

## Simple and Efficient Peptide MAM Analysis Using Fast Trypsin Digestion and the BioAccord™ LC-MS System

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### Abstract

With the increasing prevalence of multi-attribute method (MAM) workflows in the biopharmaceutical industry comes the desire for higher throughput and reduced burdens for data analysis. Successful MAM analyses heavily rely on consistent and reproducible protein digestion yielding minimal sample preparation induced modifications, which increase complexity and can lead to increased effort for data review. RapiZyme™ Trypsin is a new, commercially available, highly active autolysis-resistant enzyme that provides quick, clean, and complete mAb digestions in just 30 minutes. The shorter the digestion time, the less likely that artificial modifications may occur during the sample preparation prior to LC-MS analysis. This is highly desirable, as MAM analysis typically focuses on tracking levels of post-translational modifications (PTMs) such as deamidation and oxidation, which exhibit higher artifactual occurrence during longer sample prep incubations. In addition, the 30-minute digestion allows for faster turn-around time for results and more flexibility of analysts' time in the laboratory. This study evaluates the use of RapiZyme Trypsin in a MAM workflow applied to thermally stressed infliximab innovator and three approved biosimilars.

### Benefits

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- Autolysis-resistant RapiZyme Trypsin allows for use of high E:P ratio digestions that shorten sample preparation time and reduce sample prep-induced modifications
  - Reproducible digestion for long-term method success
  - BioAccord LC-MS System for simplified system setup and data acquisition
  - Compliance-ready Peptide MAM App workflow within the waters\_connect™ Informatics Platform
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## Introduction

Multi Attribute Method (MAM) assays using LC-MS peptide mapping workflows are becoming more commonplace in the biopharmaceutical industry for product quality attribute (PQA) analysis, cGMP stability testing, and QC release. These assays provide a wealth of site-specific information for biopharmaceutical product variation to support drug development and stability assessments. MAM analyses are also quite useful in the development of biosimilar mAb drug products, as key and critical PQAs are known and it is required that biosimilar have “no clinically meaningful differences from the FDA-approved reference product”.<sup>1</sup> MAM studies can provide comparative information about the innovator and biosimilar drug products, as well as confirming comparable degradation pathways during stress or storage.

The quality, reproducibility, and speed of protein digestion are critical for a successful MAM workflow. RapiZyme Trypsin (Waters Corporation) is a novel homogenously methylated porcine trypsin that has capabilities of producing quick, clean, complete, and reproducible digestions resulting from its high activity and autolysis resistance, especially at high enzyme:protein (E:P) ratios. When used in a 1:5 (w/w) ratio with reduced, alkylated, and desalted mAb samples, RapiZyme Trypsin can produce a complete tryptic digestion in just 30 minutes with minimal sample preparation-induced artifacts. These same conditions using other leading MS-sequencing grade trypsins generally produce digestions that contain noticeable levels of trypsin autolysis peptides, missed cleavages, and other unknown species (Figure 1), as previously reported.<sup>2</sup> RapiZyme Trypsin is, therefore, an attractive enhancement for current peptide mapping workflows, as it provides the capability to reduce sample preparation time without sacrificing data quality.

One of the most crucial aspects of MAM experiments is reproducibility. The primary goal of MAM analysis is to monitor the levels of PQAs and critical quality attributes (CQAs) that were determined from studies with the reference material. For simplified analysis, the mass and retention time information of the modified and

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unmodified peptides is used for targeted quantification (monitoring) of attributes. This requires method reproducibility in both the chromatographic separation, as well as the protein digestion. First, to ensure chromatographic reproducibility, this study utilizes an ACQUITY™ Premier Peptide CSH™ C18 Column with MaxPeak™ High Performance Surfaces (HPS) technology.<sup>3-4</sup> Second, the success of this workflow relies heavily on the generation of consistent digested peptide forms in every sample. Many successful MAM workflows have been established using leading commercially available MS-sequencing grade trypsin enzymes.<sup>4-11</sup> Following the launch of RapiZyme Trypsin with its capability of quicker, cleaner digestions, we have evaluated its applicability when used in an MAM workflow.

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## Experimental

### Sample Preparation - Stress Study

Remicade™, Inflectra™, Avsola™, and Renflexis™ (innovator and three approved biosimilar infliximab) samples were subjected to stressed conditions to facilitate the MAM study. Samples were thermally stressed at 37 °C and aliquots were frozen at the 1-week and 2-week timepoints for later digestion and MAM analysis.

### Sample Preparation - Peptide Mapping

The reference (T0), 1-week (T1), and 2-week (T2) samples (150 µg) were diluted to 1 µg/µl in 5.2 M guanidine hydrochloride containing 3 mM dithiothreitol (DTT) for 30 minutes at room temperature for denaturation and reduction. Iodoacetamide (IAM) was then added to a final concentration of 7 mM and incubated for 20 minutes at room temperature. All samples were buffer exchanged via 7K MWCO gel filtration device into 100 mM Tris HCl, 10 mM CaCl<sub>2</sub>, pH 7.5 (p/n: [186010111 <https://www.waters.com/nextgen/global/shop/standards--reagents/186010111-quick-prep-tris-cacl2-buffer-salts-ph-75-4-pk.html>](https://www.waters.com/nextgen/global/shop/standards--reagents/186010111-quick-prep-tris-cacl2-buffer-salts-ph-75-4-pk.html) ) for digestion.

RapiZyme Trypsin (p/n: [186010108 <https://www.waters.com/nextgen/global/shop/standards--reagents/186010108-rapizyme-trypsin-ms-grade-4-pk.html>](https://www.waters.com/nextgen/global/shop/standards--reagents/186010108-rapizyme-trypsin-ms-grade-4-pk.html) ) was added to each sample at an enzyme-to-protein (E:P) ratio of 1:5 (w/w). Protein digestion was carried out at 37 °C for 30 minutes. The trypsin was subsequently inactivated with 10% acetic acid (final concentration 0.1%) and the digested sample further diluted with mobile phase A (to 0.2 µg/µl) for analysis via LC-MS. Free methionine was added to each sample to a final concentration of 3 mM to stifle potential artificial oxidation while samples are queued in the autosampler for LC-MS analysis. The MassPREP™ Peptide Mixture (p/n: [186002337 <https://www.waters.com/nextgen/global/shop/standards--reagents/186002337-massprep-peptide-mixture-100-pk.html>](https://www.waters.com/nextgen/global/shop/standards--reagents/186002337-massprep-peptide-mixture-100-pk.html) ) was added to each sample at a final concentration of 100 µg/ml.

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<https://www.waters.com/nextgen/global/shop/standards--reagents/186002337-massprep-peptide-mixture.html>

> ) was used as the System Suitability Test (SST) sample for Peptide MAM App processing and was prepared as directed in the Care and Use Manual, with 5  $\mu$ L injected for analysis.<sup>12</sup>

## LC Conditions

LC system:	ACQUITY UPLC™ I-Class PLUS
Detection (optical):	ACQUITY TUV (214 nm)
Plate:	Skirted 96-well PCR plate (Thermo Scientific, p/n: AB0800)
Column(s):	ACQUITY Premier Peptide CSH C <sub>18</sub> Column, 130 Å, 1.7 $\mu$ m, 2.1 x 100 mm (p/n: 186009488)
Column temperature:	60 °C
Sample temperature:	6 °C
Injection volume:	10 $\mu$ L (2 $\mu$ g on column)
Flow rate:	0.2 mL/min
Mobile phase A:	0.1% (v/v) Formic Acid in Water
Mobile phase B:	0.1% (v/v) Formic Acid in Acetonitrile
Gradient:	Initial hold at 1 %B for 1 minute, 1–35 %B over 50 minutes, 35–85 %B over 6 minutes, 85 %B for 4 minutes, 85–1 %B over 6 minutes, hold at 1 %B for 13 minutes

## MS Conditions

MS system:	BioAccord System (ACQUITY RDa™)
Ionization mode:	ESI Positive, Full Scan MS with Fragmentation
Acquisition range:	m/z 50–2000
Capillary voltage:	1.2 kV
Collision energy:	60–120 V (low/high energy ramping)
Cone voltage:	30 V
Desolvation temperature:	350 °C
Intelligent data capture:	On

## Data Management

LC-MS acquisition:	UNIFI™ v 2.1.2.14 operated under waters_connect v 1.6.2
Data processing:	Peptide MAM v 1.5.0.13 operated under waters_connect v 2.2.0

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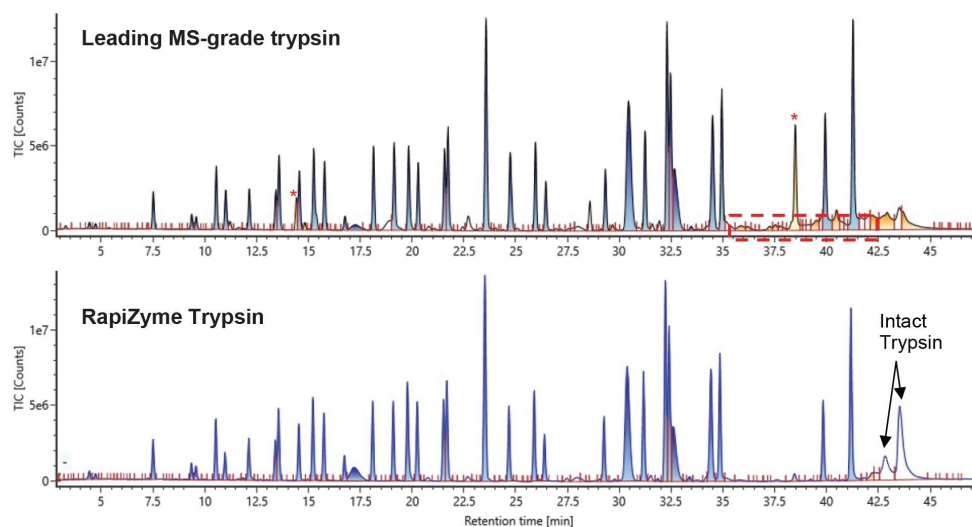
## Results and Discussion

### MAM Results

Using the 1:5 (w/w) E:P ratio for a 30-minute incubation provides near-complete digestion, as shown in the representative TIC chromatograms for digestions of Remicade reference material with a leading MS-grade

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trypsin and RapiZyme Trypsin (Figure 1). The peaks in blue represent peptides with expected tryptic cleavage. Peaks in white represent peptides with missed cleavages or non-specific cleavages. Remaining peaks (displayed in yellow) are comprised of unknown species, including trypsin autolysis peptides. Major autolysis species are observed for the leading MS sequencing grade trypsin (top panel) and are noted with the red box or asterisks. A 98% reduction of autolysis species and 78% reduction of missed cleavage species are observed for the digestion with RapiZyme Trypsin (bottom panel). These impurities increase complexity of the data, and may confound data analysis, especially if they coelute with peaks of interest.



*Figure 1. Representative chromatograms (TIC) for 1:5 (w/w) enzyme:protein ratio digestions of Remicade T0 with another leading MS-grade trypsin (top) and RapiZyme Trypsin (bottom). Red asterisks (\*) and box highlight significant trypsin autolysis species. Peaks shown in blue are identified peptides with expected tryptic cleavage. Peaks colored in white represent peptides with missed or non-specific cleavages, and peaks which are colored yellow represent unmatched species.*

Overall, 90–92% sequence coverage (Figure 2A) is observed for innovator and biosimilar digestions. (Sequence coverage is based on filtered data for confident fully tryptic peptide matches. For a confident peptide match, the precursor mass must be within 10 ppm mass accuracy, and it must have at least 3 b/y fragment ions assigned.) A representative sequence coverage map (Figure 2B) is displayed for an infliximab sample digested with RapiZyme Trypsin. Minor variations in sequence coverage arise from small peptides that sometimes do not meet the

threshold for b/y ions detected. All injections were processed via the Peptide MAM App in waters\_connect Informatics Platform, in a manner described previously.<sup>4</sup>

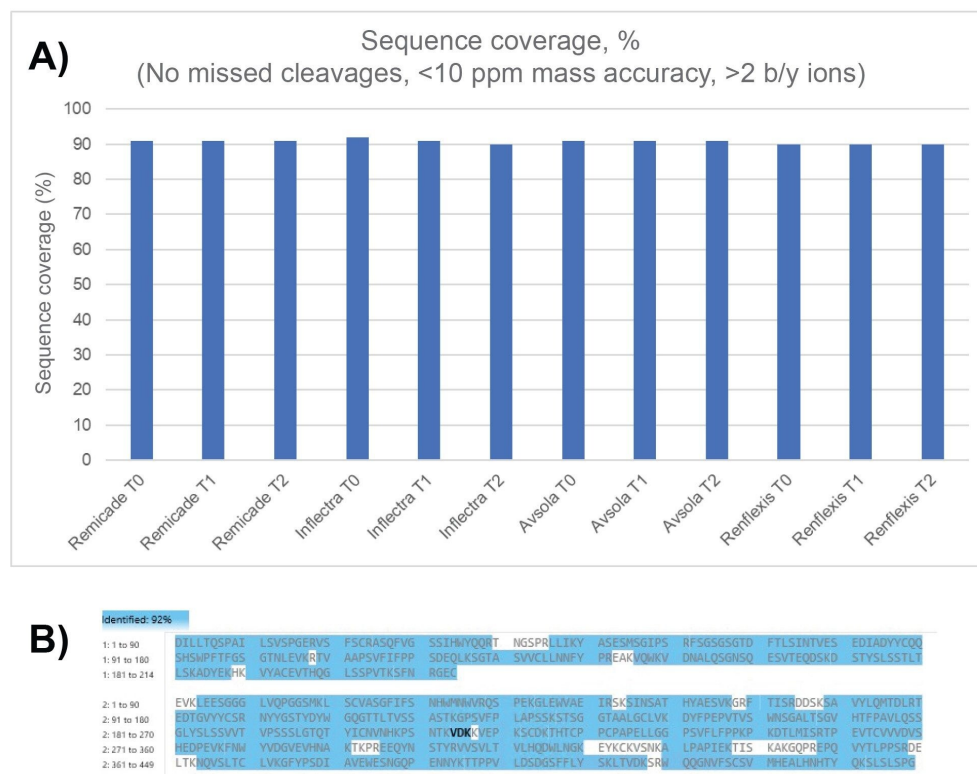


Figure 2. A) Summary of sequence coverage (%) resulting from each infliximab biosimilar sample digested with RapiZyme Trypsin. B) Example coverage map for infliximab digest. Sequence coverage is based on filtered peptides for <10 ppm mass accuracy and >2 b/y fragment ions for positive match and excludes missed cleavages, nonspecific cleavages, and in-source fragmentations/adducts.

Fourteen attributes were monitored, including N-linked glycosylation, Met oxidation, deamidation, and unprocessed C-terminal lysine. Figure 3 displays a subset of monitored PQAs for the stressed infliximab biosimilars. Samples 1–3 (blue) represent Remicade (infliximab) T0, T1, and T2, respectively, samples 4–6 (orange) represent Inflectra (infliximab) T0, T1, and T2 respectively, samples 7–9 (yellow) represent Avsola (infliximab) T0, T1, and T2 respectively, and samples 10–12 (green) represent Renflexis (infliximab) T0, T1, and T2 respectively. As expected, the C-terminal lysine variant level remained constant throughout the thermal stress,

though there are notable differences between the infliximab biosimilars. The PQAs with a clear increasing trend in this study were heavy chain peptide T7, T37, and T38 (HT07, HT37, HT38) deamidation. Heavy chain peptide T11 (HT11) oxidation remained stable at low levels (0.4–0.5%) for all biosimilars and timepoints. The oxidation on heavy chain peptide T2 (HT02) was at a relatively low level but did exhibit a slow increase over time for each biosimilar.

#### MAM results for all stressed biosimilars using RapiZyme Trypsin

■ = Remicade (infliximab)      ■ = Avsola (infliximab)  
 ■ = Inflectra (infliximab)      ■ = Renflexis (infliximab)

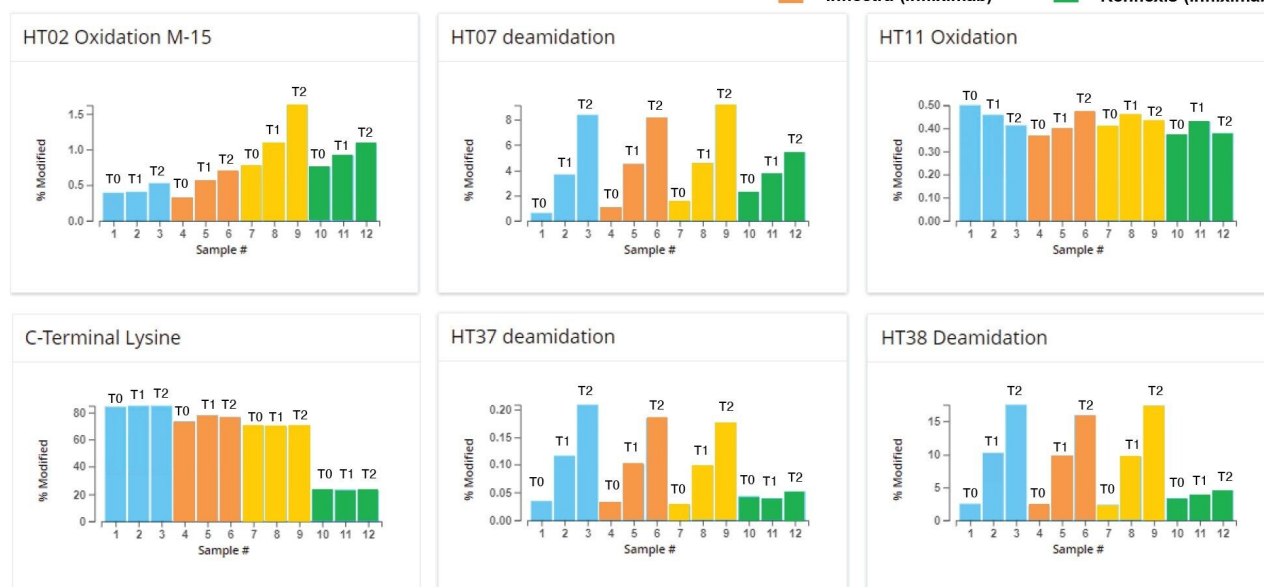


Figure 3. Selected product quality attributes for Remicade (infliximab) and three biosimilars subjected to thermal stress (all digested with RapiZyme Trypsin), T0, T1 (1 week), and T2 (2 weeks). Injections 1–3=Remicade (infliximab), 4–6=Inflectra (infliximab), 7–9=Avsola (infliximab), and 10–12=Renflexis (infliximab).

## New Peak Detection (NPD)

The Peptide MAM App includes New Peak Detection (NPD) functionality that enables the MAM analysis to serve as an assay monitoring product purity as well as the targeted attributes. Each of the timepoints (T1 and T2) were processed for NPD using its respective infliximab T0 sample as the reference. For example, Remicade T1 and T2 were processed with Remicade T0 as the reference injection. Following suit, the Inflectra T1 and T2 samples were processed for NPD with Inflectra T0 as its reference injection. Each of the biosimilars was processed in the same manner. Only five new peaks were detected using the set of NPD filtering criteria (Figure 4). They are classified



as follows: potential clipping species are shown in blue, small peptides (possibly unretained by RPLC in in reference mAb analysis) are shown in purple, and miscleaved peptides are shown in yellow. This again highlights the criticality of robust, highly reproducible digestions. Even missed cleavages can be flagged as new peaks if they are present at significantly different levels between samples. RapiZyme Trypsin has been extensively tested for digestion completion repeatability and batch-to-batch comparison, and consistently provides reproducible results.<sup>1</sup> As shown, this results in fewer new peaks and less risk of false positives due to digestion inconsistency, simplifying the data review process, and de-risking the use of MAM LCMS analysis in regulated environments.

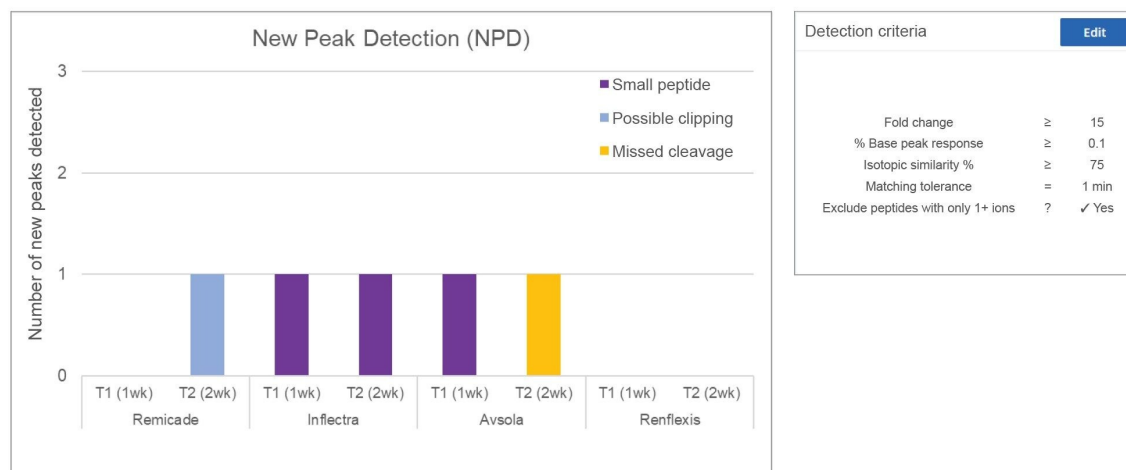


Figure 4. Summary and classification of new peaks detected for stressed infliximab innovator and biosimilars. (Note: each stressed sample is compared to its respective T0 sample.) Low number of new peaks is detected.

## Conclusion

LC-MS based peptide MAM analysis is becoming a critical assay during the development and commercialization of protein biopharmaceuticals. As such, the samples prepared for this assay must be robust and reproducible. In some applications (e.g., formulations and stability) these results must be achievable with higher throughput sample processing workflows. The use of RapiZyme Trypsin in high E:P ratio provides quick, complete, and highly reproducible digestions without sacrificing data quality, which is proven successful in monitoring of PQAs

in this MAM study of stressed infliximab biosimilars. In addition, a low number of new peak species was found with NPD processing in the Peptide MAM application, showing the digestion with RapiZyme Trypsin does not generate a background of false positive new peaks due to digestion impurities. Therefore, the use of RapiZyme Trypsin provides a faster, cleaner sample for deploying peptide MAM based LCMS analysis of biopharmaceutical products.

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720008051, September 2023



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