Thermo Scientific™ Vanquish™ Flex UHPLC system coupled to the Thermo Scientific™ Orbitrap Exploris™ MX mass detector enables the generation of highly reproducible and consistent data to meet quality control implementation requirements.
- The method provides excellent repeatability, intermediate precision, and reproducibility across laboratories for targeted product quality attribute monitoring of NISTmAb and rituximab.
- The non-targeted processing method for new peak detection in Thermo Scientific™ Chromeleon™ CDS can consistently detect all PRTC peptides in spiked rituximab samples down to 0.01% (w/w) level.

Goal

Demonstrate that the end-to-end Thermo Scientific™ MAM 2.0 workflow enables reproducible and robust CQA monitoring and new peak detection from instrument to instrument, and site to site.

Introduction

In recent years, LC-HRAM-MS based multi-attribute method (MAM) has emerged as the method of choice to meet Quality by Design (QbD) principle requirements for improving product and process understanding during development and manufacturing of biotherapeutics, ensuring product quality, safety and efficacy.[1-3] Since its introduction, biopharmaceutical companies have been actively working with regulatory agencies to

thermo scientific

investigate the potential and benefit of deploying MAM to replace multiple conventional assays, not only during product and process development, but also for stability and product release testing in commercial quality control (QC) environments.[4,5] Successful implementation of MAM as a commercial QC release assay requires overcoming challenges in QC laboratories, such as lack of mass spectrometry expertise or stringent compliance requirements, as well as consistent system-to-system performance that provides highly reproducible monitoring of critical quality attributes (CQAs) and robust new peak detection (NPD) of potential additional process-related and/or product-related impurities.

We recently introduced the end-to-end Thermo Scientific™ MAM 2.0 workflow to purposefully address these challenges. The implementation of an integrated LC-MS system performance evaluation test (SET) and seamless method transfer from development to QC labs enabled by the MAM 2.0 workflow were discussed in recently published application notes.[6,7] In this work, we focused on the compliant-ready monitoring portion of the workflow. We conducted an interlaboratory study involving two labs in Dublin, Ireland and San Jose, CA, USA to demonstrate the repeatability, intermediate precision, reproducibility, and linearity analysis of relative quantification of the selected critical quality attributes (CQAs) as well as the robustness of NPD. As shown in Figure 1, the MAM workflow was performed using tryptic digests of NISTmAb and rituximab, separated on a Thermo Scientific™ Hypersil GOLD™ Vanquish C18 UHPLC column installed on a Vanquish Flex UHPLC system, and followed by mass detection using a Full MS only instrument method on an Orbitrap Exploris MX mass detector with the entire LC-MS system fully operated under Chromeleon CDS software. Three types of samples were prepared: 1) unstressed NISTmAb and unstressed rituximab digest, 2) heat stressed rituximab digest, and 3) rituximab digest spiked with four different amounts of peptide retention time calibration (PRTC) standard. Each sample type was pooled first before aliquoting for distribution to the different sites. The MAM workflow was executed on three systems across both sites for targeted mAb CQA quantitation and NPD analysis. This MAM solution exhibits exceptional precision with reproducibility of less than 10% for selected CQAs that are above 1% for both unstressed and heat stressed rituximab digests. In addition, with optimized NPD retention time window, mass tolerance, NPD MS signal, and peak area threshold, all 15 PRTC peptides for all PRTC spiked samples on all systems were consistently detected.

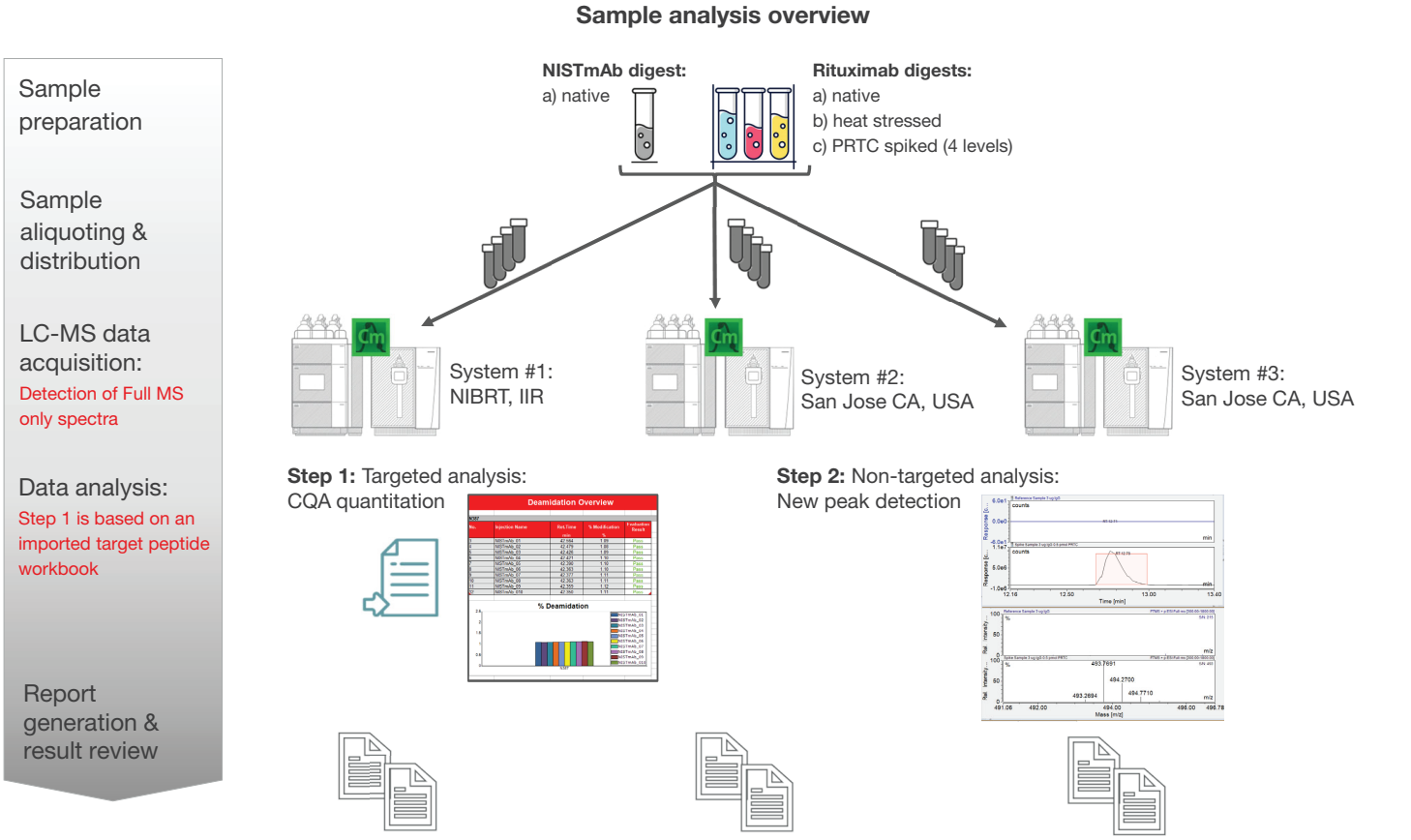


Figure 1. Interlaboratory evaluation of mAb CQA quantitation and new peak detection, applying the monitoring part of the MAM workflow, which is based on Full MS only data acquisition and data processing using a target peptide workbook derived from an earlier characterization step that was not part of this study

Experimental

Reagents and consumables

- NISTmAb Humanized IgG1κ Monoclonal Antibody Lot 14HD-D002 (NIST, RM 8671)
- Rituximab monoclonal antibody
- Thermo Scientific™ Pierce™ Peptide Retention Time Calibration (PRTC) Mixture 200 µL (P/N 88321)
- 8.0 M Guanidine hydrochloride solution (Sigma, P/N G7294-100ML)
- Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 (P/N 15567027)
- Sodium hydroxide concentrate (Sigma, P/N 43617-1L)
- Sodium iodoacetate (IAC) BioUltra > 98% purity (Sigma, P/N I-9148)
- DL-Dithiothreitol (DTT) BioXtra ≥ 99% purity (Sigma, P/N D-5545)
- Thermo Scientific™ Pierce™ Formic acid, LC-MS grade (P/N 28905)
- Thermo Scientific™ Pierce™ Trypsin Protease MS grade (P/N 90058)
- Bio-Spin™ P-6 gel columns, Tris buffer (Bio-Rad, P/N 732-6227)
- Eppendorf™ Protein LoBind™ Microcentrifuge Tube 0.5 mL (P/N 022431064)
- Eppendorf™ Protein LoBind™ Microcentrifuge Tube 1.5 mL (P/N 022431081)
- Thermo Scientific™ Hypersil GOLD™ Vanquish C18 UHPLC column, 1.9 µm, 2.1 × 150 mm (P/N 25003-152130-V)
- Thermo Scientific™ Water, UHPLC-MS grade (P/N W8-1)
- Thermo Scientific™ Acetonitrile, UHPLC-MS grade (P/N A956-1)
- Thermo Scientific™ Methanol, UHPLC-MS grade (P/N A458-1)
- Fisher Chemical™ Formic acid (FA), 99.0%, Optima™ LC-MS grade (P/N 10797488)
- Thermo Scientific™ Screw vial caps and seals (P/N 10656984)
- Thermo Scientific™ 9 mm glass autosampler inserts 400 µL (P/N 11911563)

Monoclonal antibody (mAb) preparation

For this study, NISTmAb, unstressed, and heat-stressed rituximab samples were prepared and digested using the following stock reagents.

Solution 1: 7.0 M Guanidine HCl, 100 mM Tris (pH 8.3)
87.5 mL of 8 M guanidine HCl and 10 mL of 1 M Tris-HCl pH 7.5 were added to a 100 mL volumetric flask and mixed thoroughly through inversion. The pH was measured using a pH meter and adjusted to a pH of 8.3 with sodium hydroxide concentrate. LC-MS grade water was added to bring the total volume to 100 mL.

Solution 2: 500 mM DTT in solution 1
50 mg of DTT were freshly weighed into a 1.5 mL Eppendorf tube and 649 µL of solution 1 were added. The solution was mixed by vortex until the solution became clear.

Solution 3: 500 mM IAC in solution 1
50 mg of IAC were freshly weighed into a 1.5 mL Eppendorf tube and 481 µL of solution 1 were added. The solution was mixed by vortex until the solution became clear. It is important to store solution 3 in the absence of light.

Solution 4: 50 mM DTT in solution 1
100 µL of solution 2 were added to 900 µL of solution 1 in a fresh 1.5 mL Eppendorf tube and mixed thoroughly by vortex.

Solution 5: 50 mM Tris (pH 7.9)
10 mL of 1 M Tris-HCl pH 7.5 were added to a 200 mL volumetric flask and brought to a final volume of 200 mL using LC-MS grade water. The flask was mixed through inversion and the pH was checked using a pH meter.

Solution 6: 10% Formic acid
1 mL of LC-MS grade formic acid was added to 9 mL of LC-MS grade water in a 15 mL tube and mixed by vortex.

Generation of heat-stressed samples

Heat-stressed samples were generated by adding 100 µg of rituximab to individual 0.5 mL LoBind Eppendorf tubes and incubated for 120 hours at 50 °C with low level of mixing. After 120 hours of incubation, the samples were spun down briefly with a mini centrifuge prior to sample preparation.

Sample reduction and alkylation

100 µg of mAb were diluted to 1 mg/mL with solution 1 to give a final volume of 100 µL. 2 µL of solution 2 were added to the sample and mixed by vortex. Reduction was carried out by allowing the samples to stand at room temperature for 30 minutes. 4 µL of solution 3 were added to each sample and mixed by vortex. Alkylation was carried out by allowing the sample to stand at room temperature for 20 minutes in the absence of light. Alkylation was quenched by adding 4 µL of solution 4 to each sample and mixed by vortex.

Buffer exchange

BioSpin-6 columns were conditioned by breaking off the tip and inserting the bottom of the column into a 2 mL collection tube. The columns were centrifuged at 1,000 × g for 2 minutes, and the flow through was discarded. 500 µL of solution 5 were gently pipetted to the bed of the BioSpin-6 column and centrifuged at 1,000 × g for 2 minutes. The flow through was discarded. This step was repeated three more times for a total of four washes. Following the washing procedure, the conditioned column was placed in a fresh 1.5 mL LoBind Eppendorf tube. 110 µL of the reduced and alkylated sample were added to the bed of the column. The column was then centrifuged at 1,000 × g for 4 minutes and the flow through was collected.

Sample digestion

Pierce trypsin protease was reconstituted by adding 100 µL of LC-MS grade water to the vial. 10 µL of trypsin were added to 100 µL of buffer exchanged mAb sample with a ratio of 1:10 v/v. The samples were mixed briefly by vortex before incubation at 37 °C for 30 minutes. 11 µL of solution 6 were added to the sample post digestion to quench any residual trypsin and was briefly mixed by vortex.

Preparation of PRTC spiked samples

PRTC mixture was reconstituted to a concentration of 5 pmol/µL using LC-MS grade 0.1% formic acid in water. Four different concentrations of PRTC-spiked rituximab digest samples were prepared using Table 1.

Chromatography

The Vanquish Flex UHPLC system was used for the applied gradient detailed in Table 2. The modules included in the system are listed in Table 3. Unless otherwise stated, 4 µL containing a total of 4 µg digest samples were injected onto a Hypersil GOLD Vanquish C18 UHPLC column using the LC gradient and conditions outlined in Table 2.

Table 1. Preparation of PRTC spiked rituximab digest samples. Various amounts of PRTC peptides are added to rituximab digests to represent different impurity levels, which are expressed as % relative (w/w). More volumes of PRTC peptides and rituximab digests were required for the preparation of Spiked B samples as these were used in both injection sequence 2 and 3 (see Table 5).

Sample	PRTC concentration (pmol/µL)	% relative to rituximab digest (w/w)	Volume of PRTC at 5 pmol/µL (µL)	Volume of LC-MS grade water (µL)	Volume of digested rituximab at 1 mg/mL (µL)
Spike A	0.0625	0.01	7	49	504
Spike B	0.125	0.02	40	120	1440
Spike C	0.25	0.04	28	28	504
Spike D	0.5	0.08	56	0	504

Table 2. LC and autosampler conditions

Parameter	Value	
UHPLC column	Hypersil GOLD Vanquish C18 UHPLC column, 150 × 2.1 mm, 1.9 µm (P/N 25002-152130-V)	
Column temperature	25 °C	
Flow rate	0.25 mL/min	
Solvent A	H_2O + 0.1% FA	
Solvent B	ACN + 0.1% FA	
Gradient	Time (min)	%B
	0.0	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
Injection volume	4 µL	
Needle wash solution	10% MeOH with 0.1% FA	
Seal rinse solution	10% MeOH	
Autosampler temperature	6 °C	
Thermostatting mode	Still Air	
Needle wash option	Before and after injection	
Wash speed and time	34 µL/s for 10 s	

Table 3. Vanquish Flex UHPLC system modules and part numbers

Modules	Vanquish Flex (P/N)
Vanquish System Base F/H	VF-S01-A-02
Vanquish Binary Pump F	VF-P10-A-01
Vanquish Split Sampler FT	VF-A10-A-02
Vanquish Column Compartment H	VF-C10-A-03

Mass spectrometry

The interlaboratory study involved the monitoring of selected rituximab CQAs using a full MS scan method on three Orbitrap Exploris MX mass detectors across two sites (First site: NIBRT, Dublin, Ireland; second site: San Jose, CA, USA). Detailed instrument methods and source parameters for the mass detector are summarized in Table 4.

Table 4. Instrument method parameters for the Orbitrap Exploris MX mass detectors. (Note: Unless otherwise indicated, default parameters were used.)

Instrument	Orbitrap Exploris MX
Source parameters	
Positive ion (V)	3,500
Sheath gas (Arb)	30
Aux gas (Arb)	10
Sweep gas (Arb)	1
Ion transfer tube temperature (°C)	225
Vaporizer temperature (°C)	200
Full MS scan settings	
Expected LC peak width (s)	6
Resolution at 200 *m/z*	120,000
Scan range (*m/z*)	200–2,000
RF Lens (%)	70
Time range (min)	0–70
AGC targets*	5E5, 1E6, 3E6
Maximum injection time (ms)	100

* Three AGC targets were first assessed against different sample loads for linearity and dynamic range evaluation; the AGC target of 1E6 showed the best performance across all monitored peptides and was used for remainder of the studies.

Injection sequences

Three injection sequences were created for this study. Sequence 1 was created to assess dynamic range for targeted quantitation of rituximab CQAs under stressed conditions. Sequence 2 was created for repeatability, intermediate precision, and reproducibility analysis of selected rituximab CQAs. Sequence 3 was created for new peak detection of PRTC spiked samples. With the exception of sequence 1, both sequence 2 and 3 were collected on all three systems used in this study. Detailed sequence information is outlined in Table 5. All data were uploaded to a single processing computer for data analysis and report.

Table 5. Detailed sequence information for interlaboratory study

Sequence 1		Sequence 2		Sequence 3	
Sample	**Injection volume (µL)**	**Sample**	**Injection volume (µL)**	**Sample**	**Injection volume (µL)**
Blank_1	4	Blank_1	4	Blank_1	4
NISTmAb_1		NISTmAb_1		NISTmAb_1	
NISTmAb_2		NISTmAb_2		NISTmAb_2	
Blank_2		Blank_2		Blank_2	
Stressed rituximab	1	Rituximab (3 replicates)		Rituximab (3 replicates)	
	4	Stressed Rituximab (3 replicates)		Spiked A (3 replicates)	
	7	Blank_3		Spiked B (3 replicates)	
	2	Spiked B (3 replicates)		Blank_3	
	10	Blank_4		NISTmAb_3	
	7	NISTmAb_3		Blank_4	
	10			Spiked C (3 replicates)	
	4			Spiked D (3 replicates)	
	1			Blank_5	
NISTmAb_3	4			NISTmAb_4	
Blank_3					
Stressed rituximab	2				
	1				
	4				
	7				
	2				
	10				
Blank_4	4				
NISTmAb_4					
System #2		**All systems**			

Targeted processing methods for CQA quantitation in Chromeleon CDS

Three targeted processing methods were created based on the MS quantitative template: 1) for NISTmAb injections as a system suitability check, 2) for rituximab injections for targeted quantitation of CQAs, and 3) for PRTC spiked samples for retention time (RT), peak area, and peak height evaluation of PRTC peptides. All processing methods apply the following settings: MS detection algorithm ICIS, manually defined mass tolerance 5 ppm, inhibit integration for TIC channel, Gaussian smoothing 5 points.

Composite scoring using isotopic dot product, mass accuracy, and peak apex alignment options were applied with scoring results to pass only if three criteria were met. A target peptide workbook was created in BioPharma Finder 5.0 software like the previously published work,[7] and it contained a list of associated peptides for selected post-translational modifications as shown in Table 6. The peptide list was imported to the MS component table of the respective processing method, RT of individual components were adjusted, and peak integration parameters were optimized to ensure accurate component detection and consistent peak integration across datasets.

Non-targeted processing method for new peak detection in Chromeleon CDS

A non-targeted processing method was created based on the same algorithm that was used for sequence identification during peptide mapping analysis in BioPharma Finder 5.0 software. This method was applied to all PRTC spiked sample injections (e.g., Spiked A, B, C, and D) in sequences 2 and 3 as shown in Table 5. For new peak detection, the first rituximab digest sample (i.e., injection #5) served as the control injection. "Auto-compute basic parameters" feature was used to determine the absolute MS signal threshold for each test injections. Once the threshold value was determined, the S/N threshold was adjusted so the calculated MS signal threshold was around 3E5. For consistency, all test injections collected across three systems were evaluated using 3E5 as the MS signal threshold. False positives were eliminated by applying the following filter rules: charge state equals to 2, MS area ratio is not between 0 to 10, and MS area is greater than 1E5. New peaks were visually examined, and only peaks that were detected consistently across all three replicates were counted as "new peaks".

Identification of new peaks in BioPharma Finder software

BioPharma Finder software was used for identification of new peaks using full MS scan data only. For accurate detection and identification of the new peaks observed from non-targeted processing of the test injection, the same peak detection parameters were used, which included a manually adjusted S/N threshold to obtain 3E5 as the MS signal threshold. Identification of the new peaks was done by matching the observed RT, *m/z*, and charge state.

Software

Chromeleon CDS 7.3.1 was used for all data acquisition, MAM data processing, and reporting. BioPharma Finder software 5.0 was used for NISTmAb and rituximab CQA selection, target peptide workbook creation, and new peak identifications.

Results and discussion

The aim of this interlaboratory study is to evaluate the monitoring portion of the MAM 2.0 workflow for relative quantitation of selected CQAs, as well as to demonstrate the robustness of NPD for consistent and accurate impurity detection and identification. For this study, rituximab, which is a chimeric IgG1 mAb recombinantly produced in Chinese hamster ovary cells, was used as the originators (MabThera® and Rituxan®) and its biosimilars have been well characterized in multiple published studies.[8–11] Asparagine deamidation and succinimide formation are important CQAs to monitor as these have been widely reported in recombinant mAbs, in either CDR regions or in the constant regions, which can affect mAb structure, stability and functions.[12,13] Oxidation at two conserved methionine residues, in the Fc region, have been reported to decrease thermal stability, protein A binding, and circulation half-life of IgG1 antibodies.[14–16] Glycation, which occurs during cell culture, formulation and storage, has been reported to increase the propensity of aggregation of recombinant mAbs.[17] Glycosylation is often considered one of the main CQAs as it plays an important role in the function, efficacy, *in vivo* half-life, and immunogenicity of an antibody.[18,19] Since the focus of this study is targeted CQA monitoring, a list of CQAs were selected to represent commonly monitored quality attributes for rituximab (Table 6). These CQAs are deamidation (e.g., N388 + N393, N33), succinimide formation (e.g., N290, N55), oxidation (e.g., M256, M81, and M34), glycation (e.g., K189, K330), isomerization (D284), and N-glycosylation (A2G0F, A2G1F, A2G2F, A1G0F, A2S2F, A2G0, M5 unglycosylated). A quick comparison of CQA results between the native and heat-stressed rituximab samples revealed that with the exception of glycosylation and K330 glycation, the rest of the CQAs all had a noticeable increase in % relative abundance to heat-stress (see Figure 2 and Table 6). N33 deamidation, N388/393 deamidation, N55 and N290 succinimidation, and D284 isomerization showed at least a 2-fold increase in % relative abundance in response to heat-stress. These observed trends are consistent with the previously reported forced degradation studies of rituximab.[20] Among those CQAs, N388/393 deamidation, N55 succinimidation, M256 oxidation, and D284 isomerization are known hotspots and have been shown to affect the safety and efficacy of the mAb drug product.[12–20] Hence, these four CQAs were chosen for additional method precision and linear dynamic range evaluation.

Table 6. List of CQAs and peptide sequences used to calculate each attribute for native and stressed rituximab digest. For all sequence components, the peak area for the four most abundant isotopes with charge states ranging from +1 to +5 were summed. % attribute was calculated by dividing the peak area sum of the modified component by the total peak area sum. % attribute averages (N = 18) were reported and compared between the native and heat-stressed rituximab digests. Four CQAs, as indicated by red stars, were selected for method precision and linear dynamic range evaluation.

CQA	Sequence	Native (N = 18)	Stress (N = 18)
N33 Deamidation	ASGYTFTSYN[Deamid]MHWVK (2 peaks) ASGYTFTSYNMHWVK	0.01%	0.20%
N388/393 Deamidation ★	GFYPSDIAVEWESN[Deamidation]GQPENNYK GFYPSDIAVEWESNGQPEN[Deamidation]NYK GFYPSDIAVEWESNGQPENNYK	1.4%	2.9%
N55 Succinimidation ★	GLEWIGAIYPGN[NH3 loss]GDTSYNQK GLEWIGAIYPGNGDTSYNQK	0.90%	2.2%
N290 Succinimidation	FNWYVDGVEVHN[NH3 loss]AK FNWYVDGVEVHNAK	0.05%	0.28%
M34 Oxidation	ASGYTFTSYNM[Oxidation]HWVK ASGYTFTSYNMHWVK ASGYTFTSYNMHWVKQTPGR	0.64%	0.74%
M81 Oxidation	SSSTAYM[Oxidation] QLSSLTSEDSAVYYC[Carboxymethylation]AR SSSTAYMQLSSLTSEDSAVYYC[Carboxymethylation]AR	2.4%	2.6%
M256 Oxidation ★	DTLM[Oxidation]ISR DTLMISR	2.7%	3.9%
K189 Glycation	HK[Glycation]VYAC[Carboxymethylation]EVTHQGLSSPVTK HKVYAC[Carboxymethylation]EVTHQGLSSPVTK	0.19%	0.21%
K330 Glycation	VSNK[Glycation]ALPAPIEK VSNK	0.33%	0.09%
D284 Isomerization ★	FNWYVDGVEVHNAK_Iso FNWYVDGVEVHNAK	0.09%	6.5%
N300 Glycosylation A1G0F	EEQYN[A1G0F]STYR	0.77%	0.77%
N300 Glycosylation A2G0F	EEQYN[A2G0F]STYR	39.0%	38.3%
N300 Glycosylation A2G1F	EEQYN[A2G1F]STYR	44.8%	45.5%
N300 Glycosylation A2G2F	EEQYN[A2G2F]STYR	11.0%	11.2%
N300 Glycosylation A2S2F	EEQYN[A2S2F]STYR	0.71%	0.74%
N300 Glycosylation A2G0	EEQYN[A2G0]STYR	1.27%	1.18%
N300 Glycosylation M5	EEQYN[M5]STYR	1.65%	1.55%
N300 Unglycosylated	EEQYN[Unglycosylated]STYR	0.80%	0.75%

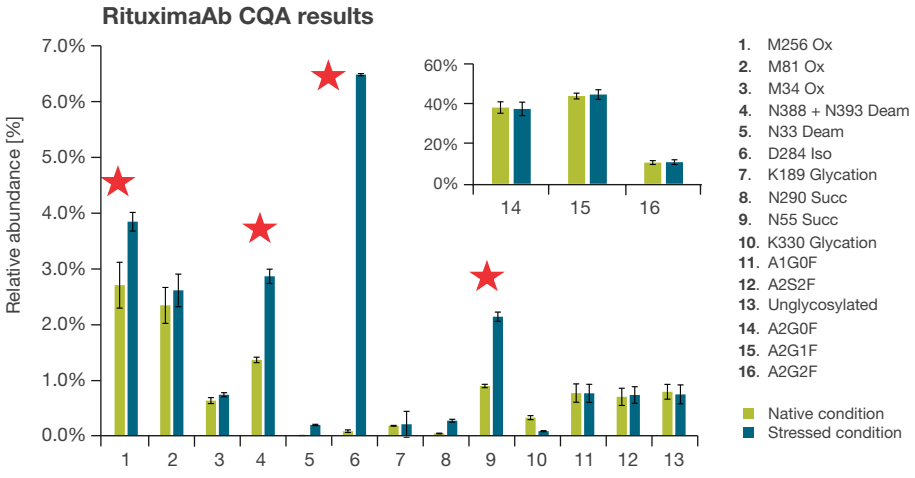


Figure 2. Result summary obtained from selected CQAs comparing native and heat-stressed (50 °C for 5 days) rituximab sample (N = 18, 6 injections per system). The peak area of the master peptide (sum of peak areas of all four isotopes) was used for the calculation of relative abundance % of the modification. The averages of 18 pooled injections were plotted for selected CQAs, and reproducibility of less than 10% CV was observed for CQA results that are above 1%. Four CQAs, as indicated by red stars, were selected for method precision and linear dynamic range evaluation.

Precision evaluation of rituximab CQAs

Since mass spectrometry has not been used regularly as a QC test for therapeutic proteins, precision including repeatability, intermediate precision, and reproducibility measurements are important for establishing method suitability in this context. For this study, seven control strategies were placed to minimize result variability:

1. All samples were prepared by the team at NIBRT, and samples with the same condition were first pooled into a master mix before aliquoting into low bind vials and distributed equally across the two sites.

2. Samples were shipped using a temperature-controlled logistics system to preserve sample integrity during the transit. A temperature sensor was placed inside the shipping container for real time monitoring of sample temperature, and dry ice was replenished during transit to keep the sample within -78 to -82 °C throughout the shipment period.

3. For all injection sequences, fresh aliquots were used, and samples were only thawed once for 30 minutes while placed in the Vanquish autosampler that was kept at 6 °C. Thawed samples were quickly vortexed to achieve homogeneity prior to injection.

4. For consistency, samples were injected using identical LC-MS configurations for all systems involved in this study (e.g., column from same lot, post-column connections, ion source x/y/z positions).

5. All systems had to pass a system performance evaluation test[6] prior to mAb sample analysis.

6. NISTmAb injections were placed at the beginning and the end of each sequence. These injections were used as check standards to bracket sample injections (e.g., unstressed, heat-stressed rituximab sample, and PRTC-spiked rituximab samples) that are used for precision and NPD performance evaluation. System performance metrics such as retention time, peak area, peak width at 10% height reproducibility, absolute peak height, and composite scoring for a few selected NISTmAb peptides were measured against pre-defined acceptance criteria, and the test sequence was qualified only if all metrics passed.

7. All sample data from qualified injection sequences were consolidated and uploaded to a single processing PC. Data were processed using the same processing methods for consistent output.

With all the control strategies in place, repeatability, intermediate precision, and reproducibility analysis of selected CQAs for both native and heat-stressed rituximab samples were evaluated. Similar to the published approach,[21] repeatability was calculated for each CQA, system, and week. Intermediate precision was calculated for each CQA and system. Reproducibility was calculated for each CQA across three systems from both laboratories. Table 7 provides a precision evaluation summary for M256 oxidation, N388/393 deamidation, N55 succinimidation, and D284 isomerization results obtained from heat-stressed rituximab. We observed less than 10% CV for repeatability and intermediate precision for data from the same system, and less than 10% CV for reproducibility across three systems for CQA results that were greater than 1% (Figure 3). An exception to note, for CQA results that are between 0.01% and 1%, the reproducibility is typically less than 20% CV (data not shown). The measured precision was comparable to the observed precision from our previous interlaboratory study of an optimized peptide mapping workflow using automated trypsin digestion for monitoring monoclonal antibody CQAs,[22] and also well within the published acceptance criteria by the biotherapeutics analytical development group of The Janssen Pharmaceutical Companies of Johnson & Johnson for implementing an LC-HRMS method in QC laboratories for release and stability testing of commercial antibody product.[21]

Table 7. Repeatability, intermediate precision, reproducibility evaluation of M256 oxidation, N388/393 deamidation, N55 succinimidation, and D284 isomerization across three systems. For repeatability analysis, mean % relative abundance and %CV of three replicate injections of heat-stressed samples were calculated. Two injection sequences were collected one week apart on each system. For intermediate precision analysis, mean % relative abundance and %CV of six pooled injections from each system were calculated. For reproducibility analysis, mean % relative abundance and %CV of 18 pooled injections for three systems were calculated.

RituximAb CQA		M256 Oxidation						N388/393 Deamidation					
System		#1		#2		#3		#1		#2		#3	
Week		1	2	1	2	1	2	1	2	1	2	1	2
Repeatability	Mean %	3.69	3.59	3.92	4.04	3.96	3.94	2.74	2.95	2.72	3.00	2.98	2.85
	%CV	0.47	0.80	0.68	0.55	0.29	1.45	1.62	1.16	1.73	0.40	5.32	0.19
Intermediate Precision	Mean %	3.64		3.98		3.95		2.84		2.86		2.91	
	%CV	1.95		2.13		0.43		5.17		6.97		3.15	
Reproducibility	Mean %	3.85						2.87					
	%CV	4.88						1.30					

RituximAb CQA		N55 Succinimidation						D284 Isomerization					
System		#1		#2		#3		#1		#2		#3	
Week		1	2	1	2	1	2	1	2	1	2	1	2
Repeatability	Mean %	2.16	2.12	2.19	2.29	2.10	2.03	6.53	6.22	6.81	6.70	6.42	6.30
	%CV	0.35	1.16	0.58	1.06	0.50	0.90	0.35	1.16	0.58	1.06	0.50	0.90
Intermediate Precision	Mean %	2.14		2.24		2.06		6.38		6.76		6.36	
	%CV	1.30		3.00		2.20		3.5		1.1		1.3	
Reproducibility	Mean %	2.15						6.5					
	%CV	4.10						3.5					

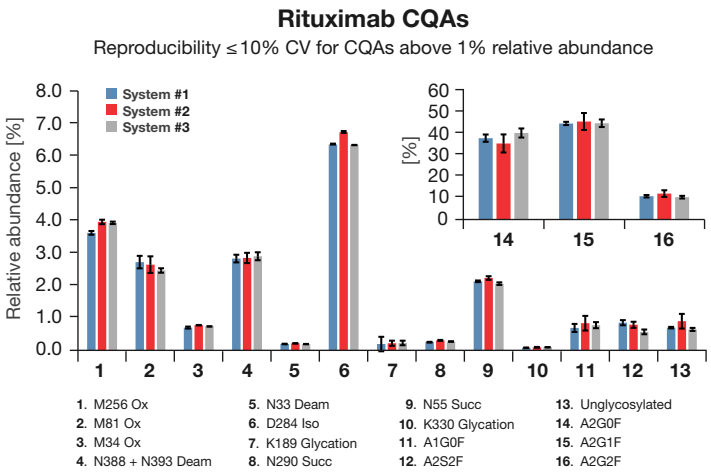


Figure 3. Interlaboratory evaluation of selected CQAs on three systems across two sites using stressed rituximab samples. Average % relative abundances (N = 18) were plotted for the selected CQAs.

Linearity and dynamic range evaluation of rituximab CQAs

For this study, variable amounts of stressed rituximab digest samples were injected to evaluate the MS response of all monitored peptides for the selected CQAs. The aim was to find the optimal Automatic Gain Control (AGC) target value such that we could consistently relatively quantify low abundant modified peptides with a %CV of less than 20% for the lowest injected amount (e.g., 1 µg) while maintaining linearity, up to 10 µg of injected sample, with a coefficient of determination or R^2 value of greater than 0.99 for the highest abundant peptide. This assessment was performed by running sequence 1 three times, each with a different AGC value ranging from 5E5 to 3E6. An AGC value of 1E6 was found to give the best precision even for CQAs below 0.1% (e.g., K330 Glycation) while maintaining linear MS response for the most abundant peptide, FNWYVDGVEVHNAK, which is used for the analysis D284 isomerization and N290 succinimide formation. As shown in Figure 4, although the loaded sample amount only varies from 1 µg to 10 µg (i.e., 1 order of magnitude), the MS peak area spans across over 4 orders of magnitude. For instance, the peak area for VSNK[Glycation]ALPAPIEK obtained from 1 µg sample load is only around 3.2E4, whereas the peak area for FNWYVDGVEVHNAK with 10 µg of sample loaded, is > 7E8. Therefore, it is essential to select an AGC target that is well balanced between precision and dynamic range for quantitation of CQAs. As shown in Figure 4, with the AGC target set to 1E6, we could consistently achieve a linear regression coefficient of greater than 0.99 for both modified and unmodified peptides that are used for the quantitation of CQAs. Despite the various injection volumes tested, quantitation of selected CQA was consistent across all injection volumes with reproducibility of less than 20% (data not shown). This would enable a lower sample load for QC testing if needed.

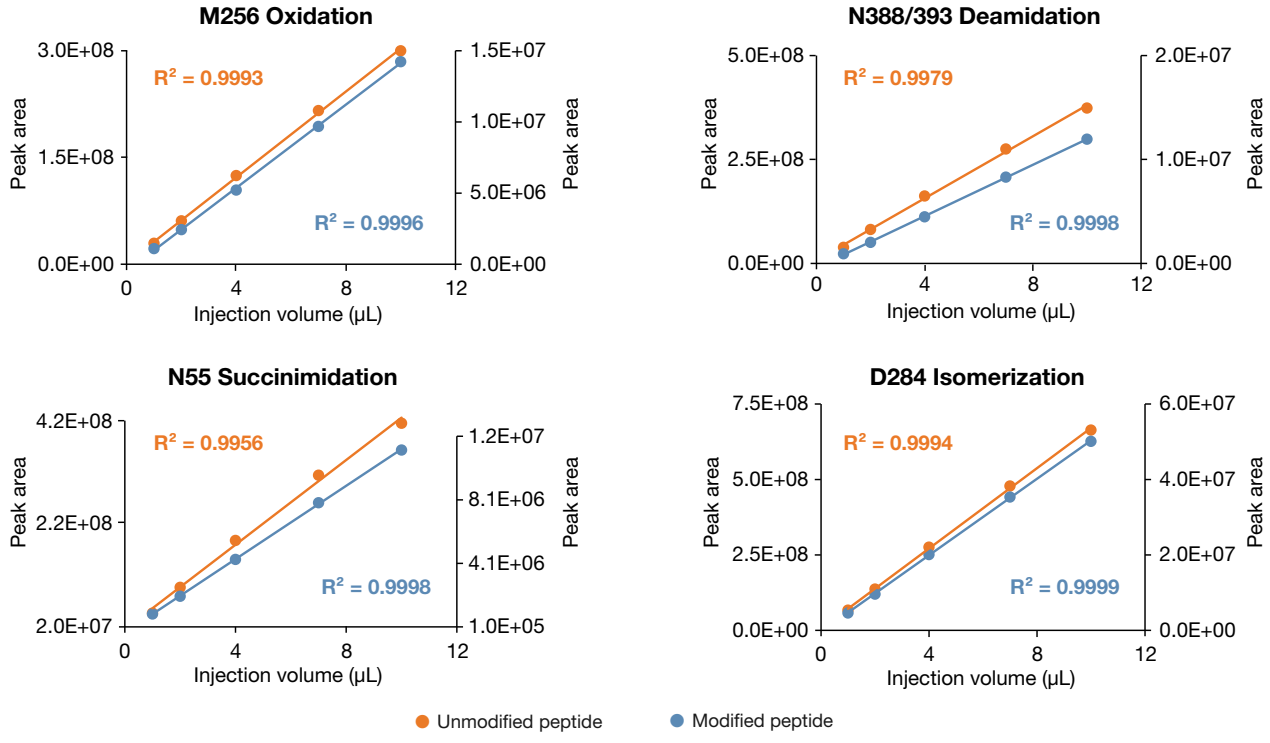


Figure 4. Linearity and dynamic range analysis of unmodified and modified peptides for selected CQAs. Average peak area (N = 3) of unmodified peptide is plotted on the left axis and average peak area of modified peptide (N = 3) is plotted on the right axis.

Detection of impurities using NPD

Another major aspect of MAM for QC testing is NPD, which is a comparative analysis between a reference data set and data obtained from a new sample, using a peak detection algorithm. The goal of the analysis is to assess if any unexpected new peaks or peaks with unexpected high or low abundance levels arise. Since MS methods are more sensitive than UV methods for detection of impurities, critical NPD parameters such as RT window, mass tolerance window, NPD signal threshold, and peak area threshold should be optimized to reduce detection of false positives and negatives. To assess these critical parameters for NPD, the peptide retention time calibration (PRTC) standard sample comprising a mixture of 15 synthetic peptides was spiked with amounts ranging from 0.25 to 2 pmol with respect to 4 µg of rituximab digests (i.e., 0.01 to 0.08% w/w) as nonspecifically digested products to mimic potential degradants.

As part of the control strategy to reduce variability, identical LC-MS configurations were used at both sites. As a result, excellent RT precision was observed across 36 injections of PRTC-spiked rituximab digest (12 injections per each system) as shown in Figure 5. RT reproducibility of 15 PRTC peptides ranges from 0.13% to 2.89%, covering the gradient from RT of 9.4 min to 48 min. The maximum standard deviation of the measured RT was 0.42 min for peptide TASEFDSAIAQDK; therefore, the minimum RT window for consistent detection of all PRTC peptides using NPD should be set to 0.84. If a smaller RT window was set for NPD, false negatives occurred as some PRTC peptides would be detected as "new peak" even if we had included all of them as target attributes.

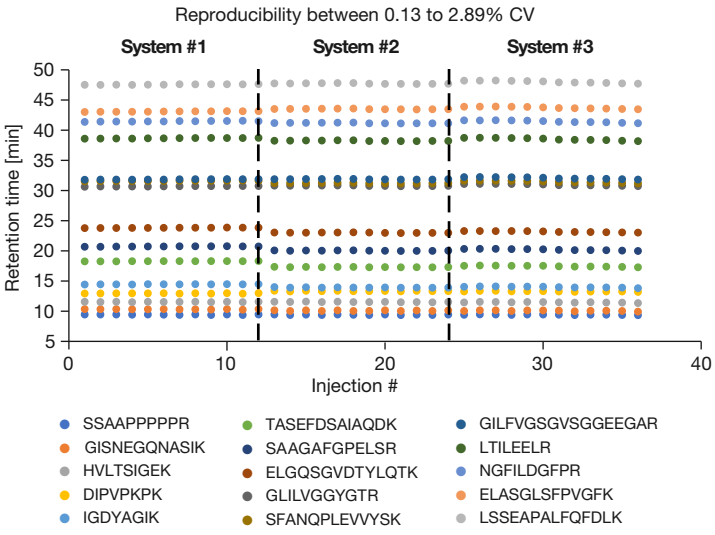


Figure 5. Interlaboratory evaluation of RT reproducibility of PRTC peptides

Since accurate detection of new peaks depends on how close the observed *m/z* value in the test sample is relative to the control sample, a tighter mass tolerance window would provide higher confidence and reduce the number of false positives in the report. As shown in Figure 6, with the Orbitrap Exploris MX mass detector, we were able to achieve less than 2 ppm mass accuracy for the most abundant ion of PRTC peptides. Such level of accuracy would provide unambiguous component identification with high confidence.

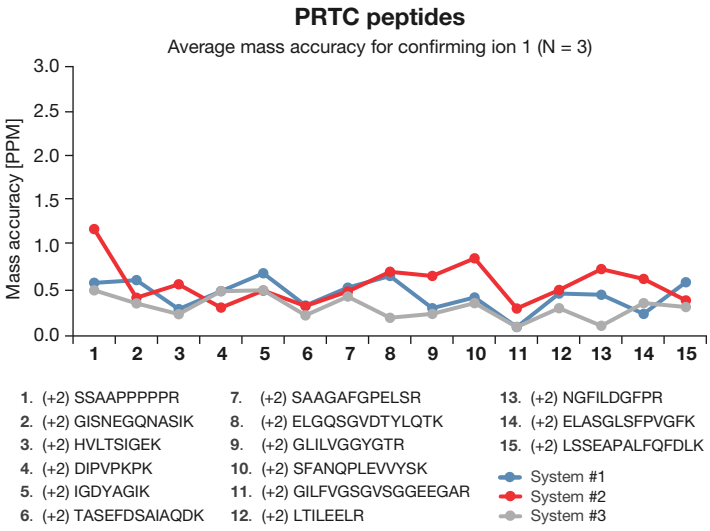


Figure 6. Interlaboratory evaluation of mass accuracy for all PRTC peptides. Average mass accuracy (N = 3, spiked A samples) obtained for the +2 charge state of the most abundant ion, also referred to as confirming ion 1.

Among all NPD parameters, NPD signal and peak area thresholds are the most critical parameters for avoiding detection of false positive and negatives. For this study, different amounts of PRTC peptides were spiked into rituximab digests for consistent detection of all PRTC peptides across two sites. We therefore had to set the NPD signal and peak area thresholds below the peak intensity and area minimum observed across three systems. As shown in Figure 7, the minimum observed peak height was 5E5 for peptide DIPVPKPK on system #2, and the minimum observed peak area was 1.5E5 for the same peptide on the same system. With the exception of DIPVPKPK, peak height and peak area reproducibility across the three systems were less than 20% CV, demonstrating excellent instrument precision, considering the method involves absolute quantitation without internal standard correction.

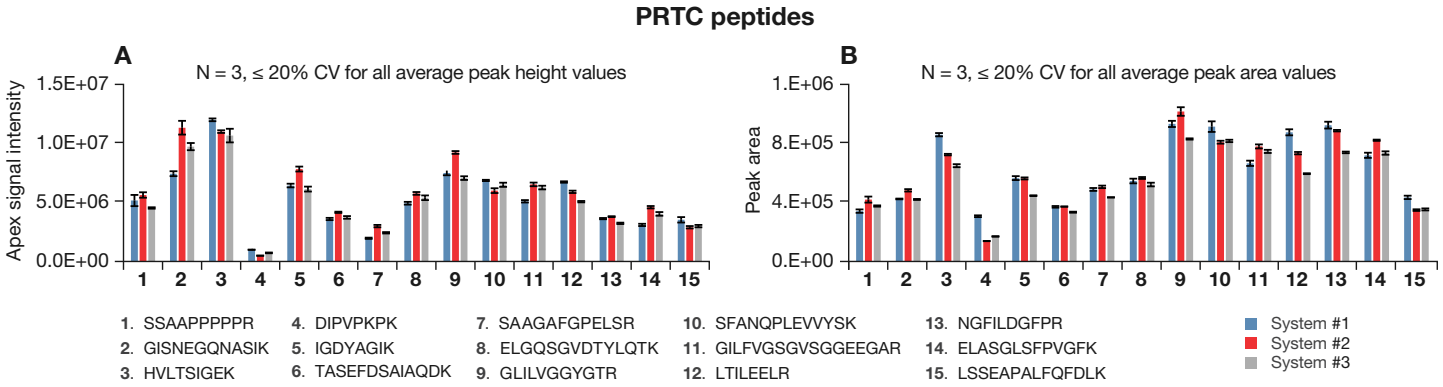


Figure 7. Interlaboratory evaluation of PRTC peptide. (A) Peak height comparison for spiked A sample across three systems and (B) peak area comparison for spiked A sample across three systems. Average peak height and area (N = 3, spiked A samples) are plotted for each PRTC peptide. Peak height and peak area reproducibility are less than 20% CV.

Using the spiked A sample data, the NPD signal threshold was set to 3E5 and the peak area threshold was set to 1E5. These thresholds were calculated using the average peak height and peak area values minus 3 standard deviations, respectively, to account for 99.7% of the sample population that may arise from instrument-to-instrument variations. Using the first unspiked rituximab digest injection as a reference, NPD was performed against two other unspiked rituximab digest injections as a negative control test using the above set thresholds and applied filter rules as described in the method section. As expected, no new peak was detected on three systems.

Similarly, NPD was performed against 12 PRTC-spiked rituximab digest injections (four concentrations, three replicates per each concentration) as a positive control test using the above set thresholds. We could consistently detect all 15 PRTC peptides for all concentrations of PRTC spiked samples, even for spiked A samples, which represented only about 0.01% (w/w) impurity. In addition to the 15 PRTC peptides, we also found “new peaks” in those spiked samples. As shown in Table 8, all 15 PRTC peptides were detected across three systems as the observed *m/z* matches with the theoretical *m/z* value. In addition to 15 PRTC peptides, three impurities from the PRTC samples were detected, and these were identified using BioPharma Finder software.

Systems #1 and #2 consistently detected all three PRTC impurities whereas system #3 detected only two. PVPKPK, a clipped version of DIPVPKPK peptide, was present in all three spiked A samples collected from system #3; however, the peak area of this low abundant specie was lower than the set threshold, and thus not detected as a “new peak”. For higher concentrations of PRTC-spiked samples, the NDP MS signal and peak area threshold need to be adjusted appropriately to avoid detection of false positives. In addition, to set thresholds and applied filter rules, cross checking “new peaks” between replicate injections for the same spiked sample can also assist in eliminating false positives. For “new peaks” to be true, these should appear in all replicate injections, and even be present in spiked samples with higher PRTC concentrations.

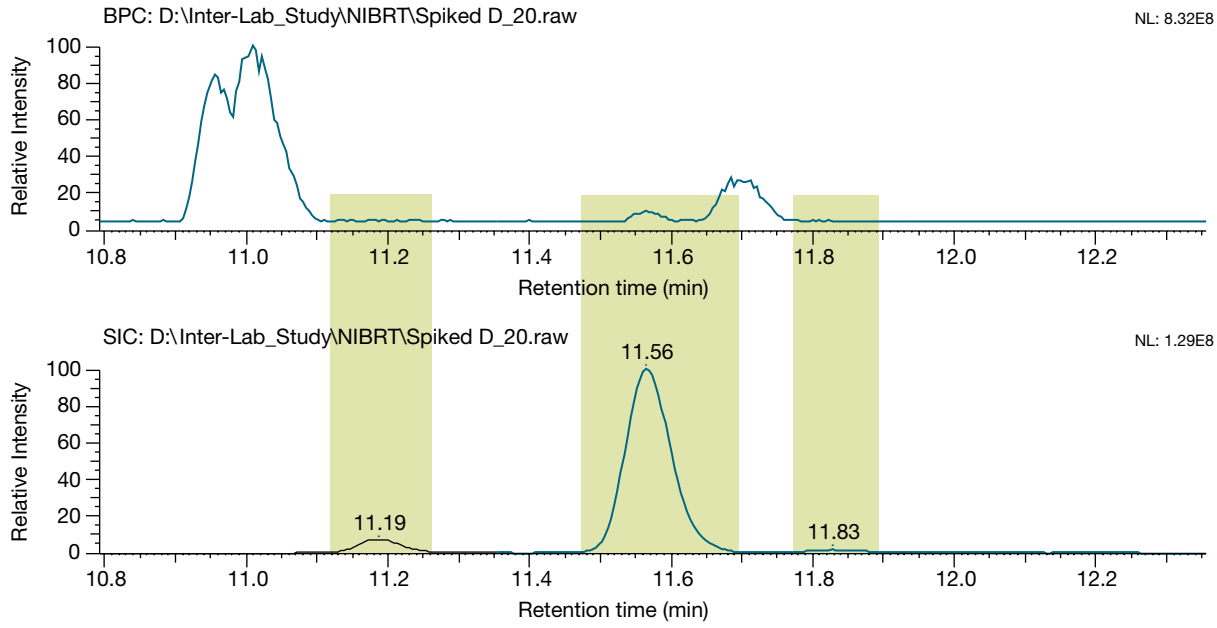
Table 8. Interlaboratory evaluation of NPD for detection of PRTC peptides across three systems. NPD results of spiked A rituximab samples, collected on three systems, are shown here.

Observed *m/z*	Identification	System #1	System #2	System #3
493.768	SSAAPPPPPR	✓	✓	✓
613.317	GISNEGQNASIK	✓	✓	✓
496.287	HVLTSIGEK	✓	✓	✓
451.283	DIPVPKPK	✓	✓	✓
422.736	IGDYAGIK	✓	✓	✓
695.832	TASEFDSAIAQDK	✓	✓	✓
586.800	SAAGAFGPELSR	✓	✓	✓
773.902	ELGQSGVDTYLQTK	✓	✓	✓
558.325	GLILVGGYGTR	✓	✓	✓
745.392	SFANQPLEVVYSK	✓	✓	✓
801.411	GILFVGSGVSGGEEGAR	✓	✓	✓
498.802	LTILEELR	✓	✓	✓
573.302	NGFILDGFPR	✓	✓	✓
680.375	ELASGLSFPVGFK	✓	✓	✓
787.421	LSSEAPALFQFDLK	✓	✓	✓
496.287*	HVLTSIGEK*	✓	✓	✓
337.228	PVPKPK	✓	✓	×
564.297	NGFILD[H_2O loss]GFPR	✓	✓	✓

* Isomers of HVLTSIGEK were detected as impurities; it has the same *m/z* but at different RT.

Identification of new peaks using BioPharma Finder software

BioPharma Finder software was used for identification of new peaks with full MS scan. With Chromeleon 7.3.1 software, NPD uses the same BioPharma Finder software algorithm for component detection. The harmonized algorithm facilitates accurate identification of new peaks by comparing the observed retention time and *m/z* ratio to the previously identified peptide sequences in BioPharma Finder software using MS/MS data. Without MS/MS confirmation, new peaks can be identified with high confidence only if the observed retention time and *m/z* ratio are within the set tolerance. As a proof of demonstration, the three detected new peaks in spiked A samples were identified using BioPharma Finder software with full MS data only. One of them is an isomer of HVLTSIGEK as it has the same *m/z* ratio but elutes at a different RT as shown in Figure 8. There are total of three isomers for HVLTSIGEK, the last of which elutes at ~11.8 minutes, detected consistently at higher concentrations of PRTC-spiked samples (e.g., spiked C and D samples). The observed retention time for three isomers and *m/z* ratio match perfectly with the previously identified peptide sequences using MS/MS data in BioPharma Finder software (data not shown). The ability to accurately identify product- and process-related impurities using NPD and BioPharma Finder software during the process development is crucial prior to batch and stability testing at the QC stage, particularly if these impurities affect the safety and efficacy of the biotherapeutic drug product after conducting risk assessments.



No.	Identification	Peptide Sequence	Mod	Site	Δ ppm	Conf. Score	Best ASR	ID Type	Integration Type	RT	M/Z	Charge St.	Mono Mass Exp.	Avg Mass Exp.
=	= (NonBlanks)	Aa (Custom)	Aa	Aa	=	=	=	Aa	Aa	=	=	= 2	=	=
354	3:H1-K9 = 990.5589m	HVLTSIGEK	(HeavyK)	(K9)	-0.48	0.0	0.0	Full	Automatic	11.19	496.286	2	990.5585	990.86
363	3:H1-K9 = 990.5589m	HVLTSIGEK	(HeavyK)	(K9)	-0.48	0.0	0.0	Full	Automatic	11.56	496.286	2	990.5585	990.86
409	3:H1-K9 = 990.5589m	HVLTSIGEK	(HeavyK)	(K9)	-0.35	0.0	0.0	Full	Automatic	11.83	496.287	2	990.5586	990.87

Figure 8. Identification of HVLTSIGEK isomers using BioPharma Finder software based on Full MS only data of spiked D sample.
The top panel represents the base peak chromatogram (BPC) for spiked D sample, and the bottom panel the selected ion chromatogram (SIC) for the monitored peptide. Three HVLTSIGEK isomers were detected and shown in the component table.

Conclusion

Here, we have developed a highly reproducible and robust LC-HRAM-MS-based MAM workflow that is suitable for QC implementation. This MAM workflow was applied to three systems across two sites for rituximab CQA monitoring and NPD evaluation. Full MS scan datasets generated on three systems for CQA monitoring were combined and the results were analyzed for repeatability, intermediate precision, reproducibility, and linearity evaluations. In addition, NPD analysis was conducted on all PRTC-spiked rituximab injections to demonstrate the robustness of NPD in a QC workflow.

- MAM executed on three systems exhibited excellent repeatability and intermediate precision for the analysis of rituximab CQAs.
- MAM executed on three systems demonstrated highly reproducible results for the analysis of rituximab CQAs.
- MAM demonstrated up to four orders of magnitude of linear dynamic range for consistent quantitation of rituximab CQAs.
- Non-targeted MS processing using NPD in Chromeleon CDS detected all PRTC peptides consistently across the three systems for rituximab sample "Spiked A", which represented an impurity level down to 0.01% (w/w).

References

1. Rathore, A.S. Roadmap for implementation of Quality by Design (QbD) for biotechnology products, *Trends Biotechnol.* **2009**, *27*, 26–34.
2. Rogers, R.S. et al., Development of a quantitative mass spectrometry multi-attribute method for characterization, quality control testing and disposition of biologics, *MAbs.* **2015**, *7*(5), 881–890.
3. Song, Y.E. et al., Automated mass spectrometry multi-attribute method analyses for process development and characterization of mAbs, *J. Chromatogr. B*, **2021**, *1166*, 122540.
4. Hao, Z., et al., Multi-attribute method performance profile for quality control of monoclonal antibody therapeutics, *Journal of Pharmaceutical and Biomedical Analysis.* **2021**, *205*(2), 114330.
5. Rogstad S., et al., Multi-attribute method for quality control of therapeutic proteins, *Anal. Chem.* **2019**, *91*(22), 14170–14177.
6. Thermo Fisher Scientific, Application Note 000336: An integrated LC-MS system performance evaluation test for peptide mapping and monitoring. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-000336-lcms-protein-digest-peptide-mapping-monitoring-an000336-na-en.pdf
7. Thermo Fisher Scientific, Application Note 000463: The MAM 2.0 workflow enables seamless transition from development to quality control.
8. Li, C. et al. Comparability analysis of anti-CD20 commercial (Rituximab) and RNAi-mediated fucosylated antibodies by two LC-MS approaches, *mAbs.* **2013**, *5*(4), 565–575.
9. Visser, J. et al. Physicochemical and functional comparability between the proposed biosimilar rituximab GP2013 and originator rituximab, *BioDrugs.* **2013**, *27*, 495–507.
10. Montacir, O. et al. Comparability study of Rituximab originator and follow-on biopharmaceutical, J. *Pharm. Biomed.* **2017**, *140*, 239-251.
11. Di Marco, F. et al. Simultaneous monitoring of monoclonal antibody variants by strong cation-exchange chromatography hyphenated to mass spectrometry to assess biosimilarity of Rituximab-based biotherapeutics, *Int. J. Mol. Sci.* **2021**, *22*(16), 9072.
12. Yan, Q. et al. Structure Based Prediction of Asparagine Deamidation Propensity in Monoclonal Antibodies, *MAbs.* **2018**, *10*(6), 901–912.
13. Yan, B. et al. Succinimide formation at Asn 55 in the complementarity determining region of a recombinant monoclonal antibody IgG1 heavy chain, J. *Pharm. Sci.* **2009**, *98*, 3509–21.
14. Mo, J. et al. Understanding the impact of methionine oxidation on the biological functions of IgG1 antibodies Using hydrogen/deuterium exchange mass spectrometry, *Anal. Chem.* **2016**, *88*, 19, 9495–9502.
15. Liu, D. et al. Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation, *Biochemistry.* **2008**, *47*(18), 5088–100.
16. Wang, W. et al. Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies, *Molecular Immunology.* **2011**, *48*(6–7), 860-6.
17. Banks, D.D. et al. The effect of sucrose hydrolysis on the stability of protein therapeutics during accelerated formulation studies, *J. Pharm. Sci.* **2009**, *98*, 4501–10.
18. Kayser, V., et al. Glycosylation influences on the aggregation propensity of therapeutic monoclonal antibodies, B*iotechnol. J.* **2011**, *6*, 38–44.
19. Goetze, A.M. et al. High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans, *Glycobiology*, **2011**, *21*, 949–59.
20. Nowak, C. et al. Forced degradation of recombinant monoclonal antibodies: A practical guide, M*Abs.* **2017**, *9*(8), 1217–1230.
21. Millán-Martín, S. et al. Inter-laboratory study of an optimised peptide mapping workflow using automated trypsin digestion for monitoring monoclonal antibody product quality attributes, *Analytical and Bioanalytical Chemistry.* **2020**, *412*, 6833–6848.
22. Sokolowska, I., et al. Implementation of a high-resolution liquid chromatography–mass spectrometry method in quality control laboratories for release and stability testing of a commercial antibody product, *Anal. Chem.* **2020**, *92*, 2369–2373.

Learn more at **thermofisher.com/mam**

AN000753-en 0822S