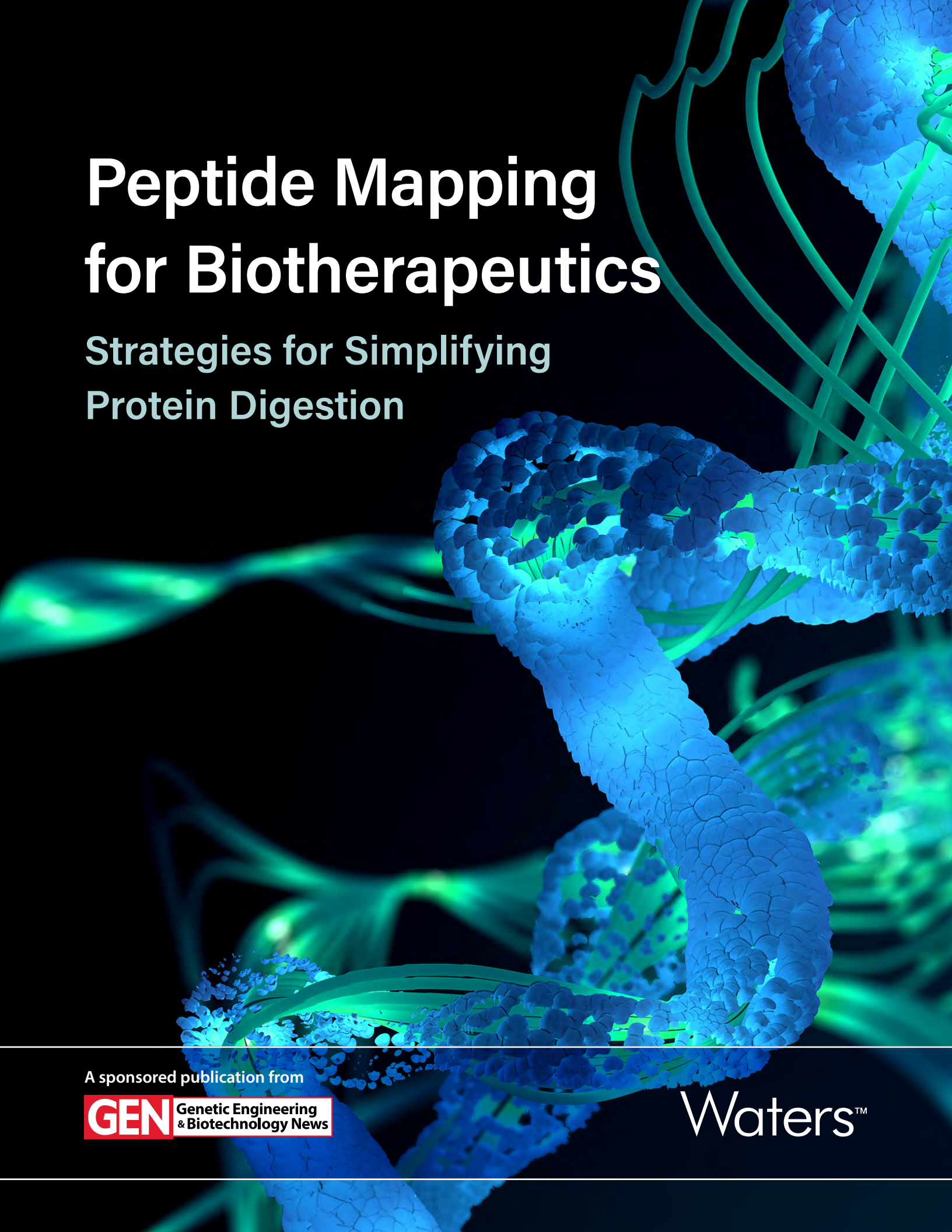
Peptide Mapping for Biotherapeutics

Strategies for Simplifying Protein Digestion



A sponsored publication from

GEN Genetic Engineering & Biotechnology News

Waters™

Waters™

Peptide Mapping Doesn't Need to Be Complex

Introducing PeptideWorks™ Tryptic Protein Digestion Kits

PeptideWorks Tryptic Protein Digestion Kits uniquely deliver automatable, high efficiency, reproducible peptide maps in under **2.5 hours**, **4x faster** than average home-brew methods. The result has **78% reduction** in missed cleavages, **98% reduction** in contaminating autolysis peaks which can further complicate data processing.

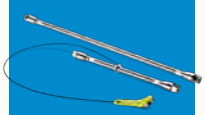
- Robust peptide mapping sample preparation in under 2.5 hours
- Automatable desalting step
- Reproducible and efficient salt removal with high protein recoveries
- Efficient 30-minute tryptic digestion with 10-fold decrease in missed cleavages*
- One comprehensive kit for manual or automated workflows
- No need to source multiple reagents

For maximum performance, pair PeptideWorks Tryptic Digestion Kits with Waters best-in-class consumables for peptide mapping and proteomics.

For more information please scan the QR code.

ACQUITY™ and XSelect™ Premier Peptide CSH™ C_{18} Columns

The best peak shape, peak capacity, mass load, and sensitivity for peptides at any scale regardless of ion-pairing reagent.



RapiGest™ SF

MS compatible, enzyme friendly surfactant to solubilize and denature proteins for quicker in solution digestion.

QuanRecovery™ Vials and Plates

Enhanced sensitivity from reduced sample losses due to non-specific binding.

Peptide Standards

Improved productivity, troubleshooting, and method development support.

IonHance™ DFA

Minimized salt adducts, improved MS signal over TFA, and improved optical peak shape over formic acid, from high purity LC-MS grade DFA.

* When compared to the leading Immoblized Trypsin Kit.

Find out more at **waters.com/PeptideWorks**

Table of Contents

Peptide Mapping for Biotherapeutics

Strategies for Simplifying Protein Digestion

Cover: Christoph Burgstedt/Getty Images

Introduction

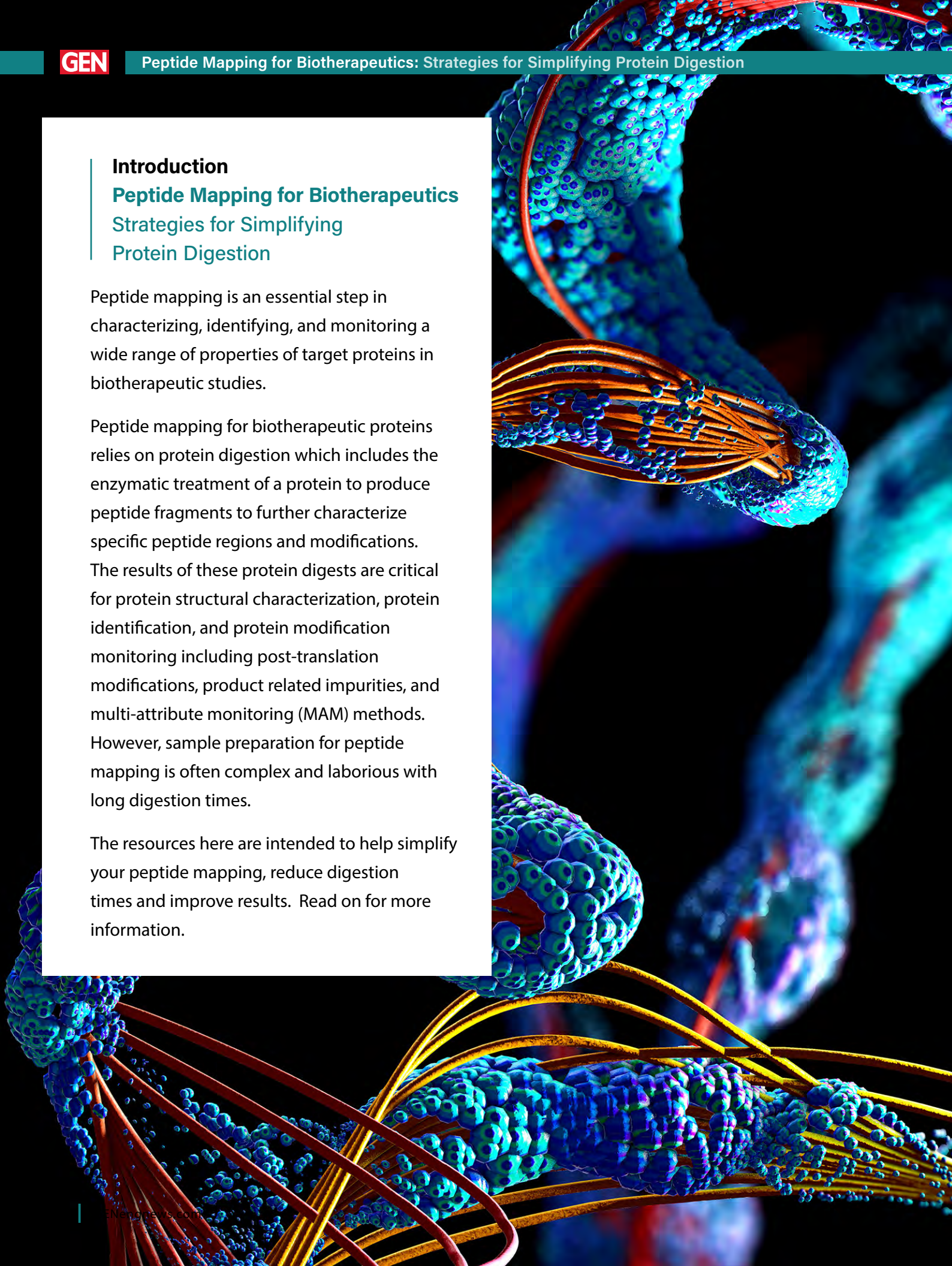
Peptide Mapping for Biotherapeutics

Strategies for Simplifying Protein Digestion

Peptide mapping is an essential step in characterizing, identifying, and monitoring a wide range of properties of target proteins in biotherapeutic studies.

Peptide mapping for biotherapeutic proteins relies on protein digestion which includes the enzymatic treatment of a protein to produce peptide fragments to further characterize specific peptide regions and modifications. The results of these protein digests are critical for protein structural characterization, protein identification, and protein modification monitoring including post-translation modifications, product related impurities, and multi-attribute monitoring (MAM) methods. However, sample preparation for peptide mapping is often complex and laborious with long digestion times.

The resources here are intended to help simplify your peptide mapping, reduce digestion times and improve results. Read on for more information.



Quick & Robust Sample Preparation for Tryptic Peptide Mapping with the PeptideWorks Kit using Simple, Automatable Workflows

Caitlin M. Hanna, Jonathan P. Danaceau, Stephan M. Koza, Steve Shiner, Mary Trudeau

Waters Corporation

Abstract

Here, we present an automatable sample preparation protocol for peptide mapping using the PeptideWorks Tryptic Protein Digestion Kit. The kit provides a reproducible tryptic protein digest developed for regulated and research peptide mapping applications.

Benefits

- Comprehensive kit and protocol for preparation of tryptic protein digests intended for quality control (QC), bioprocess, analytical development, and research environments
- Automatable preparation of 24 tryptic peptide mapping samples in under 2.5 hours
- Reproducible preparation of tryptic peptide mapping samples without sacrificing digestion completion or generating high levels of method-induced peptide modifications
- 93% reduction in missed cleavages and 55% reduction in non-specific cleavages compared to a leading immobilized trypsin digest kit

Introduction

Peptide mapping is used in QC, bioprocess, analytical development, and research environments to deliver comprehensive information about the primary structure of biotherapeutic proteins.[1] Generally, the peptide map of a test article is compared to that of a reference material; sample-to-sample reproducibility is imperative for detection of real changes between the test article and reference material. Reliable day-to-day sample preparation is also a critical factor in generating an effective peptide mapping method. Sample preparation for peptide mapping is complex. Prior to analysis, protein samples are treated in alkaline buffer at high temperatures and enzymatically digested to generate peptides. These conditions can result in method-induced peptide modifications, over- or under-digestion of the protein, and autolysis of the proteolytic enzyme, each of which complicate data analysis and interpretation.

Waters™ PeptideWorks Tryptic Protein Digestion Kits deliver fast and reliable sample preparation for routine peptide mapping of therapeutic proteins. The sample preparation kit is centered around RapiZyme™ Trypsin, Waters' homogeneously methylated, recombinant porcine trypsin. RapiZyme Trypsin enables speed, digestion fidelity, and low levels of

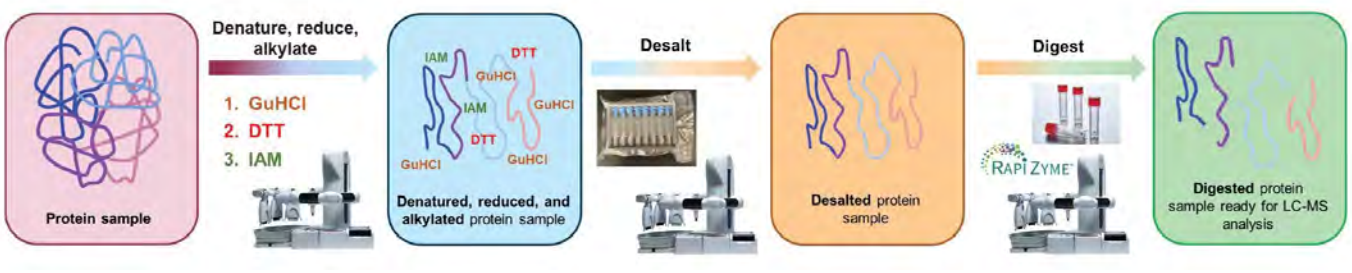


Figure 1. Workflow for the preparation of tryptic protein digests using the PeptideWorks Tryptic Protein Digestion Kit. The protocol can be performed manually or with automation on the Andrew+™ Pipetting Robot.

trypsin-derived peptides through its autolysis resistance, purity, and high activity.[2,3,4] RapiZyme Trypsin can be used at high concentrations to achieve faster digestions without requiring high temperatures or sacrificing digestion completeness. The PeptideWorks workflow is shown in Figure 1. Reagents and reaction conditions used in the PeptideWorks kit were optimized to enable fast and complete digestion of proteins by RapiZyme Trypsin.

Experimental

Reagent Preparation

The NIST Monoclonal Antibody (NISTmAb) Reference Material® was obtained from NIST (p/n: 8671), Tris HCl (1 M, pH 7.5) was obtained from ThermoFisher Scientific (p/n: 15567027), and formic acid was obtained from Fisher (p/n: A117-50). All other reagents and required devices are included in the PeptideWorks Tryptic Protein Digestion Kit (p/n: 176005311) and were prepared following the protocol outlined in the PeptideWorks Tryptic Protein Digestion Kit Care and Use Manual 720007980.

Sample Preparation

NISTmAb tryptic digest samples were prepared both manually and with automation using the procedure outlined below. Automated sample preparation was performed on the Andrew+ Pipetting Robot equipped with the Extraction+ Connected Device. NISTmAb samples (10 mg/mL) were denatured and reduced in a solution containing 5 M guanidine hydrochloride (GuHCl) and 5 mM dithiothreitol (DTT) for 30 minutes at room temperature. Iodoacetamide (IAM) was then added to a final concentration of 10 mM and the samples were incubated for 30 minutes at room temperature in the dark. Samples were desalted with Sep-Pak™ SEC Desalting Cartridges and buffer exchanged with digestion buffer (10 mM $CaCl_2$ and 100 mM tris HCl, pH 7.5). The concentration of desalted samples was measured with a UV plate reader and normalized to 0.1 mg/mL using the digestion buffer as a diluent. RapiZyme Trypsin was added to each sample at a 1:5 enzyme:protein ratio and digestion proceeded for 30 minutes at 37 °C. Finally, the reaction was quenched with 1% formic acid to a final concentration of 0.1%.

LC Conditions

LC system:	ACQUITY™ UPLC I-Class PLUS
Sample plate:	Eppendorf twin.tec® PCR Plate 96-well, skirted, green (p/n: 951020443)
Column:	ACQUITY Premier Peptide CSH™ C_{18} Column 1.7 µm, 2.1 x 150 mm (p/n: 186009489)
Column temp.:	65 °C
Sample temp.:	6 °C
Injection volume:	10 µL
Mobile phase A:	0.1% Formic Acid in H_2O
Mobile phase B:	0.1% Formic Acid in Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.25	99	1	Initial
5	0.25	99	1	6
65	0.25	60	40	6
68	0.25	30	70	6
70	0.25	30	70	6
71	0.25	99	1	6
85	0.25	99	1	6

ACQUITY RDa Detector Settings

Mass range:	50–2000 *m/z*
Mode:	Full scan with fragmentation
Ionization mode:	ESI+
Sample rate:	2 Hz
Cone voltage:	20 V
Fragmentation cone voltage:	60–120 V
Desolvation temperature:	350 °C
Capillary voltage:	1.20 kV
LockMass:	waters_connect™ LockMass solution

Data Management

Chromatography software:	waters_connect

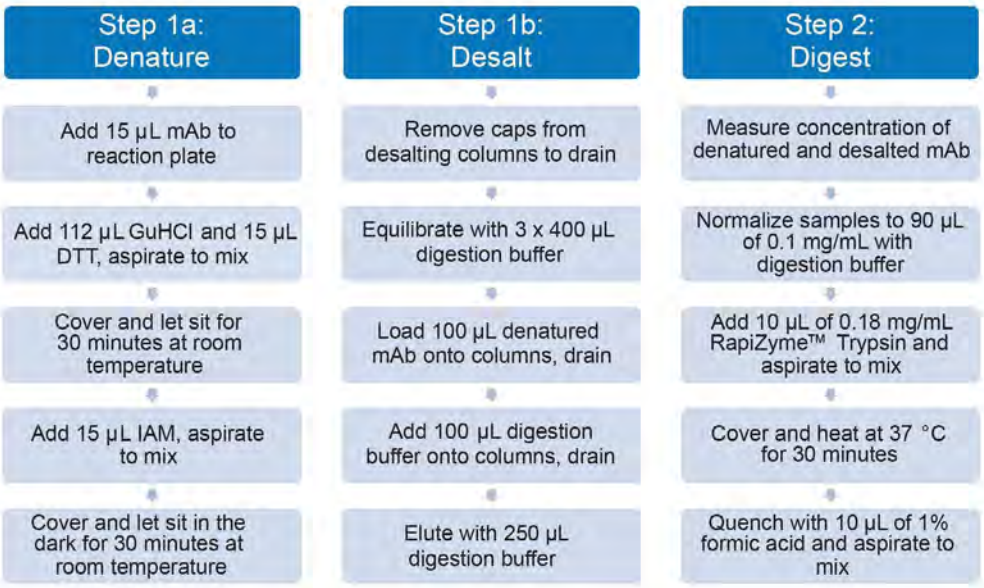


Figure 2. Flow diagram outlining the PeptideWorks sample preparation workflow. Both manual and automated sample preparation follow the depicted workflow.

Results and Discussion

The PeptideWorks Workflow

The PeptideWorks workflow was optimized to enable fast and complete digestion of proteins by RapiZyme Trypsin. The workflow can be performed manually or with automation on the Andrew+ Pipetting Robot; both manual and automated workflows follow the steps detailed in Figure 2.

The automated PeptideWorks workflow is split into two protocols: protocol A executes denaturation, reduction, and alkylation and protocol B executes concentration normalization and digestion. The automated workflow can accommodate up to 24-samples. The deck layouts for a full 24-sample workflow are shown in Figure 3. While concentration normalization is recommended to ensure accurate enzyme:protein ratios during digestion, an automated workflow without concentration normalization was also developed. The total execution time for 24-samples is 2 h 40 m with concentration normalization and 2 h 30 m without concentration normalization.

Manual vs Automated Sample Preparation

The PeptideWorks Tryptic Protein Digestion Kit was used to digest NISTmAb manually and with automation on the Andrew+ Pipetting Robot. NISTmAb digests were analyzed via UPLC-MS; chromatograms of NISTmAb digests prepared using the manual and automated PeptideWorks workflow are shown in Figure 4. Both manual and automated sample preparation yield comparable chromatographic results with high sequence coverage (>88% expected peptides). Additionally, the relative abundances of modified peptides are consistent between manual and automated sample preparation workflows, as discussed below.

A.

Position	Domino
1, 2	Tip Insertion Domino
3	Microplate Domino
4	Storage Plate Domino
5	Extraction+
6	Deepwell Microplate Domino
7	Microtube Domino
8	Deepwell Microplate Domino

B.

Position	Domino
1	Tip Insertion Domino
2, 3	Microplate Domino
4	Deepwell Microplate Domino
5	Microtube Domino
6	96-PCR Plate Peltier+

Figure 3. Deck layouts for the 24-sample automated PeptideWorks workflow. Protocol A executes denaturation, reduction, and alkylation and protocol B executes concentration normalization and digestion.

The chromatography data were processed in waters_connect using the Peptide Map workflow. Relative levels of missed and non-specific cleavages were calculated using published methods.[5] Figure 5 displays the relative missed and non-specific cleavage results for NISTmAb digests prepared using manual and automated PeptideWorks workflows. Both PeptideWorks workflows deliver NISTmAb digests with less than 5% missed and non-specific cleavages, indicating high digestion efficiency without over-digestion of the protein. Missed and non-specific

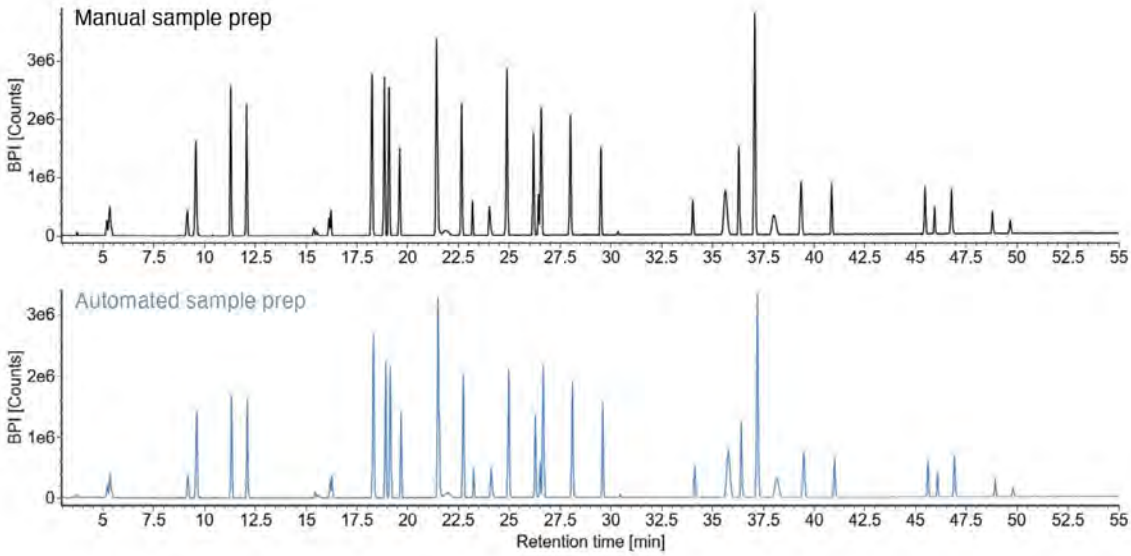


Figure 4. BPI chromatograms of NISTmAb digests prepared using the manual and automated PeptideWorks workflows, showing equivalent results.

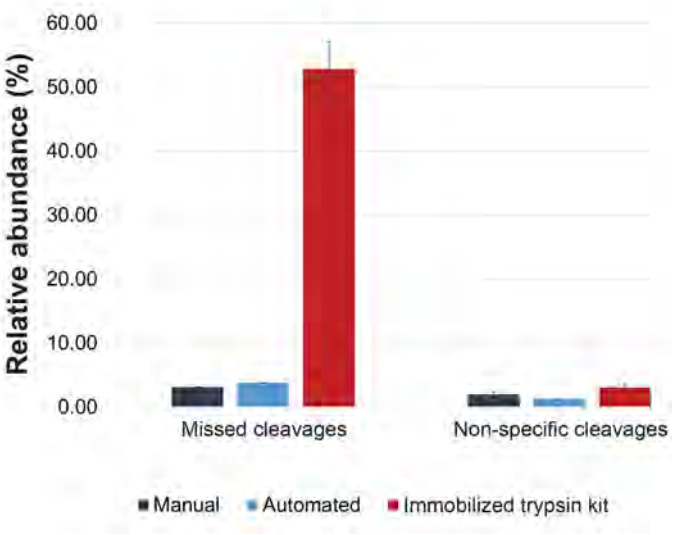


Figure 5. Relative abundance of missed and non-specific cleavages for NISTmAb digests prepared using the manual and automated PeptideWorks workflows and a leading immobilized trypsin kit. Error bars represent one standard deviation. PeptideWorks delivers a more complete and specific digestion.

cleavage results for NISTmAb digests prepared using a leading immobilized trypsin digest kit are based on published results and shown in red in Figure 5.[6] PeptideWorks yields a 93% reduction in missed cleavages and 55% reduction in non-specific cleavages compared to the immobilized trypsin digest kit. In total, PeptideWorks delivers a far more complete and specific digestion, increasing reliability, and enabling faster data processing.

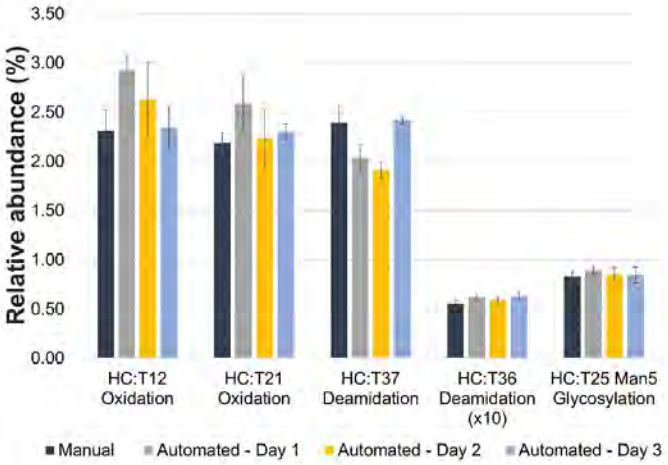
Peptide Modification Characterization and Reproducibility

The relative abundance of select peptide modifications for three batches of NISTmAb digests prepared across three days with the automated PeptideWorks workflow are shown in Figure 6; results for NISTmAb digests prepared with the manual workflow are also shown to demonstrate consistency between manual and automated sample preparation. The values reported in Figure 6 are well within range of published NISTmAb tryptic digest data.[6–9] The consistency of the modification values reported here with those reported in the literature highlight the efficacy of PeptideWorks. PeptideWorks enables fast preparation of tryptic peptide mapping samples without sacrificing digestion completion or inducing high levels of method-induced deamidation or oxidation.

Variability for unmodified peptide abundance and modified peptide relative abundance, expressed as relative standard deviation (RSD), are detailed in the table in Figure 6. The day-to-day variability of the unmodified peptide abundance was less than 15% for each peptide, demonstrating consistent peptide recovery and therefore consistent quantification limits when using PeptideWorks for sample preparation. Day-to-day variability of the modified peptide relative abundance was less than 10% for each peptide modification.

Conclusion

PeptideWorks Tryptic Protein Digestion Kit is a comprehensive kit for the preparation of tryptic peptide mapping samples suitable for QC, bioprocess, analytical development, and research environments. The PeptideWorks protocol can be performed manually or with automation, yielding 24 digested samples in under 2.5 hours. As demonstrated with NISTmAb, PeptideWorks enables reproducible preparation of tryptic peptide mapping samples without sacrificing digestion completion or inducing high levels of peptide modifications.



Peptide (modification)	% RSD	
	Unmodified peptide abundance	Modified peptide relative abundance
HC:T12 (Ox.)	11.80	8.06
HC:T21 (Ox.)	6.02	6.79
HC:T37 (Deam.)	8.09	8.82
HC:T36 (Deam.)	4.62	3.01
HC:T25 (Man5)	6.47[a]	2.12

[a] The combined abundance of the G0F, G1F, and G2F glycoforms of HC:T25 were used in place of the unmodified peptide abundance.

Figure 6. Left: Bar chart representing the relative abundance of select peptide modifications for three batches of NISTmAb digests prepared using the automated PeptideWorks workflow. Results for NISTmAb digests prepared with the manual workflow are also shown to demonstrate consistency between the manual and automated sample preparation. Error bars represent one standard deviation. Right: Table outlining the %RSD of the unmodified peptide abundance and modified peptide relative abundance to demonstrate consistent peptide recovery and therefore consistent quantification limits when using PeptideWorks for sample preparation.

References

1. Hada V., Bagdi A., Bihari Z., Timari S. B., Fizil A., Szantay Jr. C. Recent Advancements, Challenges, and Practical Considerations in the Mass Spectrometry-Based Analytics of Protein Biotherapeutics: A Viewpoint From the Biosimilar Industry. *Jour. of Pharm. and Biom. Anal*. 2018, 161, 214–2382.
2. Ippoliti S., Zampa N., Yu, Y. Q., Lauber M. A. Versatile and Rapid Digestion Protocols for Biopharmaceutical Characterization Using RapiZyme™ Trypsin. Waters Application Note. 720007840. January 2023.
3. Finny A. S., Zampa N., Addepalli B., Lauber M. A. Fast and Robust LC-UV-MS Based Peptide Mapping Using RapiZyme™ Trypsin and IonHance™ DFA. Waters Application Note. 720007864. February 2023.
4. Yang H., Koza S. M., Yu Y. Q. Automated High-Throughput LC-MS Focused Peptide Mapping of Monoclonal Antibodies in Microbioreactor Samples. Waters Application Note. 720007885. April 2023.
5. Mouchahoir T., Schiel J. E. Development of an LC-MS/MS Peptide Mapping Protocol for the NISTmAb. *Anal. Bioanal. Chem*. 2018, 410, 2111–2126.
6. Millan-Martin S., Jakes C., Carillo S., Buchanan T., Guender M., Kristensen D. B., Sloth T. M., Orgaard M., Cook K., Bones J. Inter-Laboratory Study of an Optimised Peptide Mapping Workflow using Automated Trypsin Digestion for Monitoring Antibody Product Quality Attributes. *Anal. Bioanal. Chem*. 2020, 412, 6833–6848.
7. Dong Q., Liang Y., Yan X., Markey S. P., Mirokhin Y. A., Tchekhovskoi D. V., Bukhari T. H., Stein S. E. The NISTmAb Tryptic Peptide Spectral Library for Monoclonal Antibody Characterization. *MABS*, 2018, 10, 354–369.
8. Arndt J. R., Wormwood Moser K. L., Van Aken G., Doyle R. M., Talamantes T., DeBord D., Maxon L., Stafford G., Fjeldsted J., Miller B., Sherman M. High-Resolution Ion-Mobility-Enabled Peptide Mapping for High-Throughput Critical Quality Attribute Monitoring. *J. Am. Mass. Spec*. 2021, 32, 2019–2032.
9. Jalili P., Turner J., Ray K., Dube M. An Optimized Protocol for Peptide Mapping of Therapeutic Monoclonal Antibodies with Minimum Deamidation and Oxidation Artifacts. *Analytix Reporter*, 2022, 11.

Automated High-Throughput LC-MS Focused Peptide Mapping of Monoclonal Antibodies in Microbioreactor Samples

Hua Yang, Stephan M. Koza, Ying Qing Yu
Waters Corporation

Abstract

To facilitate cell line selection and bioprocess optimization, an automated high-throughput (HT) sample preparation method and fast LC-MS peptide mapping for Critical Quality Attribute (CQA) peptide measurement was developed for low volume and low concentration microbioreactor samples. For this method, 30 µL of a 1 mg/mL sample of a monoclonal antibody (mAb) in neutralized Protein A affinity chromatography elution buffer was buffer exchanged and trypsin digested using Andrew+™ automation. The resulting peptides were separated on an ACQUITY™ Premier Peptide CSH™ C_{18} Column and detected by a BioAccord™ LC-MS System. Effective analysis was demonstrated for selected CQA peptides including N-glycosylated, deamidated, and oxidized peptides. The automated preparation of 48 samples takes 3.5 hours, with a two-hour digestion time, and LC-MS analysis time was ten minutes per sample. Besides Protein A purified mAb, this method could also be adapted to the analysis of other samples with limited amounts of protein.

Benefits

- Consistent results for the relative quantification of CQA peptides from 30 µg of mAb even at low concentrations
- An automated trypsin digestion protocol with the Andrew+ robotic platform and using a new commercialized trypsin (RapiZyme™ Trypsin) that is highly resistant to autolysis
- High-throughput (HT) LC-MS analysis of CQA peptides using an ESI-ToF BioAccord LC-MS and automated data analysis using the waters_connect™ Peptide MAM application software

Introduction

Recombinant protein biopharmaceuticals, such as monoclonal antibodies (mAbs), have benefited patients for many years. Development and production of recombinant protein can be costly, and one potential bottleneck in developing an efficient process producing high quality product can be host cell-line selection and bioprocess optimization. To reduce the timeline for cell line selection and process optimization, microbioreactors have been used in early process development.[1] In recent years, product quality has been included in cell line selection criteria, driven in part by the regulatory demands of making biosimilars. While microbioreactors provide advantages of being able to approximate the

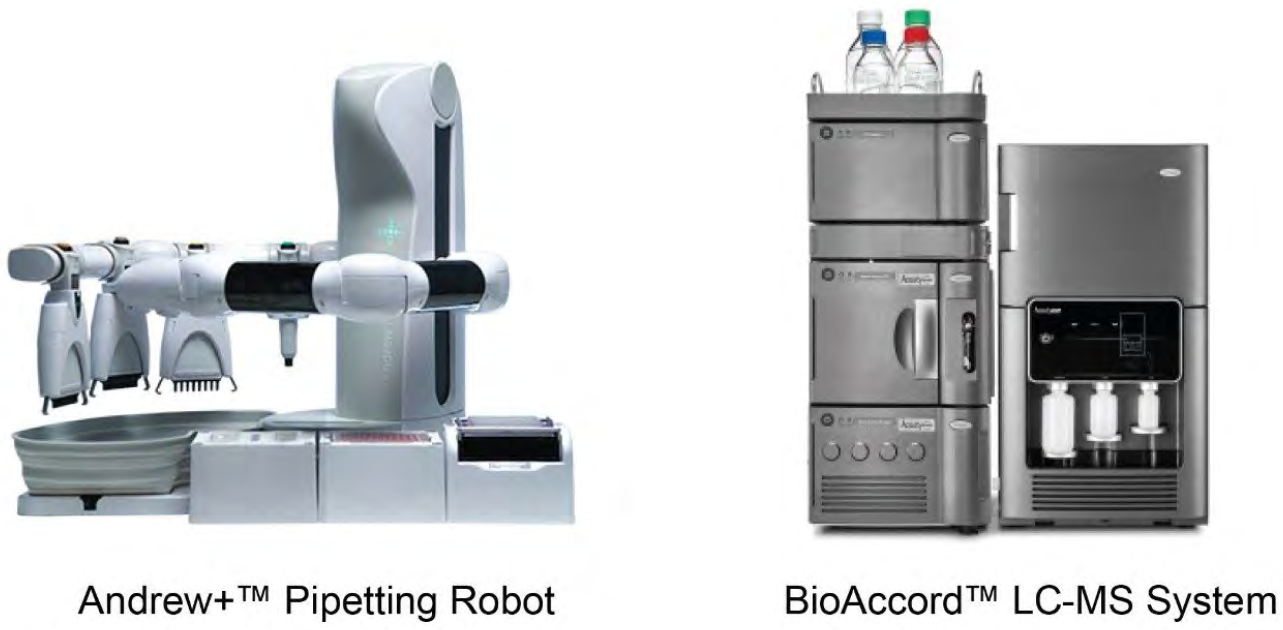


Figure 1. Image of Andrew+ Pipetting Robot and BioAccord LC-MS System.

production conditions of a large-scale bioreactor, the amount of sample taken from a microbioreactor can be analytically limiting. Another challenge is that analyzing CQAs of the sample such as site-specific modifications usually involve peptide mapping via LC-UV or LC-MS, which can be labor-intensive and low throughput.

This application note demonstrates an automated procedure using an Andrew+ robot (Andrew Alliance™) with minimum manual intervention for a CQA peptide mapping method to assist cell line selection and cell-culture optimization (Figure 1). Presented is a focused or targeted peptide mapping method for monitoring the abundances of selected CQA peptides and their modified forms. For demonstration purposes, 30 µg of 1 mg/mL mAb (infliximab) sample was reduced and trypsin digested, after which its CQA peptides were monitored using a ten minute long reversed-phase separation with MS detection. This method could also be adapted for use with mAb and other protein samples with concentrations lower than 1 mg/mL, due to a pre-concentration step in the sample preparation procedure.

Experimental

Sample Description

Infliximab (10 mg/mL) was diluted into neutralized Protein A elution buffer which contains 100 mM glycine (pH 3) and 1 M Tris (pH 7.5) in a 5:1 v/v ratio. The final infliximab concentration was 1.0 mg/mL.

For the stressed sample experiments, infliximab (10 mg/mL) was incubated in 0.005% H_2O_2 and 50 mM sodium phosphate (pH 7.6) for two weeks at 37 °C to induce oxidation and deamidation. The stressed sample was also co-mixed with original unstressed sample in 1:1 volume ratio. All samples were then diluted in the above neutralized Protein A elution buffer to 1.0 mg/mL

LC Conditions

LC system:	ACQUITY UPLC™ I-Class PLUS
Detection:	ACQUITY BioAccord MS System
Plates:	Acroprep™ Advance 350 µl Omega 10 k MWCO (p/n: PALL-8034) Eppendorf twin.tec® PCR Plate 96, skirted, 150 µL (p/n: 951020443) 6mm Pre-Slit Silicone/PTFE Cap Mat (Analytical Sales and services, p/n: 96727)
Column(s):	ACQUITY Premier Peptide CSH C_{18} 1.7 µm, 2.1 x 100 mm (p/n: 186009488)
Column temperature:	60 ˚C
Sample temperature:	10 ˚C
Injection volume:	5 µL, 10 µL
Flow rate:	0.2 mL/min, 0.4 mL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient Table (50-min gradient, 80-minute run time)

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.4	99	1	Initial
1.0	0.4	99	1	6
7.0	0.4	70	30	6
7.8	0.4	15	85	6
8.3	0.4	15	85	6
8.8	0.4	99	1	6
14.0	0	99	1	11

Gradient Table (6-min gradient, 10-minute run time)

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.2	99	1	Initial
1.0	0.2	99	1	6
51.0	0.2	65	35	6
57.0	0.2	15	85	6
61.0	0.2	15	85	6
66.0	0.2	99	1	6
90.0	0	99	1	11

ACQUITY RDa Detector Settings

Mode:	Full scan with fragmentation
Mass range:	50–2000 *m/z*
Polarity:	Positive
Sample rate:	5 Hz
Cone voltage:	30 V
Fragmentation cone voltage:	60 V – 120 V
Capillary voltage:	1.20 kV
Desolvation temperature:	350 °C

Data Management

LC-MS software:	waters_connect

Results and Discussion

General Procedure

An automated high throughput (HT) peptide mapping method using LC-MS analysis to monitor CQA peptides was successfully developed for low volume and low concentration microbioreactor mAb samples. Peptide mapping has been used for multiple attribute monitoring (MAM) by pharmaceutical industry for many years.[2] It is typical to use 100 µg of protein or more in these digestion procedures because large amount of concentrated sample is often available for product characterization studies. However, in the current application, the samples that obtained from microbioreactors usually have low concentrations and volumes. To achieve an effective focused peptide mapping sample preparation, optimization of parameters including guanidine-HCl concentration, protein to enzyme ratio, digestion time was carried out (data not shown). It is important to note that the presented methods were not developed with the intent of comprehensively mapping the mAb, as such, significant under digestion is observed for these digest conditions. Also, the sample is only disulfide bond reduced without alkylation as part of this procedure.

Figure 2A shows the major steps for the automated focused peptide mapping procedure. Thirty µg of infliximab was diluted in neutralized Protein A elution buffer to 1.0 mg/mL (See EXPERIMENTAL for details). It is assumed that the mAb is affinity-purified with Protein A affinity chromatography resin.

For this method, the first step is to buffer exchange the samples into the denaturation and reduction buffer (DRB, 6 M guanidine HCl, 1 mM methionine, 3 mM DTT, and 0.1 M Tris pH 7.5) as shown in Figure 2B. This is done using Extraction+ domino on the Andrew+ automation system and a 10 K MWCO filter plate to retain the sample. Before the samples are loaded, 50 µL water is added to the filter plate wells and vacuum applied for ten minutes at 650 mbar to drain the water through the filter. It was discovered that this conditioning step can expedite the buffer drainage in the next steps.[3] Then 20 µL of 6 M guanidine HCl, 2.5 mM methionine, 0.1 M Tris pH 7.5 solution and 30 µL of mAb (1.0 mg/mL) is added and vacuum is applied for 12 minutes at 650 mbar to drain the buffers while retaining the mAb. DRB (30 µL) is then added into the wells. To transfer samples from the filter plate to an Eppendorf 96-well PCR plate, the filter plate is inverted over the PCR collection plate and centrifuged at 500 RPM for two minutes. It is important to note that this inversion and centrifugation requires a user action and results in changing the well positions of the samples in a mirror image fashion.

After denaturation and reduction for 30 minutes at 25 °C, 100 µL 0.15 mg/mL RapiZyme Trypsin, a modified trypsin, in 1.0 mM methionine and 0.1 M Tris pH 7.5 is added to lower the guanidine-HCl concentration while digesting the mAb. The reaction mix is incubated at 37 °C for two hours, after which 10 µL of 2% acetic acid is added to stop the digestion. Finally, 0.10 % formic acid (mobile phase A) is added to dilute the reaction mix so that the final concentration of the digested protein is approximately 0.20 mg/mL.

A few points are worth noting for this procedure. First, the buffer exchange step can

potentially pre-concentrate the sample if the sample concentration is low. In current experiments, for the purpose of demonstration, 30 µL sample was loaded and 30 µL was recovered. However, if the sample concentration is low, more volume can be loaded, and recovery can be done using less volume to concentrate the sample. Secondly, comparable results were obtained with and without alkylation. Therefore, alkylation step is not included in the procedure. Thirdly, the trypsin used in this procedure has an advantage of minimal low autolysis, so it can be used at high

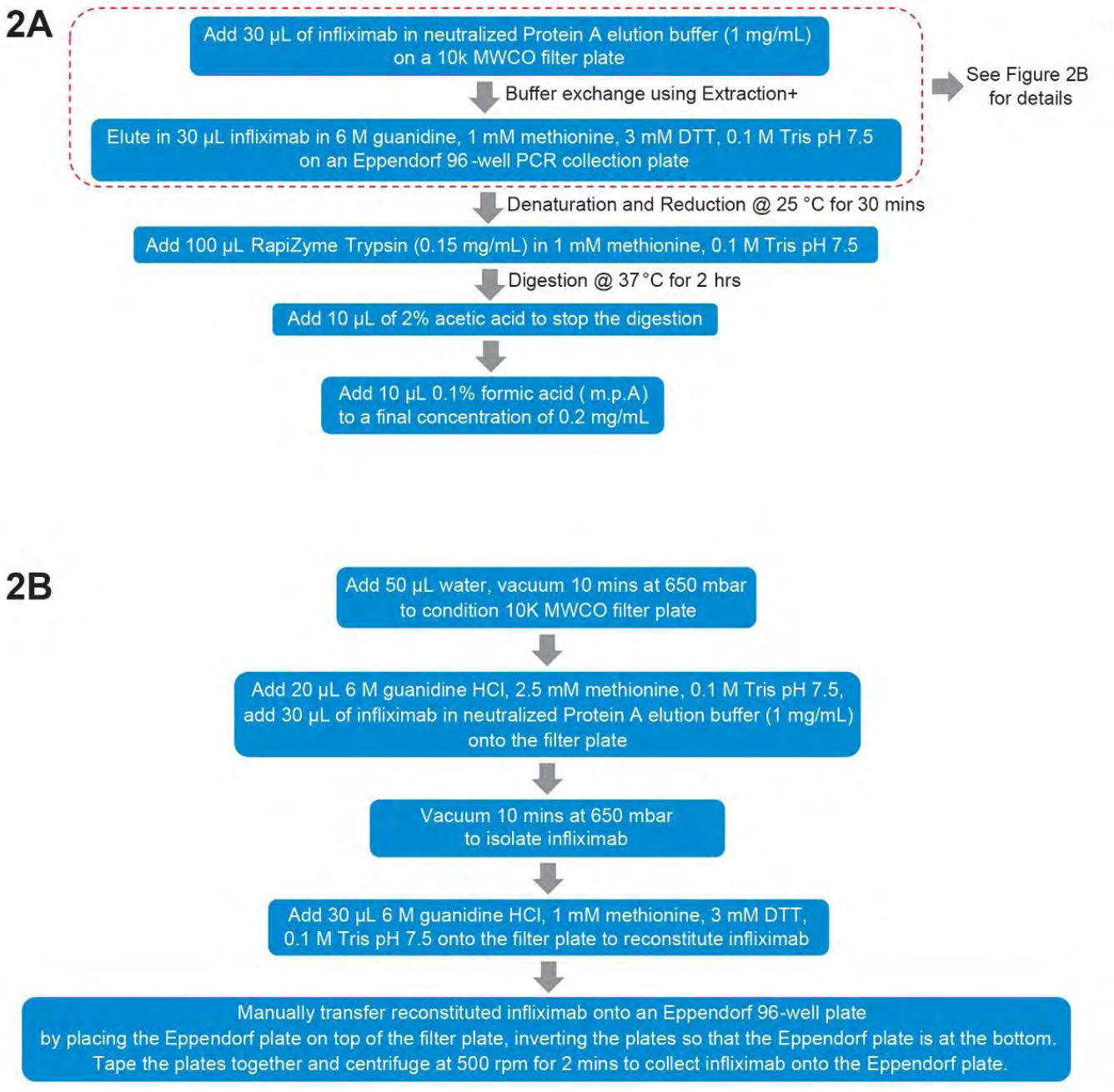


Figure 2. A. General digestion procedure using Andrew+ automation; B. Detailed procedure of buffer exchange.

concentrations to speed up the digestion rate. Figure 3A and 3B shows the blank digest and the infliximab digest run on an ACQUITY Premier Peptide CSH C_{18}, 2.1 x 100 mm Column with a 50-min gradient, respectively. Note: An ACQUITY Premier Peptide CSH C_{18} Column was selected to help ensure column to column performance consistency by QC testing each batch of synthesized CSH C_{18} particles with a tryptic protein digest rejecting those batches that do not meet performance specifications. The blank digest does not show many interfering trypsin peaks even though the Rapizyme Trypsin amount is high with a protein to enzyme ratio of 2:1.

For high throughput analysis, the digest was run with a 6-min elution gradient. As predicted, the 6-min gradient (Figure 3C) resulted in lower chromatographic resolution than the 50-min gradient (Figure 3B). Since the goal of this method is to enable relative quantitation of CQA peptides, calculated using combined ions counts from observed charge states, instead of fully characterizing the protein, it was found that the 6-min gradient was adequate.

Figure 4 shows the layout of Andrew+ for this experiment. It takes <3.5 hours to digest 48 samples using Andrew+ automation and eliminates seven manual pipetting steps.

Reproducibility

The automated sample preparation and LC-MS analysis demonstrated acceptable

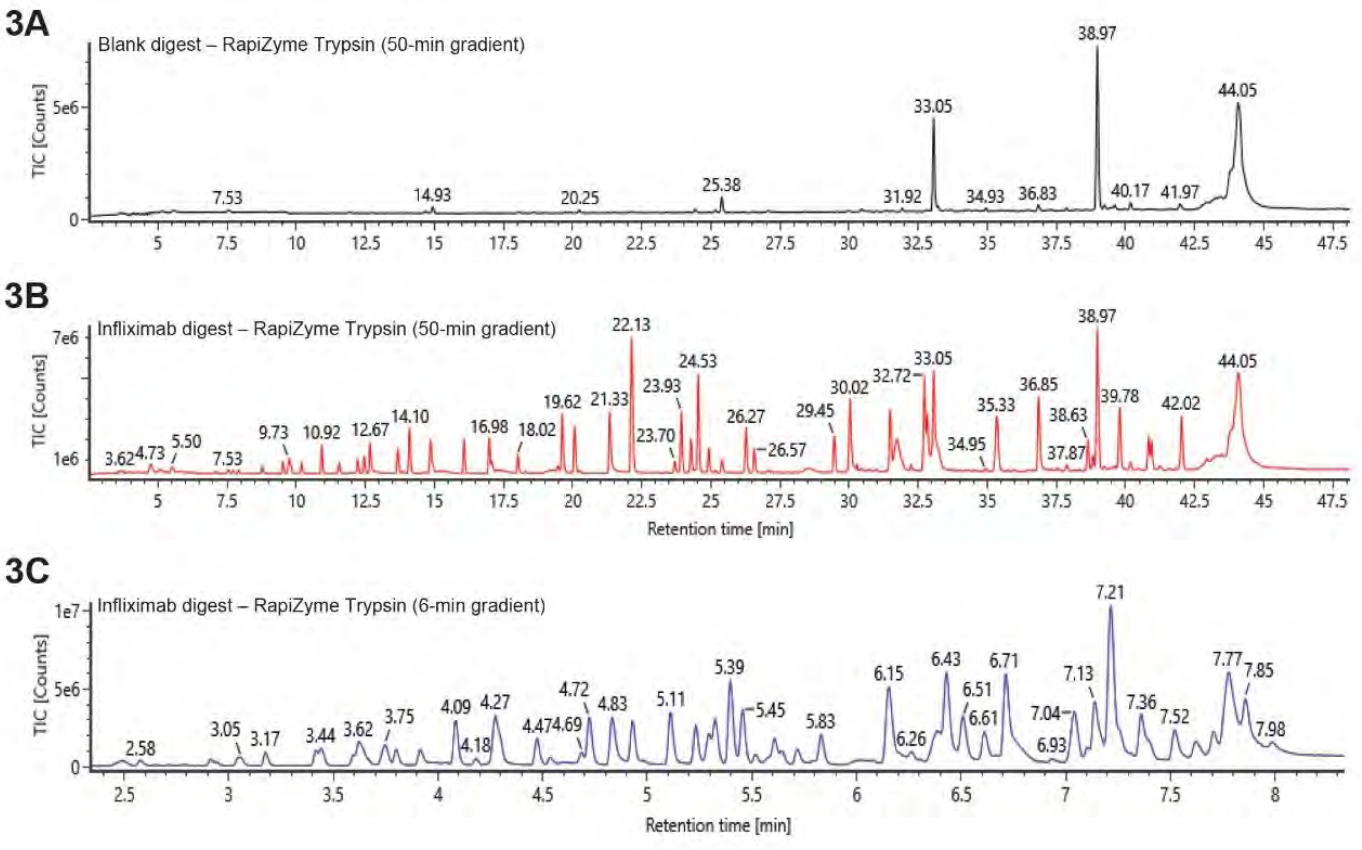


Figure 3. LC-MS chromatograms of infliximab CQA peptide mapping by RapiZyme Trypsin.
A. Blank digest; B. infliximab digest; C. infliximab digest.
For A and B, the gradient is 1–35%B in 50 mins, 0.2 mL/min. For C, the gradient is 1–30%B in 6 mins, 0.4 mL/min.

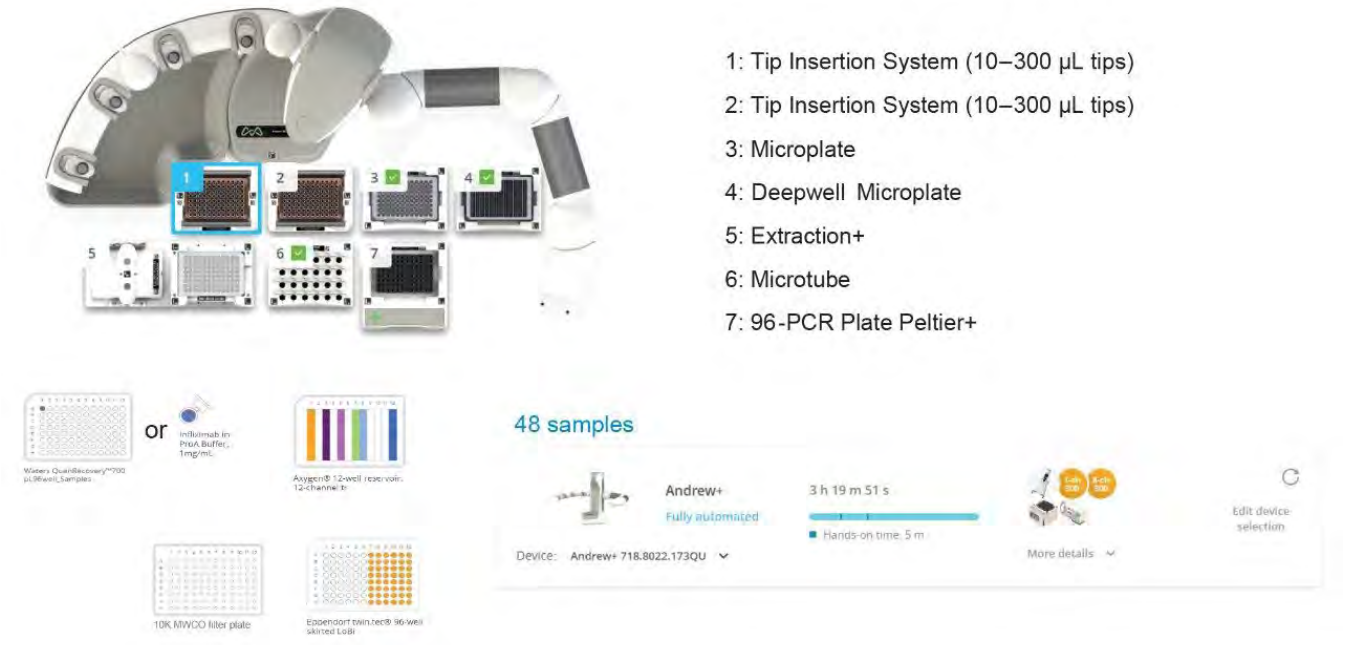


Figure 4. Andrew+ layout, dominos, and experimental time for digesting 48 samples.

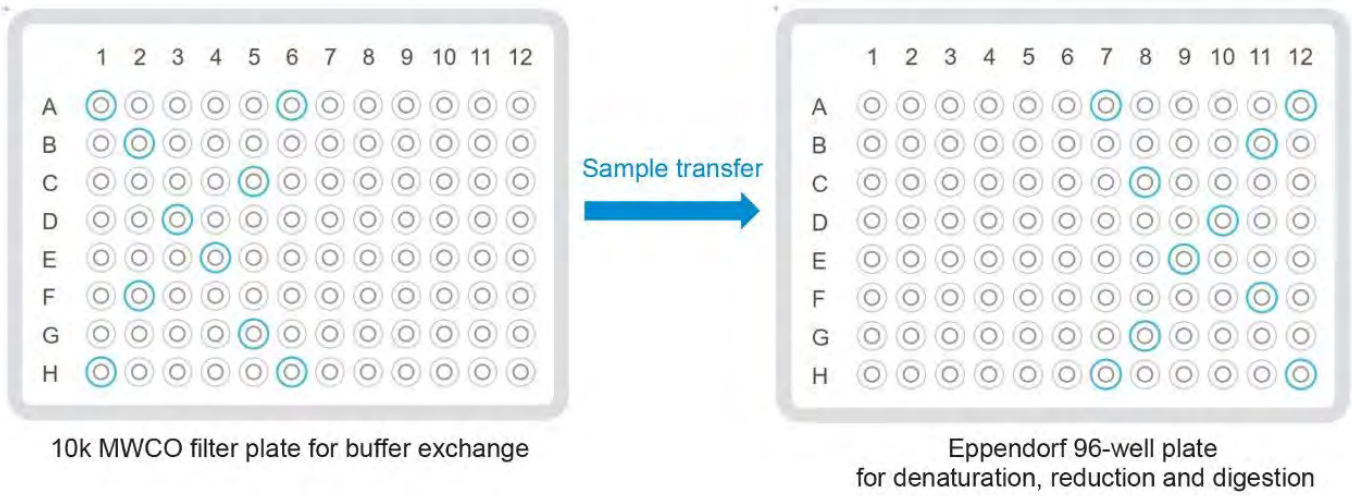


Figure 5. Positions of 10 representative wells for reproducibility study. Notice that the positions of the wells are mirror-images before and after buffer exchange.

reproducibility for a range of CQA peptides. For this study, samples of infliximab (30 µL of 1.0 mg/mL) were digested using a 48-sample protocol. Of these 10 samples representing different positions on the plate were chosen for LC-MS analysis (Figure 5). Automatic data analysis was executed using the waters_connect informatics platform and the Peptide MAM application software. Table 1 and Figure 6 show the relative abundances of the CQA peptides that were evaluated, and the relative standard deviations of those measurements. An injection volume of 10 µL (2 µg mAb) was found to result in more

Peptide number	Peptide name and modification	Average % modification	Standard deviation	%RSD
#1	HC:T2_LEESGGGLVQPGGSMK Oxidation M	0.66	0.13	19.47%
#2	HC:T3_LSCVASGFIFSNHWMNWVR Deamidation N	1.47	0.08	5.30%
#3	HC:T3_LSCVASGFIFSNHWMNWVR Oxidation M	0.69	0.06	8.87%
#4	HC:T3_LSCVASGFIFSNHWMNWVR Oxidation W, Oxidation M	0.50	0.07	14.94%
#5	HC:T7_SINSATHYAESVK Deamidation N	0.94	0.05	5.69%
#6	HC:T11_SAVYLQMTDLR Oxidation M	0.50	0.08	16.88%
#7	HC:T22_DTLMISR Oxidation M	1.66	0.27	16.04%
#8	HC:T24_FNWYVDGVEVHNAK Oxidation W	0.20	0.04	17.27%
#9	HC:T26_EEQYNSTYR G0F N	56.76	0.67	1.19%
#10	HC:T26_EEQYNSTYR G0F-GlcNAc N	3.91	0.43	10.90%
#11	HC:T26_EEQYNSTYR G1F N	31.28	0.62	1.98%
#12	HC:T26_EEQYNSTYR G2F N	3.48	0.28	8.08%
#13	HC:T26_EEQYNSTYR Man5 N	4.57	0.28	6.18%
#14	HC:T27_VVSVLTVLHQDWLNGK Deamidation N	0.55	0.03	5.33%
#15	HC:T37_NQVSLTCLVK Deamidation N	0.22	0.01	5.06%
#16	HC:T38_GFYPSDIAVEWESNGQPENNYK Deamidation N	3.87	0.14	3.58%
#17	HC:T42_WQQGNVFSCSVMHEALHNHTYQK Deamidation N	2.39	0.15	6.39%
#18	HC:T42_WQQGNVFSCSVMHEALHNHTYQK Oxidation M	1.19	0.24	19.82%
#19	HC:T43_SLSLSPG +Lysine C-TERM	53.37	0.18	0.34%
#20	LC:T6_YASESMSGIPSR Oxidation M	0.59	0.07	11.91%

Table 1. Average, standard deviation, and %RSD of relative abundances of infliximab CQA peptides (n=10).

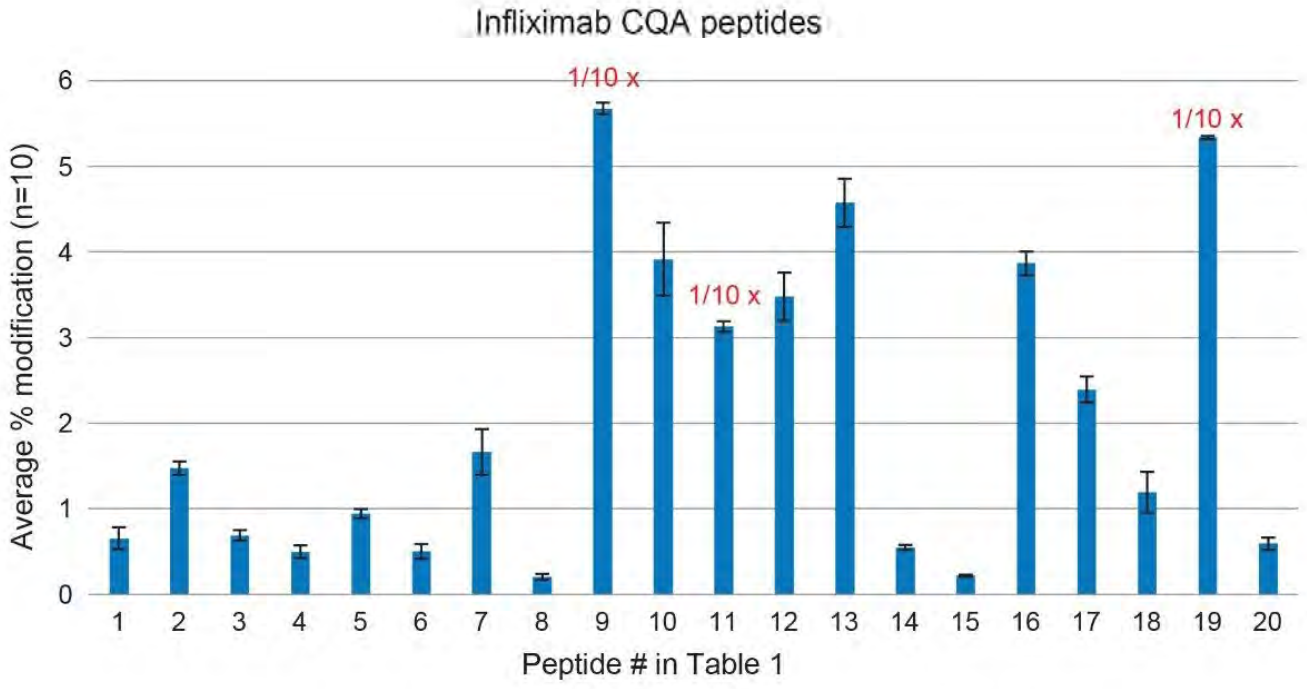


Figure 6. Average and standard deviation of relative abundances of infliximab CQA peptides (n=10). For peptide #9, #11 and #19, one tenth of the signal was plotted.

consistent results than injecting 5 µL (1 µg). In this example, injecting more than 2 µg did not improve the reproducibility results further. For all 20 CQA peptides, %RSD of the percent modification was < 20% (n=10), and of the eight CQA peptides with a %RSD between 10% to 20%, seven were at levels of 1.66% or lower. The cause of slightly higher RSD% for oxidated peptides is under investigation. On the whole, these data demonstrate that this HT LC-MS method can provide consistent results of relative abundance of site-specific modifications of a protein using limited amounts (30 µg) sample.

Stressed Sample Analysis

For cell line selection and process optimization, it is important that the method can detect changes of the percent modification of CQA peptides. For a fit for purpose demonstration, samples were stressed to increase oxidation and deamidation (please see EXPERIMENTAL for details). Stressed sample was also mixed with un-stressed sample (1:1 volume ratio). Figure 7A shows percent modification of several infliximab CQA peptides. The LC-MS data for the four oxidized and three deamidated CQA peptides evaluated were consistent with the 1:1 co-mixed sample having intermediate levels of degraded peptides. The sensitivity of this analysis to detect CQA changes is best exemplified in the results observed for the deamidated HC:T37 peptide NQVSLTCLVK (f in Figure 7A), which increased in abundance from 0.1% to 0.6%. The mass spectra of un-modified (top) and deamidated (bottom) form of this peptide are shown in Figure 7B. Based on the retention time, this deamidated form is likely aspartic acid that is converted from asparagine. Figure 7C shows results for the N-glycan modified and C-term heavy chain (HC) peptides. The HC C-terminal peptide can be present with or without a lysine residue at the C-terminus. As predicted, consistent percent modification was obtained for these stable peptide modifications. Overall, the results show that this automated HT CQA peptide mapping method is able to detect site-specific changes among different bioprocessing samples.

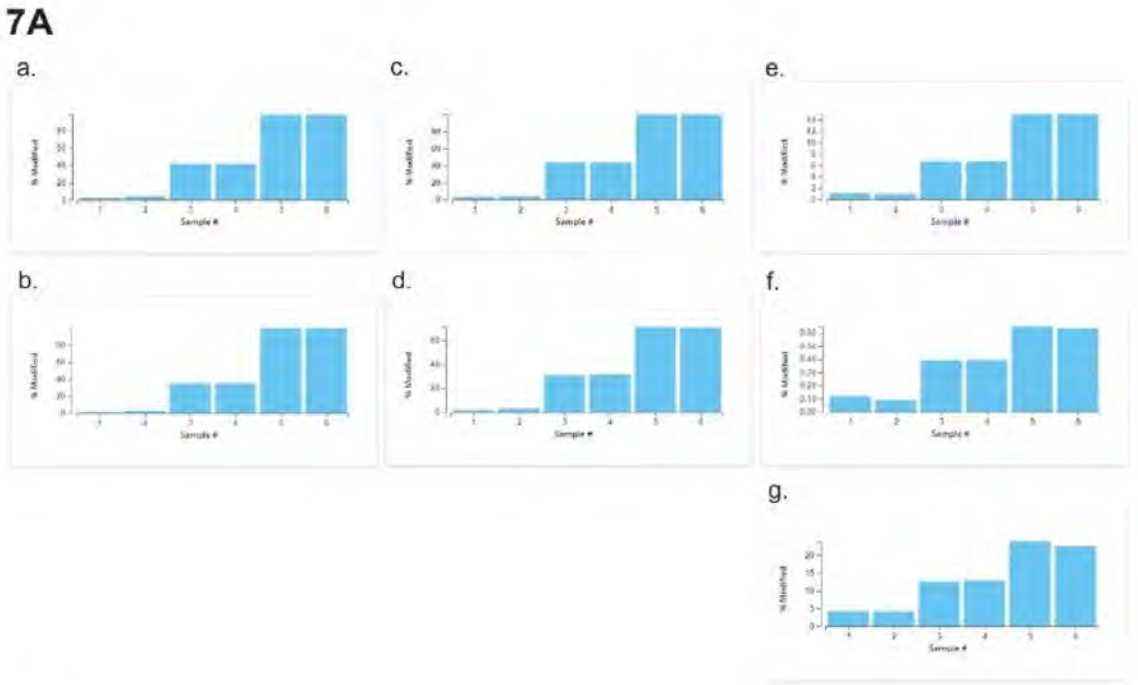
Percent modification of infliximab CQA peptides. #1 and #2 are un-stressed samples, #5 and #6 are stressed samples, while #3 and #4 are the 1:1 mixed samples.

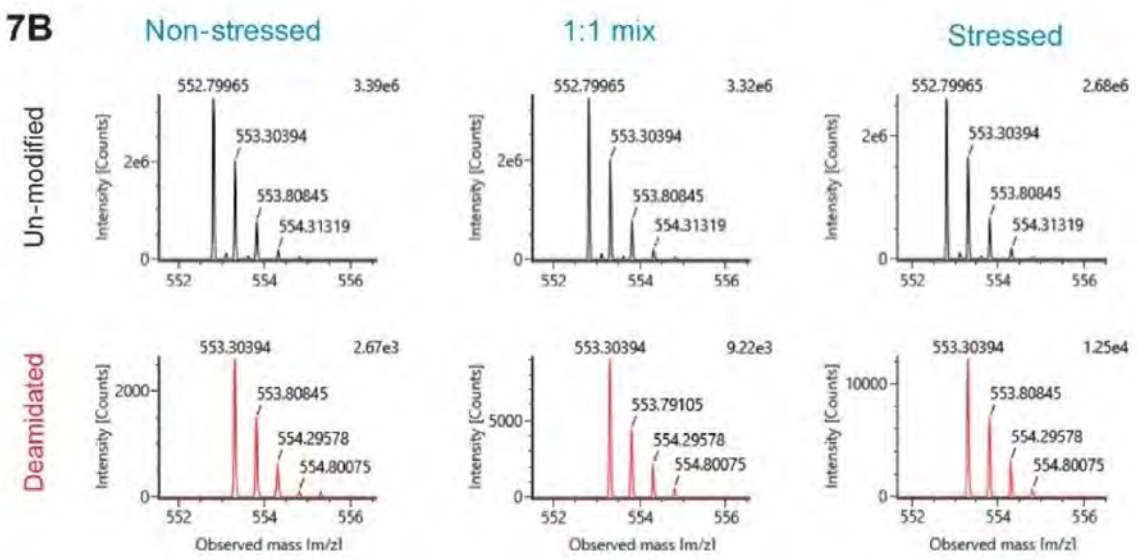
Figure 7. Percent modification of infliximab CQA peptides. #1 and #2 are un-stressed samples, #5 and #6 are stressed samples, while #3 and #4 are the 1:1 mixed samples.

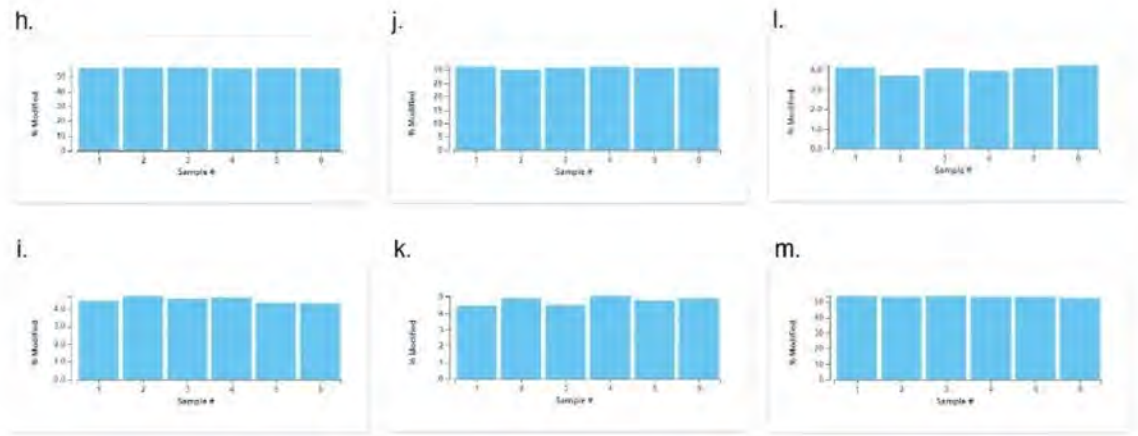
A. For CQA peptides that have oxidation or deamidation modification, the percent modification of the un-stressed and stressed sample 1:1 volume mix is approximately in the middle of the percent modification of the un-stressed sample and the stressed sample. a. HC:T2 (LEES…GSMK) Oxidation M; b. LC:T6 (YASE…IPSR) Oxidation M; c. HC:T22 (DTLMISR) Oxidation M; d. HC:T42 (WQQG…TYQK) Oxidation M; e. HC:T7 (SINS…ESVK) Deamidation N; f. HC:T37 (NQVS…CLVK) Deamidation N; g. HC:T38 (GFYP…NNYK) Deamidation N.

B. Mass spectra of HC:T37 peptide NQVSLTCLVK (f in Figure 7A). Top: un-modified form (m/z: 552.81, doubly charged); Bottom: deamidated form (m/z: 553.30, doubly charged).

C. For CQA peptides that do not have oxidation or deamidation modification, the percent modification is consistent regardless of the stress state. h: HC:T26 (EEQYNSTYR) G0F N; i: HC:T26 (EEQYNSTYR) G0F-GlcNAc N; j: HC:T26 (EEQYNSTYR) G1F N; k: HC:T26 (EEQYNSTYR) Man5 N; l: HC:T26 (EEQYNSTYR) G2F N; m. HC:T43 (SLSLSPG) +Lysine C-TERM.


7A
a.
c.
e.
b.
d.
f.
g.


7B
Non-stressed
1:1 mix
Stressed
Un-modified
Deamidated
552.79965
3.39e6
3.32e6
2.68e6
553.30394
553.80845
554.31319
2e6
Intensity [Counts]
553.30394
2.67e3
9.22e3
1.25e4
553.80845
554.29578
554.80075
553.79105
2000
5000
10000
552
554
556
Observed mass [m/z]


7C
h.
j.
l.
i.
k.
m.

Conclusion

Consistent results for the relative quantification of CQA peptides using 30 µg of mAb sample were obtained using a method featuring an automated trypsin digestion protocol with the Andrew+ robotic platform and using a new trypsin, RapiZyme Trypsin, that is highly resistant to autolysis, and offers increased activity compared to other sequencing grade trypsin products.[4, 5] This automated trypsin digest method is capable of generating 48 samples within four hours. In addition, the ESI-ToF BioAccord LC-MS method has a ten minutes total run time and automated LC-MS data processing is delivered by waters_connect using the Peptide MAM application software.

The general procedures outlined in this application note demonstrate the capabilities of the robotic platform and LC-MS used but can be readily optimized to meet the specific analytical requirements of other protein samples.

References

1. Lange I., Chhatre S., and Zoro B. Reducing Timelines in Early Process Development – Using a Multiparametric Clone-Selection and Feed-Optimization Strategy. *Bioprocess International*, November 2014.
2. Ren D., Pipes G.D., Liu D., Shih L-Y, Nichols A.C., Treuheit M.J., Brems D.N., Bondarenko P.V. An improved trypsin digestion method minimizes digestion-inducedmodifications on proteins. *Analytical Biochemistry* 392 (2009) 12–21.
3. Hanna C. M., Koza S. M., Yu Y. Q. Automated High-Throughput N-glycan Labelling and LC-MS Analysis for Protein A Purified Monoclonal Antibodies. Waters Application note. 720007854, February 2023.
4. Ippoliti S., Zampa N., Yu Y. Q., Lauber M. A. Versatile and Rapid Digestion Protocols for Biopharmaceutical Characterization Using RapiZyme™ Trypsin. Waters Application note. 720007840, January 2023.
5. Finny A. S., Zampa N., Addepalli B., Lauber M. A. Fast and Robust LC-UV-MS Based Peptide Mapping Using RapiZyme™ Trypsin and IonHance™ DFA. Waters Application note. 720007864, February 2023.

Increasing Chromatographic Performance of Acidic Peptides in RPLC-MS-based Assays with ACQUITY Premier featuring MaxPeak HPS Technology

Robert E. Birdsall, Jacob Kellett, Samantha Ippoliti, Nilini Ranbaduge, Henry Shion, Ying Qing Yu

Waters Corporation

Abstract

Metal-ion mediated adsorption of analytes as a contributing factor in poor peak shape, tailing, and diminished recovery of compounds in LC-based techniques can negatively impact data quality and assay robustness. Analytes exhibiting phosphate groups, uncharged amines, and deprotonated carboxylic acids are particularly susceptible to these phenomena and are commonly encountered in the development and manufacturing of protein-based therapeutics. Current methods for addressing analyte/surface adsorption include ion-pairing additives, hardware passivation, and high-ionic strength mobile phase. These strategies, while proven effective, can be challenging to deploy in terms of instrument/technique compatibility in the case of MS-based methods as well ensuring they can be implemented in a safe and efficient manner with respect to lengthy passivation procedures that can involve corrosive reagents. The newly introduced ACQUITY Premier brand columns with MaxPeak HPS Technology is Waters solution to these challenges. Waters ACQUITY Premier Columns are designed to deliver exceptional chromatographic performance while minimizing analyte/surface interactions of sensitive compounds. In this study, the performance gain of ACQUITY Premier Columns with MaxPeak HPS Technology is demonstrated with increased recovery, reproducibility, and robustness of RPLC-MS-based peptide mapping assays using the Waters NIST mAb tryptic digest standard. Collectively, this study establishes MaxPeak HPS Technology can be broadly applied in the development and manufacturing of therapeutic drug products to deliver the chromatographic performance expected from Waters technologies while increasing reproducibility, peak shape, and recovery of analytes prone to surface interactions.

Benefits

- Increased sensitivity through improved recovery and lower peak tailing
- Compatibility with legacy methods without the use of additional additives
- Improved method robustness through increased assay reproducibility
- Increased productivity through reduced method development time

Introduction

Analyte/surface adsorption in liquid chromatography (LC) has been established as a contributing factor in poor peak shape, tailing, and diminished recovery of compounds in LC-based techniques.[1-3] Recently, metal-ion mediated adsorption has been identified as a specific adsorption mechanism for analytes that exhibit Lewis acid/base characteristics.[4] The hypothesis being analytes bearing electron rich moieties such as phosphate groups, uncharged amines, and deprotonated carboxylic acids act as Lewis Bases which can adsorb in a non-covalent manner to electron deficient sites on the metal surface which act as a Lewis Acid. Conventional strategies to suppress metal-ion mediated adsorption include ion-pairing, hardware passivation, and high-ionic strength mobile phases. While largely successful, challenges still exist in certain instances where analytes exhibit inordinately strong interactions with metal surfaces (e.g. bearing multiple electron-rich moieties) and/or assays are performed with non-optimal conditions (e.g. weak vs. strong ion-pairing). This is particularly evident in RPLC-MS-based peptide analyses wherein peptide fragments containing aspartic acid (D) or glutamic acid (E) residues can interact with metal surfaces which can exacerbate adsorption characteristics resulting in increased tailing and reduced sensitivity of analytes prone to metal-ion mediated adsorption as shown in Figure 1. Recently, it was shown that metal chelators can be used as mobile phase additives to mitigate adsorption artifacts with notable success.[5] However, incorporation of such additives is not always ideal as they can introduce new chromatographic artifacts as well as suppress ionization in MS-based analyses. These challenges highlight the need for novel columns and instrumentation that can mitigate metal-ion mediated adsorption without the need for additional additives or lengthy passivation processes.

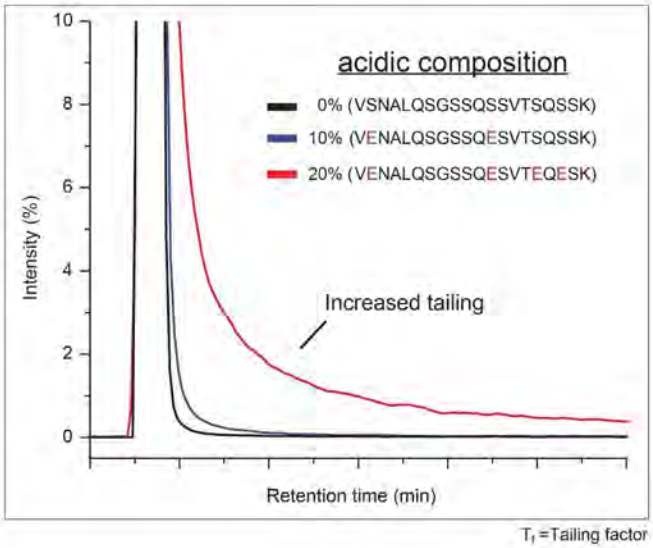


Figure 1. Synthetic acidic peptide ladder. Tailing was evaluated for 3 synthetic peptides manufactured with 0, 2, and 4 glutamic acid (E) residues representing 0%, 10%, and 20% acidic content by composition. Under isocratic conditions (MP A: 89%, MP B: 11%), tailing was observed to increase significantly for peptides containing multiple acidic residues.

The newly introduced ACQUITY Premeir brand columns with MaxPeak HPS Technology is Waters solution to these challenges. The ACQUITY Premeir Columns are designed to deliver exceptional chromatographic performance while minimizing analyte/surface interactions of sensitive compounds. The objective of this application note is to demonstrate how ACQUITY Premeir with MaxPeak HPS Technology can increase productivity in the lab and mitigate risk through increased reproducibility, recovery, and robustness of assays performed in the development and manufacturing of biopharmaceutical drug products.

Experimental

Columns were conditioned as outlined by the column care and use manual. Waters mAb Tryptic Digestion Standard (p/n: 186009126) was reconstituted in MS-grade water with 0.1% FA at a concentration of 0.2 mg/mL (SYNAPT XS) and 0.5 mg/mL (ACQUITY QDa), aliquoted and stored at -80 °C prior to use.

LC Conditions

LC system:	ACQUITY H-Class Binary Bio PLUS
Detection:	TUV, 10 mm Analytical FC, λ=214 nm
Vials:	QuanRecovery with MaxPeak HPS (300 µL/25pk, p/n:186009242)
Column(s):	CSH 130 Å C_{18} Column (2.1 x 100 mm, 1.7mm p/n: 186005297) ACQUITY Premier Peptide CSH 130 Å C_{18} Column (2.1 x 100 mm, 1.7mm p/n: 186009461)
Column temp.:	60 °C
Sample temp.:	6 °C
Injection volume:	QDa = 10 µL, Synapt XS = 1.0 µL
Flow rate:	0.200 mL/min
Mobile phase A:	H_2O, 0.1 % Formic acid
Mobile phase B:	Acetonitrile, 0.1 % Formic acid

Gradient Table (Figure 2–7)

Time	Flow (mL/min)	% A	% B	Curve
Initial	0.200	99	1	6
2.00	0.200	99	1	6
52.00	0.200	65	35	6
57.00	0.200	15	85	6
62.00	0.200	15	85	6
67.00	0.200	99	1	6
80.00	0.200	99	1	6

MS Conditions (Figure 1 and 6)

MS system:	ACQUITY QDa
Ionization mode:	ESI+
Acquisition range:	250–1250 *m/z*
Capillary voltage:	1.5 kV
Cone voltage:	10 V
Probe temp.:	600 °C

MS Conditions (Figure 2-5)

MS system:	SYNAPT XS
Ionization mode:	ESI+
Acquisition range:	50–2000 *m/z*
Capillary voltage:	2.2 kV
Cone voltage:	20 V
Source temp.:	120 °C
Desolvation temp.:	350 °C
Cone gas:	35 L/hr
Desolvation gas:	500 L/hr
Lockmass:	Glu fibrinopeptide B at 100 fmol/mL in 75/25 acetonitrile/water, 0.1% formic acid

Data Management

Chromatography software:	Empower 3 FR4
MS software:	MassLynx 4.2
Informatics:	UNIFI 1.9.4

Results and Discussion

Industry Relevance:

Peptide based analyses have proven to be an invaluable tool in the characterization and quality control of protein-based therapeutics as part of a quality lifecycle management process. As part of this process, LC-MS-based data plays a critical role in providing information used to determine primary sequence protein modifications and their related impurities. The value of mass information in these analyses has contributed in part to renewed interest in expanding the role of MS-based methods to improve productivity and data quality in the development and manufacturing of drug products. However, peptide assays with MS detection are often deployed with weaker mobile phase additives such as formic acid in favor of sensitivity over chromatographic performance. This can be problematic for trace impurities that are prone to metal-ion mediated adsorption (e.g. "acidic" peptides) as assay reproducibility and accuracy of results can vary based on the severity of the analyte/surface interaction. An example of this is shown in Figure 2A in the case of deamidation of asparagine.

Deamidation of asparagine to aspartic acid and iso-aspartic acid is a common post-translational modification of monoclonal antibodies (mAbs) that has been correlated to drug efficacy. As a result, biopharmaceutical companies invest a significant amount of resources in the control and monitoring of critical quality attributes (CQAs) such as deamidation. Of the deamidated species monitored, the "PENNYK" T:37 peptide

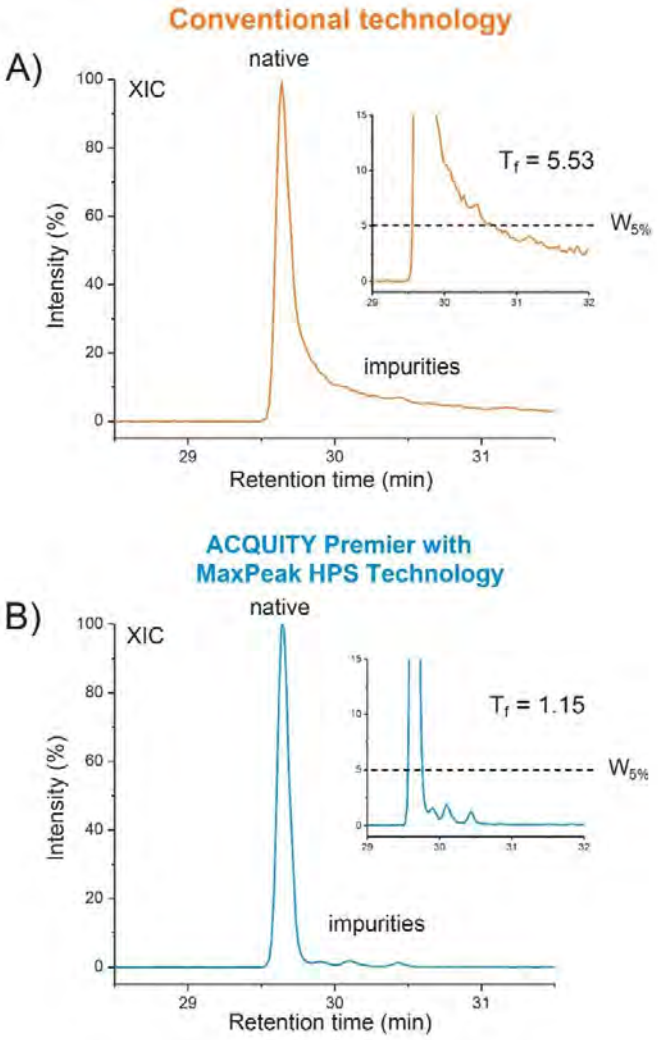


Figure 2. Critical quality attribute related T37 PENNYK peptide. A) Tailing factor was calculated at 5.53 for the T37 native peak using a conventional stainless-steel column which resulted in the inability to detect closely eluting deamidated related impurities. B) Tailing was reduced by 79% to a value of 1.15 for the native peak when using a ACQUITY Premier Column with MaxPeak HPS Technology allowing for detection of CQA related deamidated impurities.

(sequence: GFYPSDIAVEWESNGQPENNYK) is of notable interest in that it is a routinely monitored Fc domain peptide which is known to be susceptible to post translation modifications such as deamidation and contains 4 "acidic" residues (3 glutamic acid, 1 aspartic acid). As shown in Figure 2A, the impurities associated with the PENNYK peptide elute closely to the native peptide due to similar physicochemical properties making them susceptible to missidentification/integration errors due to tailing artifacts. In this example a tailing factor of 5.53 was observed for the native PENNYK peptide when performing a formic acid-based RPLC peptide map on a conventional LC system using the Waters ACQUITY UPLC CSH 130 Å C_{18} Column. This resulted in the inability to detect closely eluting impurities due to excessive tailing of the native peak. In contrast, when the same separation was performed using the Waters ACQUITY Premier Peptide CSH 130 Å C_{18} Column, tailing was reduced by 79% with a tailing factor of 1.15 observed for the native peptide. The observed performance gain allowed for the chromatographic separation of both deamidated impurities which were approximately baseline resolved from the native peak. These results demonstrate the value ACQUITY Premier with MaxPeak HPS Technology can bring to the lab to improve chromatographic performance for the development and manufacture of safe and efficacious drug products.

Increased Recovery:

The performance gains observed when using ACQUITY Premier with MaxPeak HPS Technology find merit for its application in both upstream and downstream activities. The reoccurring frequency of glutamic acid and aspartic acid residues in peptide fragments suggests all acidic residue containing peptides stand to benefit from MaxPeak HPS Technology in terms of reproducibility, peak shape, and recovery by minimizing analyte/surface interactions.

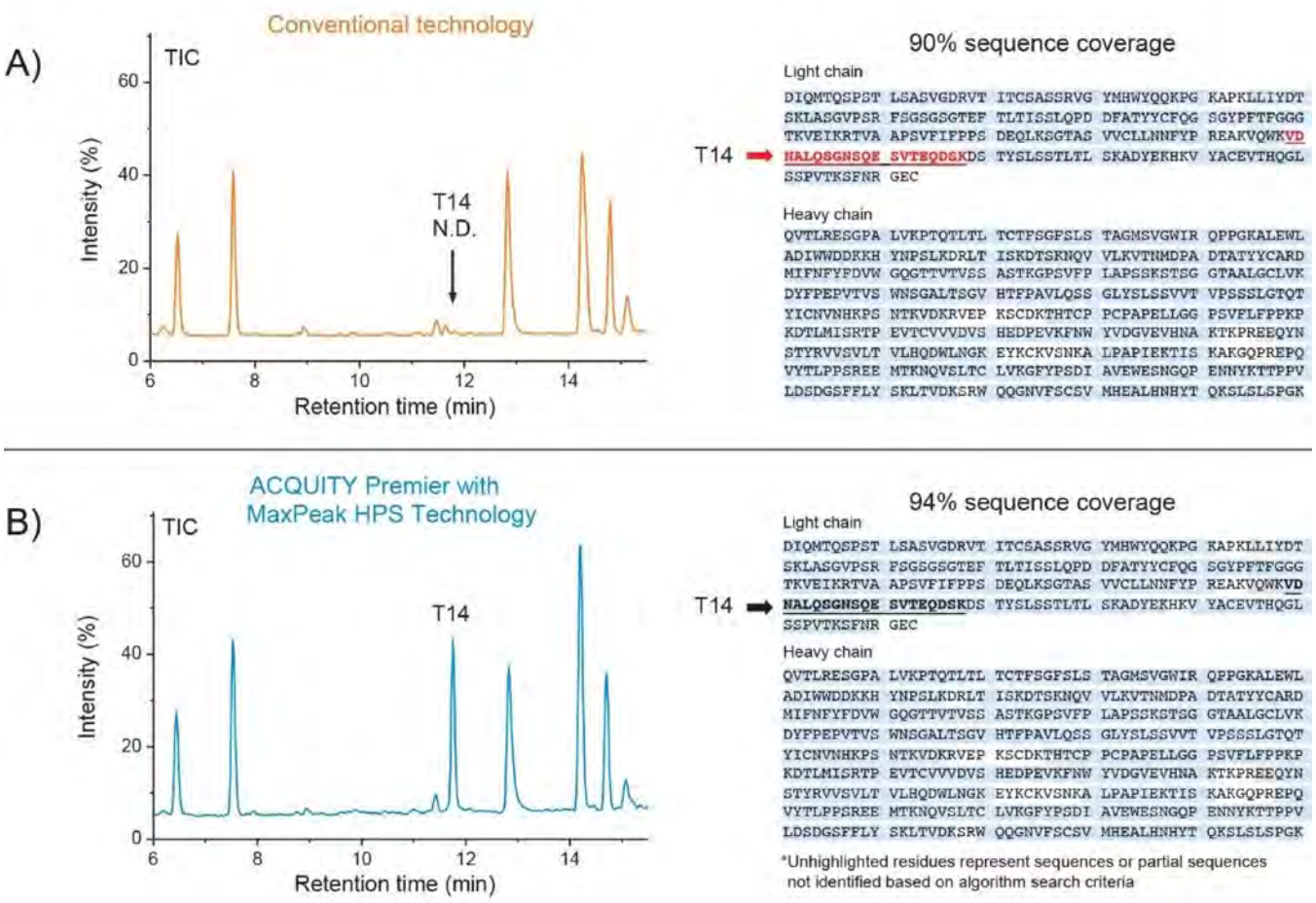


Figure 3. Increased Recovery. A) The T14 peptide fragment of the Waters NIST mAb digest standard was not detected in a LC-MS-based peptide mapping assay at a 0.2 µg mass load when using a conventional stainless-steel column, resulting in 90% sequence coverage. B) Recovery of the T14 peptide fragment when using an ACQUITY Premier Column with MaxPeak HPS Technology enabled detection and identification of the T14 peptide fragment resulting in increased sequence coverage (94%).

As shown in Figure 3, the total ion chromatogram (TIC) of the NIST mAb tryptic digest was evaluated for the recovery of the T:14 peptide fragment (sequence = VDNALQSGNSQESVTEQDSK), which contains 4 acidic residues (20%), using a Waters UPLC CSH 130 Å C_{18} Column (Figure 3A) as well as a Waters ACQUITY Premier Peptide CSH 130 Å C_{18} Column featuring MaxPeak HPS Technology (Figure 3B) with detection being performed with a Waters SYNAPT XS Mass Spectrometer. As shown in Figure 3A, the T:14 peptide fragment was not detected at a mass load of 0.2 µg resulting in a reduced sequence coverage of 90% when using the ACQUITY UPLC CSH 130 Å C_{18} Column. In contrast, the T:14 peptide was observed to have a significant increase in recovery when the same sample was separated using the same method with the ACQUITY Premier Peptide CSH 130 Å C_{18} Column (Figure 3B) resulting in 94% sequence coverage for the peptide mapping assay. The benefits afforded by ACQUITY Premier with MaxPeak HPS Technology extend beyond increased recovery alone.

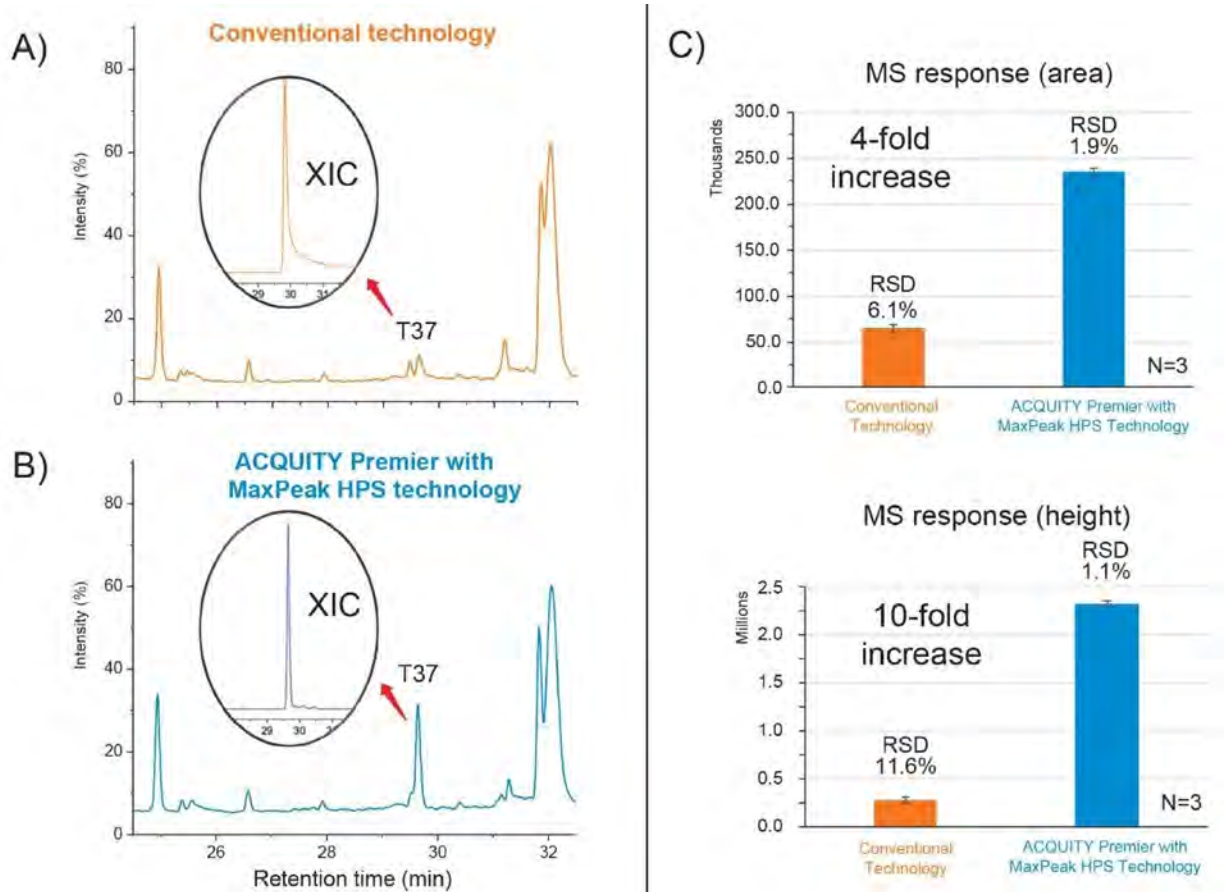


Figure 4. Increased response. Recovery of the T37 peptide fragment from a tryptic digest of the NIST reference mAb standard was evaluated for a peptide map performed on A) a conventional column (stainless-steel) as well as a B) ACQUITY Premier Column with MaxPeak HPS Technology. C) A 10-fold increase in peak height was observed due to the reduced tailing using MaxPeak HPS Technology resulting in a 4-fold increase in peak area. The observed improvement in recovery resulted in a ~90% reduction in MS-response variability (height %RSD 11.6% vs. 1.1%) across 3 replicate injections when using the ACQUITY Premier Column with MaxPeak HPS Technology.

Increased Response:

As shown in Figure 4, the T:37 peptide was evaluated concurrently with the T:14 peptide fragment using the same sample and system configuration. As shown in Figure 4A, the XIC (848.7174 *m/z*) of the T:37 peptide fragment at a 0.2 µg mass load exhibited significant tailing which impeded detection of related impurities that closely elute with the native peak as well as introducing increased variability in instrument response (Figure 4C). In contrast, the same peptide showed a 10-fold increase in peak height and a 4-fold increase in peak area as a result of reduced tailing and increased recovery when the same sample was separated using the same method on an ACQUITY Premier Column with MaxPeak HPS Technology (Figure 4B). The increased recovery and improved peak shape afforded by MaxPeak HPS Technology resulted in increased assay reproducibility with a 90% reduction in MS-response (height) variability calculated at an R.S.D. of 1.1 % for 3 replicate injections. These results demonstrate ACQUITY Premier with MaxPeak HPS Technology can be

broadly applied to improve chromatographic performance of acidic residue containing peptides for increased assay robustness and reproducibility.

Improved Peptide Characterization Data Quality:

The recovery and chromatographic performance gains observed with ACQUITY Premier and MaxPeak HPS Technology has the added benefit of improving the quality and interpretation of data. As part of upstream activities, characterization of protein-based therapeutics relies heavily on peptide mapping to determine amino acid sequence and related impurities of prospective drug candidates. In this respect, the ability of ACQUITY Premier with MaxPeak HPS Technology to reduce tailing and resolve critical species of sensitive peptides enables analysts to sequence drug candidates with increased confidence. This is demonstrated with the fragmentation profile of the T:37 peptide fragment as shown in Figure 5. Using MS^E acquisition mode (Data Independent Acquisition) on the Waters SYNAPT XS Mass Spectrometer, spectra for each of the 4 peaks (inset) related to T:37 (GFYPSDIAVEWESNGQ-PENNYK) species are shown in Figure 5. In this instance, critical y series fragmentation ions were observed and used to assign the deamidation sites for unmodified (blue) as well as deamidated or succinimide forms (red). Beginning with the y3

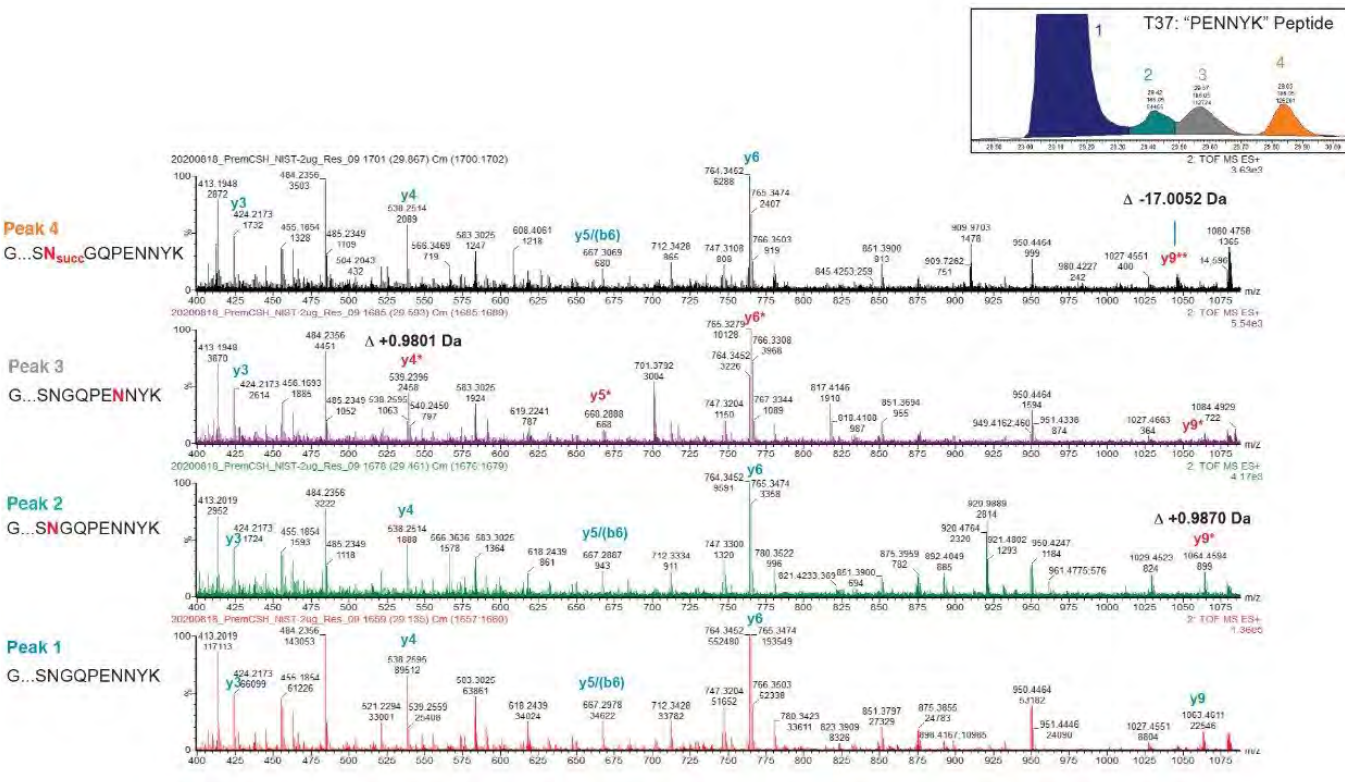


Figure 5. Enhanced data quality. MSE high energy fragment spectra (m/z 400-1150) with labeled critical y series fragmentation ions for GFYPSDIAVEWESNGQPENNYK peptide and its respective deamidation species (shown in the XIC in the top right). The bottom panel corresponds to MSE spectra of Peak 1 (unmodified), the center panels correspond to Peaks 2 and 3 (deamidated species), and the top panel corresponds to Peak 4 (a succinimide intermediate). Unmodified y ions are labeled in blue, while deamidated (*) and succinimide (**) species are labeled in red.

ion, which corresponds to N393, is unmodified in all peaks. Next, the y4 ion is unmodified in all but Peak 3, which shows a +0.9801 Da shift, pointing to deamidation on N392. The y series for Peak 3 continue to show the mass shift for deamidation, as expected. It is not until y9 ion that any mass shifts are observed for Peaks 2 and 4. The y9 ion, corresponding to N387, is observed with +0.9870 Da for Peak 2 (deamidation) and -17.0052 Da for Peak 4 (succinimide intermediate). Therefore, we can unambiguously assign these chromatographically resolved T:37 species as follows: Peak 1- unmodified, Peak 2- N387 deamidation, Peak 3- N392 deamidation, Peak 4- N387 succinimide intermediate. These results demonstrate ACQUITY Premier with MaxPeak HPS Technology not only can improve recovery and reproducibility of assays but also increase confidence in data interpretation and peptide identification through reduced tailing to resolve co-eluting peaks and increased detector response to support both upstream and downstream activities associated in the development and manufacture of therapeutic drugs.

Reproducible Chromatographic Performance:

As methods are transferred from development to manufacturing environments, reproducibility as an assay criterion is critical to ensure consistent results can be obtained in an efficient manner and accurately reflect CQAs in a drug product (a lack of which can result in costly delays and in some instances investigations when results are out of specification as in the case of regulated environments). To this end, chromatographers often incorporate passivation procedures as part of a standard operating procedure to aid in stabilizing chromatographic performance. These passivation procedures range from relatively benign practices such as repeated injections of a sample matrix to more aggressive techniques that can include the use of corrosive solvents such as nitric and/or phosphoric acid to passivate metal surfaces. While these practices are not without merit, they do take time to execute and prevent the full utilization of lab and instrument resources. ACQUITY Premier with MaxPeak HPS Technology offers users the ability to bypass these lengthy and sometimes hazardous procedures with consistent and reliable performance out-of-the box.

An example of this is shown in Figure 6 for the CQA associated PENNYK peptide. Using a newly cleaned ACQUITY UPLC System (phosphoric acid wash), tailing factor for the PENNYK peptide was monitored using a single quadrupole mass detector (ACQUITY QDa) with selected ion recording (SIR) set at a value of 849.20 *m/z* in a peptide map separation with and without ACQUITY Premier MaxPeak HPS Technology. As shown in Figure 6A, peak tailing increased in the separation without MaxPeak HPS Technology (conventional technology) with a %RSD of 8.87% over a 48-hr time period as the system approached a steady-state. In contrast to this, chromatographic performance was stable and consistent when the assay was performed on a newly cleaned system using ACQUITY Premier with MaxPeak HPS Technology with a %RSD of 1.01 % for peak tailing over the same time period. The reproducibility of MaxPeak HPS Technology in this instance translates to stable and consistent chromatographic performance. As shown in Figure 6B, when using MaxPeak HPS Technology,

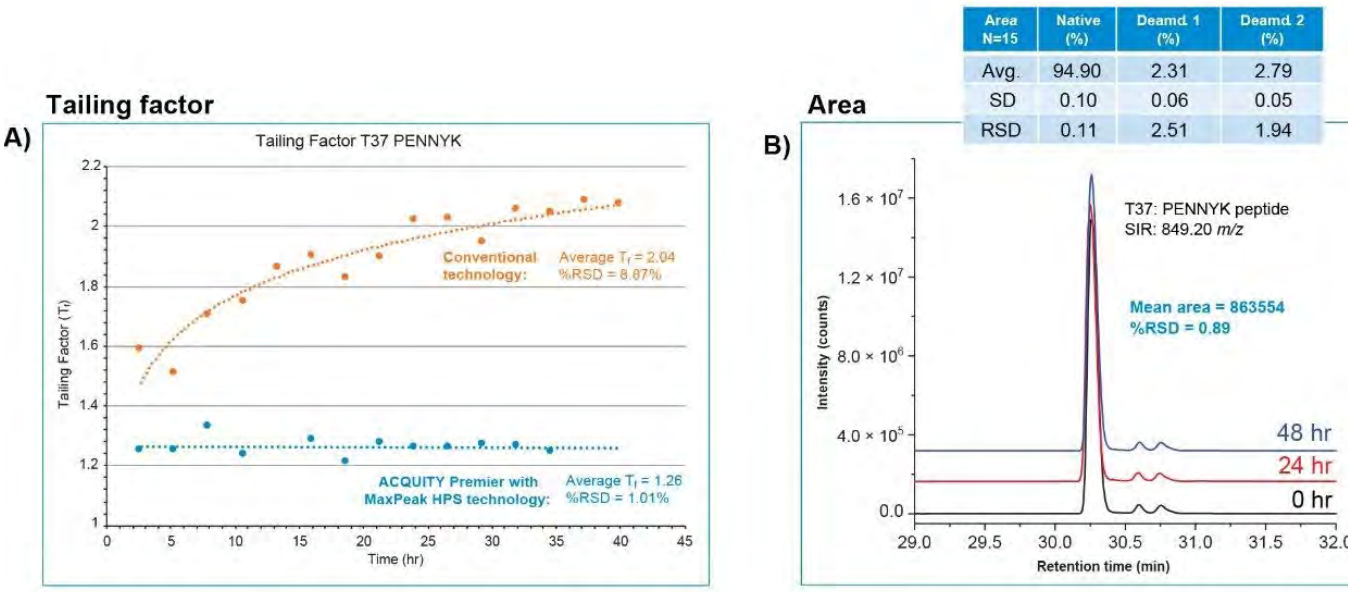


Figure 6. Reproducibility of ACQUITY Premier with MaxPeak HPS Technology. A) Tailing factor for the PENNYK peptide was monitored over a 48-hr period in a newly cleaned system with (blue data trace) and without (orange data trace) MaxPeak HPS Technology. Peak tailing % RSD was calculated at 8.87% for the separation performed without MaxPeak HPS Technology compared to 1.01% when using the ACQUITY Premier with MaxPeak HPS Technology. B) Chromatographic performance for the PENNYK peptide and associated impurities was consistent over the 48-hr injection series with %RSDs for relative abundance below 2.5% (table inset).

chromatographic performance for the PENNYK peptide was consistent and highly reproducible over the 48-hr injection series enabling accurate determination of relative abundance with %RSDs below 2.5%. These results demonstrate ACQUITY Premier with MaxPeak HPS Technology can deliver consistent and reliable results for increased productivity in the lab by reducing instrument down time and variability of results, assay qualities which are critical in ensuring safe and efficacious drug products can be brought to market in an efficient manner.

Legacy Compatibility:

Evaluation of new technology to improve the lifecycle of drug products is recommended as part of a pharmaceutical quality system. As part of the evaluation pharmaceutical companies will often perform comparability studies to determine what impact the new technology may have on existing methods deployed in the development and manufacturing of therapeutic drugs. Using these principals, retention time and selectivity was compared for a NIST mAb digest peptide map performed with and without Maxpeak Premier Technology. To ensure comparability, both data sets were generated in an identical fashion with respect to system preparation (acid washed prior to data acquisition) and column conditioning (15 injections of a mAb tryptic digest) prior to data acquisition. As shown in Figure 7A and 7B, chromatograms were analyzed between both separations to assess differences. To facilitate comparison, UV chromatograms were time aligned to adjust for dwell volume differences and plotted on

the same scale. Qualitatively, the separation performed with MaxPeak HPS Technology showed improvement in peak tailing and recovery for peptides T:14 and T:37 (insets) that were consistent with previous findings. More notably, the peptide profiles were highly consistent with each other with respect to profile and abundance indicating MaxPeak HPS Technology did not negatively impact the separation. Using a more rigorous approach peaks with a S/N ≥3 were integrated and plotted against each other as a function of relative retention time using the last eluting peak as the reference peak. As shown in Figure 7C, the orthogonal comparison indicated good retention time agreement (slope = 1.00) and a negligible time offset (y-intercept = -0.005) between the separations indicating selectivity was predominantly conserved when using MaxPeak HPS Technology. These results demonstrate ACQUITY Premier with MaxPeak HPS Technology can be applied to legacy RPLC-based methods with negligible impact to chromatographic selectivity while increasing reproducibility, peak shape, and recovery of sensitive analytes by minimizing analyte/surface interactions.

Conclusion

Analyte/surface adsorption in liquid chromatography as a contributing factor to poor peak shape, tailing, and diminished recovery can lead to increased assay variability, reduced assay sensitivity, and missinterpretation of results for analytes susceptible to surface interactions. ACQUITY Premier with MaxPeak HPS Technology is Waters, solution to these

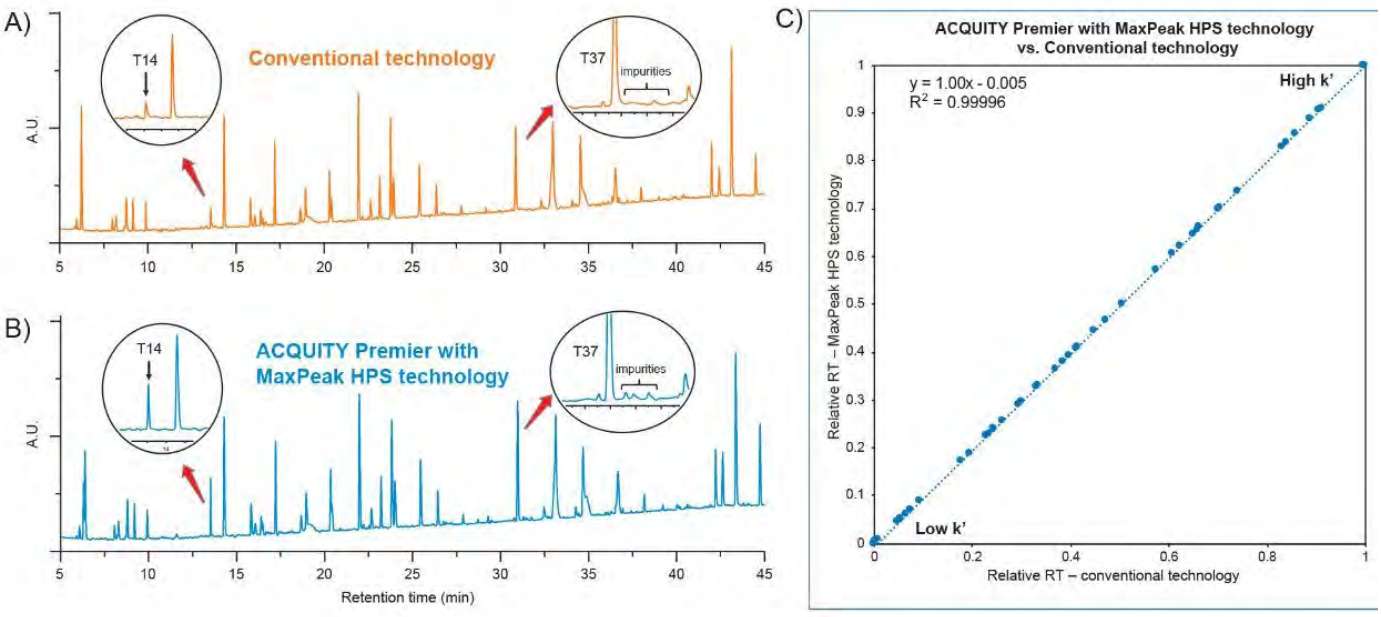


Figure 7. Comparability. Peptide profiles were highly comparable with respect to profile and abundance for a NIST mAb digest peptide map performed using A) conventional LC technology versus B) ACQUITY Premier with MaxPeak HPS Technology. C) An orthogonal comparison of relative retention time of peaks with S/N≥3 indicated good retention time agreement (slope = 1.00) and a negligible time offset (y-intercept = -0.005) indicating selectivity was predominantly conserved when using ACQUITY Premier with MaxPeak HPS Technology.

challenges. Waters, ACQUITY Premier Columns are designed to deliver exceptional chromatographic performance while minimizing analyte/surface interactions of sensitive compounds. Collectively, this study demonstrates MaxPeak HPS Technology can be broadly applied in the development and manufacturing of new or existing therapeutic drug products to deliver the chromatographic performance expected from Waters technologies while increasing reproducibility, peak shape, and recovery of analytes prone to surface interactions.

References

1. Giddings JC, Eyring H. A Molecular Dynamic Theory of Chromatography, *The Journal of Physical Chemistry*, 59 (1955) 416–421.
2. Fornstedt T, Zhong G, Guiochon G. Peak Tailing and Slow Mass Transfer Kinetics in Nonlinear Chromatography, *Journal of Chromatography A*, 742 (1996) 55–68.
3. Fornstedt T, Zhong G, Guiochon G. Peak Tailing and Mass Transfer Kinetics in Linear Chromatography, *Journal of Chromatography A*, 741 (1996) 1–12.
4. M. Lauber et al. Low Adsorption LC Technology Based on MaxPeak High Performance Surfaces. Waters White Paper 720006930EN. 2020.
5. Birdsall R, Kellett J, Yu YQ, Chen W. Application of Mobile Phase Additives to Reduce Metal-ion Mediated Adsorption of Non-phosphorylated Peptides in RPLC-MS-based Assays. *Journal of Chromatography B*, 1126–1127 (2019) 121773.

Love Employee/Getty Images

Identifying Elusive Post-Translational Modifications

Anjali Sarkar, PhD

Post-translational modifications (PTMs) provide a rapid mechanism that enables protein phenotypic diversity so that proteins can react to external and internal disturbances and regulate cellular activity. Advanced mass spectrometry (MS) analysis has allowed for the identification of over 600 distinct PTM classes jointly comprising an order of 106 unique sites but the true functional fraction is unknown.[1] Major PTM types include phosphorylation, acetylation, glycosylation, succinylation, methylation, malonylation, SUMOylation, and ubiquitination.[2]

Discoveries continue

The focus of vertebrate studies has mainly been on canonical phosphorylation, but research indicates that phosphorylation of other non-canonical amino acids also regulates integral aspects of cell biology. According to Cristina Martin-Granados, PhD, cell signaling research area scientific lead at Abcam, Claire Eyers used strong anion exchange-mediated phosphoproteomics to detect non-canonical phosphorylation. The findings indicated that non-canonical phospho-sites account for approximately one-third of the number of observed canonical phospho-sites.[3]

Non-canonical phosphorylation is highly susceptible to hydrolysis at low pH and/or at elevated temperature, therefore, standard biochemical techniques of phosphoprotein characterization are largely unsuitable for analysis of "atypical" phosphorylated amino acids.

In addition, the discovery of non-lysine ubiquitylation led to the concomitant revelation that non-proteinaceous ubiquitylation substrates such as glycogen exist.[4] "This represents a major paradigm shift in our understanding of

ubiquitylation and the breadth of biological processes that it regulates," said Martin-Granados. Over a decade ago, reports highlighting the existence and biological relevance of non-lysine ubiquitylation first appeared.[5] The number of studies has since rocketed.[6-11]

Technical challenges

"PTMs are sub-stoichiometric, highly dynamic, transient, and generally labile in nature," said Martin-Granados. "They are often present in a small subfraction of the protein population making detection by antibody-based approaches difficult."

Enrichment of a specific PTM can help tackle low stoichiometry challenges. For example, immunoprecipitation can be performed before Western blotting or MS analysis. Ion exchange, immobilized metal ion affinity, and immunoaffinity chromatography are also enrichment techniques that can be used to segregate PTM proteins/peptides from the unmodified pools, to decrease sample heterogenicity while increasing analytic efficiency and reliability.

PTMs can be cell- or tissue-specific and experiments require very stringent controls. The choice of positive and negative experimental controls is essential to correctly interpreting results. "There is evidence that some PTM modifications can block the binding site of the antibody on its target protein leading to a false negative result," said Martin-Granados. In addition, since many PTMs are the aftermath of enzymatic reactions, sample processing can affect the target if unwanted enzymatic activity is not controlled.

Characterization of PTMs relies heavily on proteomics analyses. Antibodies are essential detection and enrichment tools. "But the development of highly specific antibodies with exquisite binding affinity remains challenging due to the small size of the PTM chemical moieties, similarities in the chemical structure, and poor antigenicity. In addition, specific recognition of the PTM and surrounding sequence or a pan-PTM may be required," said Martin-Granados. Polycloncal antibodies can present drawbacks in delivering reproducible and reliable data due to strong lot-to-lot variations.

Useful tools

Computational methods for predicting PTMs are attracting considerable attention. The AI program AlphaFold is a valuable tool to predict unsolved protein structures from their amino-acid sequence.[12,13]

Unfortunately, AlphaFold2 does not consider the impact of PTMs on protein structure, but databases on protein PTMs and computational tools are available, and NMR spectroscopy and MS largely complement the limitations of AlphaFold2.[14,15] With time, it may become possible to create and integrate new algorithms into AlphaFold2-generated structures and PTM databases to achieve a comprehensive outlook for PTM prediction.[15]

Although advances in MS have enabled the mapping of individual ubiquitin modifications that generate the ubiquitin code, the intricate architecture of polyubiquitin signals has remained largely elusive. Ubiquitin-clipping is a novel methodology that has provided insight into ubiquitin chain architecture and can be useful to decipher combinatorial complexity and architecture.[16]

The discovery of ester-linked ubiquitin linkages also presents an opportunity to design new antibodies against these linkages. "This will be challenging due to the increased ability of the ester bond when compared to the canonical isopeptide linkage," said Martin-Granados.

Another recent strategy is specific uncaging-assisted biotinylation and mapping of phosphoproteome, SubMAPP, which integrates an activatable proximity labeling enzyme with an orthogonal phosphorylation enrichment scheme and LC-MS/MS. SubMAPP is a highly sensitive method to characterize the subcellular phosphoproteome in living systems with high temporal resolution.[17]

References

1. Bradley D. The evolution of post-translational modifications. Curr Opin Genet Dev. 2022 Oct;76:101956. doi: 10.1016/j.gde.2022.101956. Epub 2022 Jul 14.
2. Ramazi S and Zahiri J. Post-translational modifications in proteins: resources, tools and prediction. Database, Volume 2021, 2021, baab012. doi: 10.1093/database/baab012
3. Hardman G, et al. Strong anion exchange-mediated phosphoproteomics reveals extensive human non-canonical phosphorylation. The EMBO Journal (2019)38:e100847. doi:10.15252/embj.2018100847
4. Kelsall IR, et al. HOIL-1 ubiquitin ligase activity targets unbranched glucosaccharides and is required to prevent polyglucosan accumulation. The EMBO Journal (2022)41:e10970. doi:10.15252/embj.2021109700
5. Wang X, et al. Ube2j2 ubiquitinates hydroxylated amino acids on ER-associated degradation substrates. J Cell Biol (2009) 187 (5): 655–668. doi:10.1083/jcb.200908036
6. Pao KC, et al. Activity-based E3 ligase profiling uncovers an E3 ligase with esterification activity. Nature. 2018 Apr;556(7701):381-385. doi: 10.1038/s41586-018-0026-1. Epub 2018 Apr 11. PMID: 29643511
7. Kelsall IR, et al. The E3 ligase HOIL-1 catalyses ester bond formation between ubiquitin and components of the Myddosome in mammalian cells. PNAS. 116 (27) 13293-13298. doi:10.1073/pnas.1905873116
8. Otten EG, et al. Ubiquitylation of lipopolysaccharide by RNF213 during bacterial infection. Nature. 2021 Jun;594(7861):111-116. doi: 10.1038/s41586-021-03566-4. Epub 2021 May 19. PMID: 34012115; PMCID: PMC7610904
9. Zhu K, et al. DELTEX E3 ligases ubiquitylate ADP-ribosyl modification on protein substrates. Science Advances, 2022, 8 (40), ff10.1126/sciadv.add4253ff. ffhal-03862525f
10. Sakamaki J and Mizushima N. Protocol to purify and detect ubiquitinated phospholipids in budding yeast and human cell lines. STAR Protocols, Volume 4, Issue 1,2023, 101935, ISSN 2666-1667, doi:10.1016/j.xpro.2022.101935
11. McCrory EH, Akimov V, Cohen P, Blagoev B. Identification of ester-linked ubiquitylation sites during TLR7 signalling increases the number of inter-ubiquitin linkages from 8 to 12. Biochem J 9 December 2022; 479 (23): 2419–2431. doi:10.1042/BCJ20220510
12. Jumper J, et al. Highly accurate protein structure prediction with AlphaFold. Nature 596, 583–589 (2021). doi:10.1038/s41586-021-03819-2
13. Varadi M, et al. Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. Nucleic Acids Research, Volume 50, Issue D1, 7 January 2022, Pages D439–D444, doi:10.1093/nar/gkab1061
14. Laurents DV. AlphaFold 2 and NMR Spectroscopy: Partners to Understand Protein Structure, Dynamics and Function. Frontiers in Molecular Biosciences. Vol 9 2022. doi:10.3389/fmolb.2022.906437
15. Biehn SE and Lindert S. Protein Structure Prediction with Mass Spectrometry Data. Annual Review of Physical Chemistry 2022 73:1, 1-19. doi:10.1146/annurev-physchem-082720-123928
16. Swatek KN, et al. Insights into ubiquitin chain architecture using Ub-clipping. Nature. 2019 Aug;572(7770):533-537. doi: 10.1038/s41586-019-1482-y. Epub 2019 Aug 15. PMID: 31413367; PMCID: PMC6823057
17. Liu Y, et al. Spatiotemporally resolved subcellular phosphoproteomics. 2021 PNAS 118, e2025299118, doi:10.1073/pnas.2025299118

Proteome Maps Open an Age of Biological Discovery

Garry Killian/Getty Images

Spatial proteomics began hardly more than a decade ago, but it is already gathering momentum. Consider the progress that has been made in the subcellular mapping of proteins. In 2006, pioneering researchers from the Max Planck Institute for Biochemistry reported that they had generated a mammalian organelle map.[1] The same year, researchers from the University of Cambridge indicated that they had mapped the *Arabidopsis* organelle proteome.[2] Such efforts demonstrated what could be achieved with density gradient centrifugation and marker protein profiling, and they stimulated a proliferation of experimental techniques for determining how proteins are distributed within cells and tissue.

"Spatial proteomics has been around, in an infant way, for a little while, but I think we're learning more about the importance of identifying where different proteins are within the cell and body, and how this affects their function," says Alla Gagarinova, PhD, a postdoctoral fellow at the University of Saskatchewan. Gagarinova is among the researchers using new techniques to explore the proteome's spatial dimension—both within cells and between them. The new techniques have many applications, from basic science to therapeutic development to pandemic preparedness.

High-resolution instrumentation

Spatial proteomics has developed considerably in the last decade, according to Laurent Gatto, PhD, an associate professor of bioinformatics at the Catholic University of Louvain. He explains, "Experimental techniques, by which I mean mass spectrometry, have moved on considerably."

The instruments available today have higher resolution than the ones that were available 5 to 10 years ago. "And that's going to continue evolving," Gagarinova points out. "So, we'll be able to look at [which proteins are] in a cell, where they are, what they do, and how that can change." She adds that researchers are already shifting beyond the "lowest hanging fruit" of identifying the most abundant proteins to mapping proteins in the nucleus and organelles in cells.

Computational tools

Computational techniques have also improved, and a wider range of software available to researchers. "I have the feeling [researchers] don't fully understand the sheer range of software and approaches [available]," Gatto says. "This is a rapidly emerging field."

At the Belgium Proteomics Association Conference (cancelled due to COVID-19), Gatto planned to give a presentation on using bioinformatics to map the location of proteins inside a sample. In this presentation, Gatto would have shared an observation that he states here as follows: "You might have a complex experimental design and have collected data over weeks or months, but once you start the analysis, you only need two pieces of information."

By "two pieces of information," Gatto means two bodies of information—the first pertaining to marker proteins (that is, proteins with experimentally verified locations), the second pertaining to proteins for which location information is available only from a database.

In a dataset with 5,000 proteins, a researcher might be able to preannotate 1,000 proteins, leaving 4,000 with their location unknown. A simple analysis might then use Principal Component Analysis to see if any clusters can be identified in the data.

"If we don't see any clusters, that's not a great sign," Gatto remarks. "However, if these clusters match well-known protein markers, it's a good sign that, okay, my data or my experiment worked, and that it's possible to carry on with more complex analyses."

A researcher might proceed by using a classification algorithm to predict the location of the unknown proteins based on the characteristics of the markers, he continues. Alternatively, a researcher who didn't have enough markers for every organelle in a cell might use semisupervised machine learning to guess the locations of unknown proteins.

Essentially, the researcher is asking the algorithm a question. In Gatto's words, the question is: "There's stuff missing, please can you see if there's meaning here as well?" Many proteins are also found in multiple locations. Consequently, a researcher may want to ask an additional question, one that Gatto puts as follows: "Is my algorithm able to identify them?"

Global interrogation

Spatial proteomics refers to two different activities, explains Kathryn S. Lilley, PhD, a professor of biochemistry at the University of Cambridge. One activity is looking at different cells within a tissue slice and determining the groups of proteins associated with them. The second activity is identifying where proteins are located within a cell. Lilley says that her research focuses on the second activity.

"Our method has been around in concept for a long time," Lilley notes. "We first published [about it] in 2004." Her research was inspired, she recalls, by the difficulty of mapping proteins to specific organelles. Since 50% of proteins were present in more than one location, and many organelles had similar physical properties, even if her team could purify and enrich proteins in one niche, they wouldn't know where else each protein could be found.

Lilley's team turned to protein correlation profiling, a technique from the 1950s. Each subcellular niche is strung along a different subcellular fraction by centrifugation, and the protein amounts in each fraction can be measured. "You get rich and complex data," she says. "It's taken us a long time to come up with computational methods to deal with these data and use the method comparatively."

In a paper that recently appeared in *Nature Communications* , Lilley and colleagues described how the abundance of proteins in human immune cells changed in the 12 hours after a proinflammatory response was induced by lipopolysaccharide treatment. [3] The researchers used novel Bayesian statistical analyses to estimate the probability of a protein belonging in a location and moving to another location under this physiological stress.

"What was surprising, which we didn't expect to see at such a stark level, was that many proteins weren't changing in abundance—but they were changing in location," Lilley points out. This finding currently lacks a definite explanation. "It is not possible," Lilley and colleagues noted in the paper, "to distinguish trafficking of existing proteins from one location to another, from

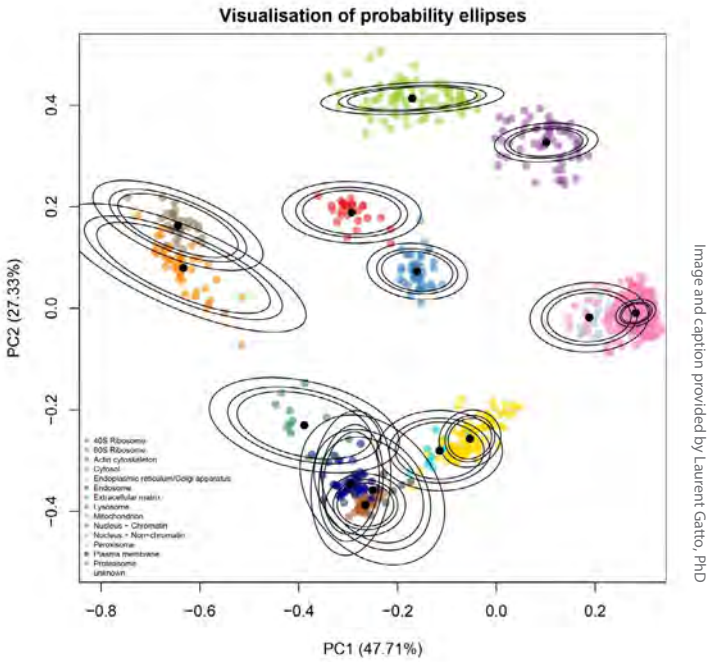


Image and caption provided by Laurent Gatto, PhD

Figure 1. Probabilistic modeling of the spatial proteome: probability ellipses are modelled using known marker proteins, and used to reliably classify proteins to one or multiple locations.

proteins being differentially degraded at one location and newly synthesized proteins locating to an alternate location."

"The paper," Lilley declares, "threw up more questions than it answered!"

Single-cell imaging

Nikolai Slavov, PhD, is also studying proteins at a subcellular level. An Allen Distinguished Investigator and Associate Professor of Bioengineering at Northeastern University College of Engineering, he says his research differs from Lilley's research in a crucial way: "She is using 10 to 100 million cells and measuring the average across them, whereas we're doing one cell at a time."

Slavov's main project is working on the Human Cell Atlas, an international collaboration to map the position, function, and characteristics of every cell type in the body. He

is using single-cell imaging to measure the abundance of RNA and proteins in single cells, and then to localize them in three dimensions within body tissue.

Speaking about the benefits of single-cell imaging, he says: "Population-wide averages have existed for more than a decade and are substantially easier to do, but human cells are different functionally, and that's why we want to have single-cell resolution."

As a secondary project, Slavov is using single-cell imaging to measure the abundance of proteins inside macrophages when these immune cells are activated by antigens. "Looking at protein abundance can help us identify cell types," he notes, "but you need the additional dimension [of single-cell analysis] to understand the molecular mechanisms behind human disease."

A major application of his work is how to make tumor-associated macrophages more proinflammatory, so they can be used to fight cancer. He is also seeking to understand the role of macrophages in autoimmune and neurodegenerative disease.

Cataloguing cell types

Charlotte Stadler, PhD, focuses on the first type of spatial proteomics—understanding the difference between cells within the same tissue type. In her research at the KTH Royal Institute of Technology and at SciLifeLab's Spatial Proteomics Facility, Stadler uses highly multiplexed imaging to look at 20–30 different proteins in each cell.

"We can look for different proteins in a single sample," she says. We can, she continues, identify cellular phenotypes and get clues about the expression and cellular function of proteins.

Stadler and colleagues use fluorescence-based methods to study the proteins. According to Stadler, these methods overcome the problems of spectral overlap between multiple fluorophores by looking at a few proteins at a time and washing away the fluorophores between each imaging step.

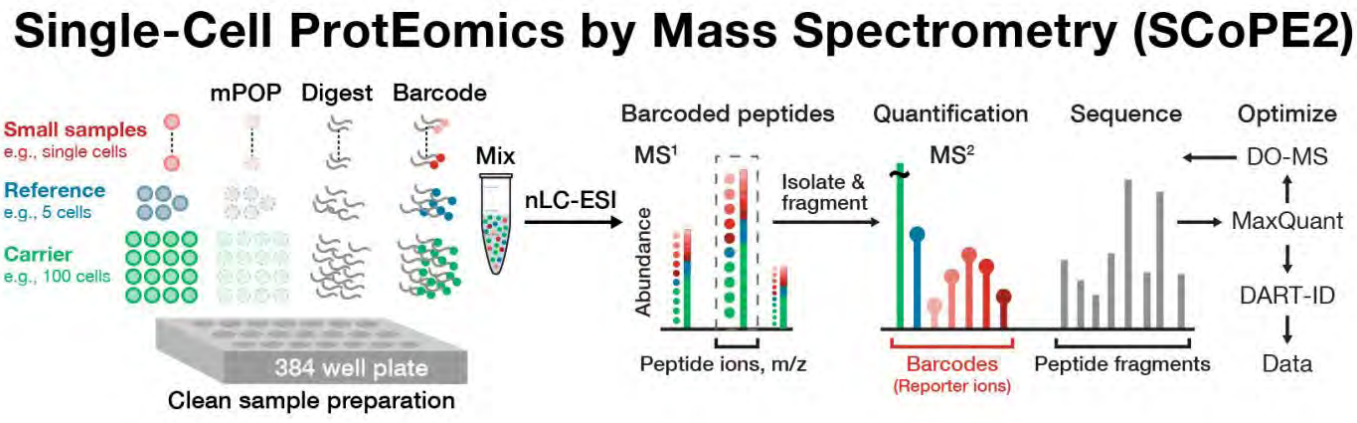
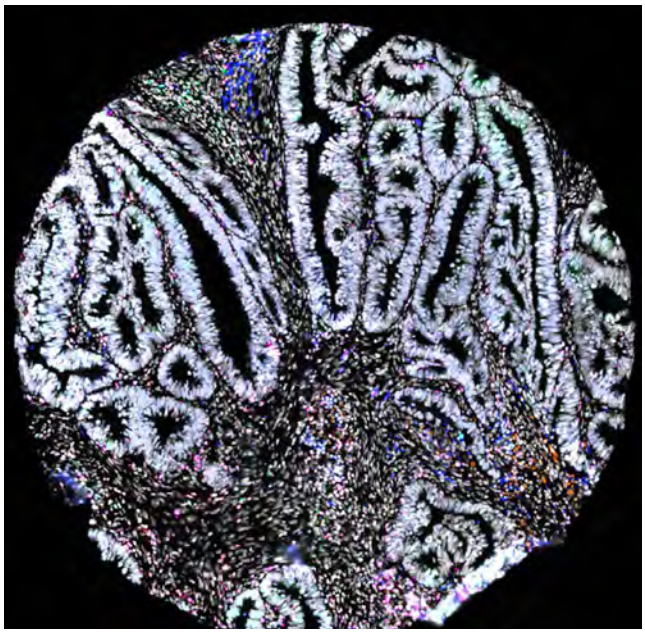


Figure 2. Optimizing and benchmarking MS analysis with bulk standards modeling SCoPE2 sets. A Conceptual diagram and work flow of SCoPE2. Cells are sorted into multiwell plates and lysed by mPOP [24]. The proteins in the lysates are digested with trypsin; the resulting peptides labeled with TMT, combined, and analyzed by LC-MS/MS. SCoPE2 sets contain reference channels that allow merging single cells from different SCoPE2 sets into a single dataset. The LC-MS/MS analysis is optimized by DO-MS [25], and peptide identification enhanced by DART-ID [26].



Spatial Proteomics unit at SciLifeLab

Figure 3. Image from a colorectal cancer core using sequential indirect immunofluorescence. Colors reflect the markers DAPI (white), Ki67 (green), CD68 (orange), CD3 (red), CD8 (pink), PARP1 (blue).

These methods allow researchers to look at multiple different proteins in the same sample. By using imaging, researchers can visualize proteins at single-cell resolution and capture cell dynamics, including rare events that would be masked if bulk samples of whole cell populations were analyzed.

Stadler emphasizes that these methods are useful for answering any biology-related question where it makes sense to study intact tissue. She gives the example of studying the immune landscape in tumors, including which immune cells are present and how they're orientated within the tissue. "Many studies," she notes, "are investigating what this means for patients, and whether it can be used to predict who'd benefit from specific therapies."

The future of spatial proteomics

Stadler predicts that researchers will use imaging in combination with other methods to combine spatial information with more in-depth data on proteins or other biomolecules. For example, researchers may use laser dissection microscopy to cut out specific cell types, which can be analyzed further using mass spectrometry.

"There are so many methods to study proteins and other biomolecules," she says. "Finding ways to combine them will tell us much more than using a single method alone." She anticipates that it will become increasingly common for researchers to combine spatial proteomics with genetic-sequencing-based methods to get a fuller picture of single cells.

For Gagarinova, meanwhile, spatial proteomics will draw upon the skills of computational biologists. "There's definitely been a demand for competent computational biologists," she observes. "And the amount of data is growing. So, we'll be looking at new ways to come up with new insights." She highlights the role of new supercomputing and data storage facilities in Canada and elsewhere.

Finally, the sky is the limit for applications of spatial proteomics beyond basic research. For example, the techniques of spatial proteomics can be used to study bacterial and viral pathogenesis. This possibility, Gagarinova points out, was emphasized in a report commissioned by the United Kingdom's Prime Minister. According to this report, antibiotic-resistant bacteria will kill more people than cancer by 2050.[4] This assertion, which was concerning when it was made in

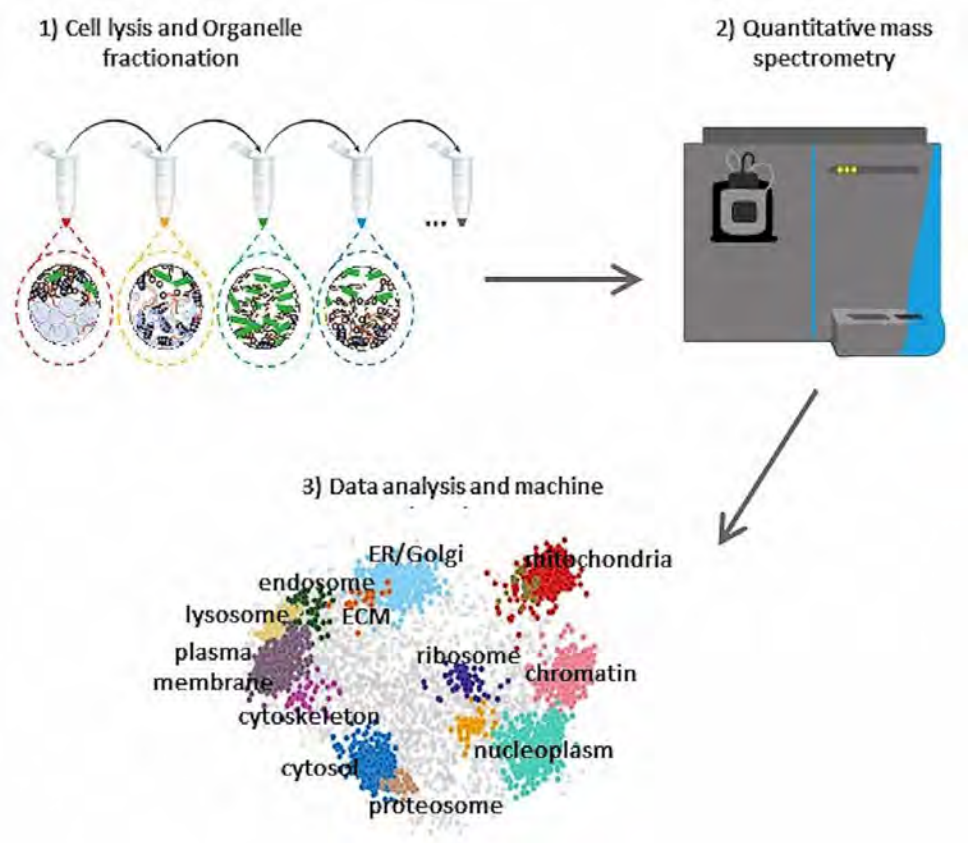


Image and caption provided by Kathryn Lilley, PhD

Figure 4. The workflow for spatial proteomics method, LOPIT (localization of proteins using isotope tagging). 1) Cells are gently lysed, so their subcellular compartments/organelles remain intact, and then partially separated from one another using a choice of centrifugation methods. 2) The amount of each protein in each fraction is measured using quantitative mass spectrometry. 3) Machine learning tools are applied to the mass spectrometry data to classify proteins into one or more subcellular compartment, and the data visualized using principal component analysis.

2016, has become only more concerning since then. And bacteria are not the only pathogens to cause worry. "COVID-19," Gagarinova remarks, "has brought the science [of viruses and other pathogens] to the forefront."

She adds, "We need to keep in front of the next pandemic, as well as understanding the effect of pollution and environmental change on the human body. [With spatial proteomics], we can study [how cells change.]"

References

1. Foster LJ, de Hoog CL, Zhang Y, et al. A Mammalian Organelle Map by ProteinCorrelation Profiling. *Cell* 2006; 125: 187–199.
2. Dunkley TPJ, Hester S, Shadforth IP, et al. Mapping the *Arabidopsis* organelle proteome. *Proc. Natl. Acad. Sci. USA* 2006; 103(17): 6518–6523.
3. Mulvey CM, Breckels LM, Crook OM, et al. Spatiotemporal proteomic profiling of the pro-inflammatory response to lipopolysaccharide in the THP-1 human leukaemia cell line. *Nat. Commun.* 2021; 12(1): 5773.
4. O'Neill, J. Tackling Drug-Resistant Infections Globally: An Overview of Our Work . *The Review on Antimicrobial Resistance*. 2016.

Versatile and Rapid Digestion Protocols for Biopharmaceutical Characterization Using RapiZyme™ Trypsin

Samantha Ippoliti, Nick Zampa, Ying Qing Yu, Matthew A. Lauber
Waters Corporation

Abstract

Complete and clean trypsin digestion is achieved with an enzyme that provides a balance of missed cleavage, non-specific cleavage, and trypsin autolysis. RapiZyme trypsin is a new homogeneously methylated, recombinant porcine trypsin which exhibits excellent thermal stability and improved levels of autolysis resistance. This application note evaluates RapiZyme trypsin in traditional peptide mapping protocols and also explores its applicability in a variety of alternative protocols utilizing various enzyme ratios, pH, incubation time, and temperatures. It was observed that RapiZyme trypsin uniquely enables the use of a high enzyme:protein (E:P) ratio (1:5, w/w) along with a rapid 30-minute digestion. The result of which is a high quality, exemplary peptide map that shows little to no autolysis peak interference despite the use of a high concentration of protease. Additionally, the use of RapiZyme trypsin has made it possible to accelerate traditional E:P ratio digestions (1–3 hours, 1:20 E:P). Furthermore, for those who prefer to digest overnight, RapiZyme trypsin is also demonstrated here to provide a complete, low artifact digestion with conditions based on a 1:100 E:P ratio, pH 6.5 buffer, and an ambient temperature incubation. Finally, this application work expands once more to consider a one-pot reaction condition, wherein RapiZyme trypsin was used in the presence of low amounts of guanidine HCl in a manner not possible with other industry-leading MS-grade trypsin.

Benefits

- Improved levels of autolysis resistance unlocks high enzyme:protein ratios that enable rapid and efficient 30-minute digestion protocols without the need for high temperature incubations
- Affords quick and confident decisions during critical data analysis because of clean baselines that minimize the number of unmatched peaks
- Demonstrated versatility with a number of unique digestion protocols
- Reproducible for long-term method success

Introduction

Peptide mapping is an essential assay for biopharmaceutical characterization and monitoring. By digesting an intact protein or monoclonal antibody (mAb) down to the peptide level, it becomes possible to establish

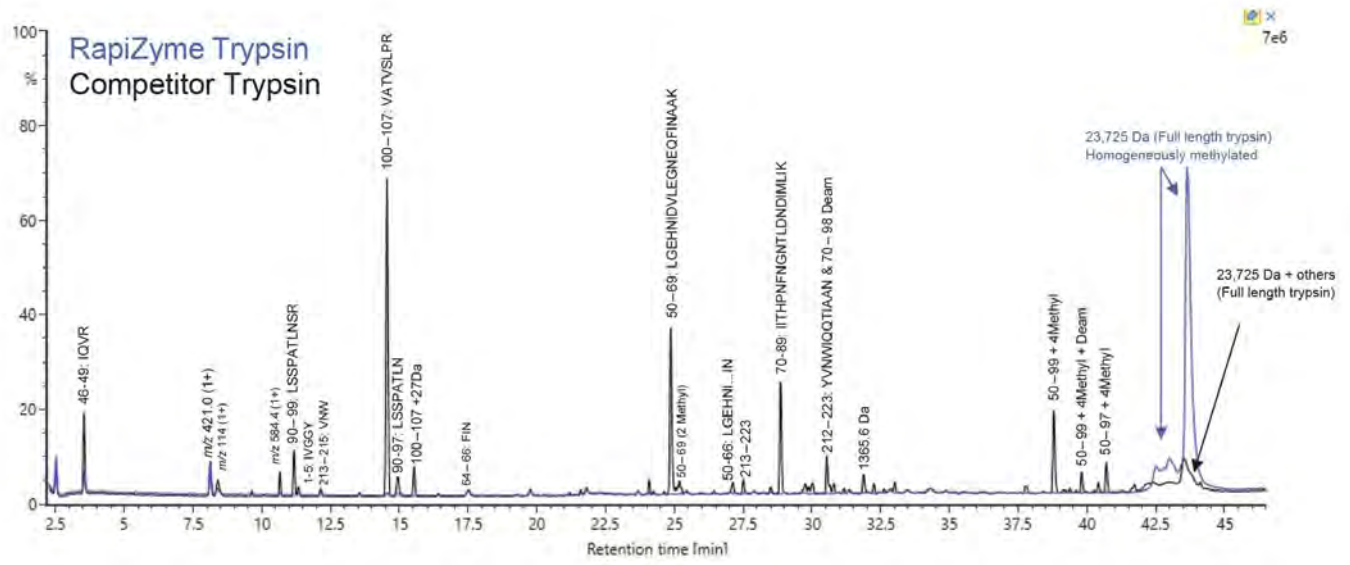


Figure 1. TIC overlay for a mock 1:5 enzyme:protein ratio sample with RapiZyme trypsin (blue trace) and another industry-leading MS-grade trypsin (black trace). These mock samples represent enzyme blanks.

protein identity via sequence coverage and investigate site-specific modifications. Peptide mapping is now being implemented as a multi-attribute monitoring (MAM) approach for quality control and release of biotherapeutics, and therefore must be robust and reliable.[1] Peptide mapping data analysis is quite complex even when thinking only about the expected peptide contents. It is further complicated when the proteolysis enzyme yields missed cleavage (under-digestion), non-specific cleavage (over-digestion), and autolysis peaks (digestion of itself). Additional peaks increase the time spent on data processing and review. Furthermore, peptide mapping is traditionally associated with long sample preparation times. Long digestion times increase sample turnaround time, possibly delaying critical decisions.

To combat these complexities, Waters™ has launched a new in-solution trypsin. RapiZyme trypsin is a homogeneously methylated recombinant porcine trypsin which is thermally stable and carefully derivatized so as to be extremely resistant to autolysis. This is demonstrated by incubating RapiZyme and another industry-leading MS-grade trypsin overnight at elevated temperature. As shown in Figure 1, RapiZyme trypsin remains almost entirely intact, while the competitor enzyme exhibits significant autolysis. This application note showcases the use of RapiZyme trypsin first in traditional digestions, then explores its applicability to a variety of other approaches to peptide mapping sample preparation.

Experimental

Traditional Sample Preparation (Desalt Before Trypsin Digestion)

Remicade™ (infliximab) samples were denatured and reduced in a 5.2 M guanidine hydrochloride solution containing 3 mM dithiothreitol (DTT) for 30-minutes at room temperature. Iodoacetamide (IAM) was then added to a final concentration

Condition	Enzyme : protein ratio	Temperature	pH	Incubation time	Buffer composition
Accelerated	1:5 (w/w)	37 °C	7.5	30 minutes	100 mM Tris, 10 mM $CaCl_2$ (Waters p/n 186010111)
Traditional	1:20 (w/w)	37 °C	7.5	1–3 hour	100 mM Tris, 10 mM $CaCl_2$ (Waters p/n 186010111)
Overnight	1:100 (w/w)	Ambient	6.5	Overnight (15–18hr)	50 mM Histidine, 10 mM $CaCl_2$
Dilution method (no desalting)	1:5 (w/w)	37 °C	7.5	2 hours	100 mM Tris, 10 mM $CaCl_2$ (Waters p/n 186010111)

Table 1. Flexible trypsin digestion conditions.

of 7 mM iodoacetamide (IAM) and incubated for 20-minutes at room temperature.Buffer Salts (p/n: via 7K MWCO gel filtration device into pH 7.5 or pH 6.5 digestion buffers. The pH 7.5 buffer was Tris $CaCl_2$ Buffer Salts (p/n: 186010111), and the pH 6.5 digestion buffer was 50 mM Histidine, 10 mM $CaCl_2$, prepared in-house. RapiZyme trypsin (p/n: 186010108) was added to each sample and incubated as outlined in Table 1. At each timepoint, sample was acidified with 10% acetic acid (final concentration 0.1%) and further diluted with mobile phase A for analysis via LC-MS.

One Pot (No Desalt) Samples Preparations

To facilitate a dilution-based one pot digestion protocol, samples were added directly to solid guanidine hydrochloride for a final denaturant concentration of 5 M. They were reduced and alkylated as described above. The samples were diluted to 0.6 M guanidine hydrochloride with the same pH 7.5 digestion buffer (Tris $CaCl_2$ Buffer Salts (p/n: 186010111) and trypsin was added at a ratio of 1:5 (w/w) for digestion. After incubation for two hours at 37 °C, samples were acidified with 10% acetic acid (final concentration 0.1%) and further diluted with mobile phase A for analysis via LC-MS.

Results and Discussion

The purpose of this study was to evaluate the performance of RapiZyme trypsin and its use in various types of protocols. It was also of interest to compare its performance to that of another industry leading MS-grade trypsin which boasts of autolysis resistance. Key parameters constituting a successful digestion include low levels of each of the following components: 1) missed and non-specific cleavages, 2) trypsin enzyme autolysis species, 3) unmatched/unknown peaks, and 4) artificial post-translational modifications (PTMs), such as deamidation or oxidation that can result from nonoptimal digestion conditions.[2–3] All of these create added complexity in the data, which puts an extra burden on the analyst. If unmatched peaks are present after standard peptide searching against an *in-silico* digest, the user would need to widen the search to include parameters such as semi-tryptic digestion (to assign non-specific cleavage), an increased number of missed cleavages, and the trypsin sequence itself (along with its derivatization) to begin matching autolysis peaks. Each of these additional parameters lengthens the time it takes the software to process the data, and the amount of time a user must spend on the review.

LC Conditions

LC system:	ACQUITY™ UPLC™ I-Class PLUS
Detection (optical):	ACQUITY TUV
Vials:	QuanRecovery™ Vials with MaxPeak™ HPS (p/n: 186009186)
Column(s):	ACQUITY Premier Peptide CSH™ C_{18}, 130 Å, 1.7 µm, 2.1 x 100 mm (p/n: 186009488)
Column temperature:	60 °C
Sample temperature:	6 °C
Injection volume:	5–10 µL (1–2 µg 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:	ACQUITY RDa™
Ionization mode:	ESI+, 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 caquisition & processing:	UNIFI™ v 3.0.0.6 operated under waters_connect™ v 2.1.0

Accelerating Traditional Trypsin Digestion

Many reference papers outlining the tryptic digestion of mAbs have called for the use of a denatured, reduced, and alkylated protein sample that gets subjected to a desalting step prior to digestion.[1–7] In these procedures, trypsin has thereafter been applied at an enzyme:protein ratio of 1:20–1:25 (w/w) and incubations have been performed for up to four hours at an elevated temperature. Such trypsin digestion protocols have been adopted by many biopharmaceutical industry labs as part of the peptide mapping analytical workflow. This is where we began the evaluation of RapiZyme trypsin. Using Remicade (an IgG1 kappa mAb) as a case study, we compared the performance of RapiZyme trypsin

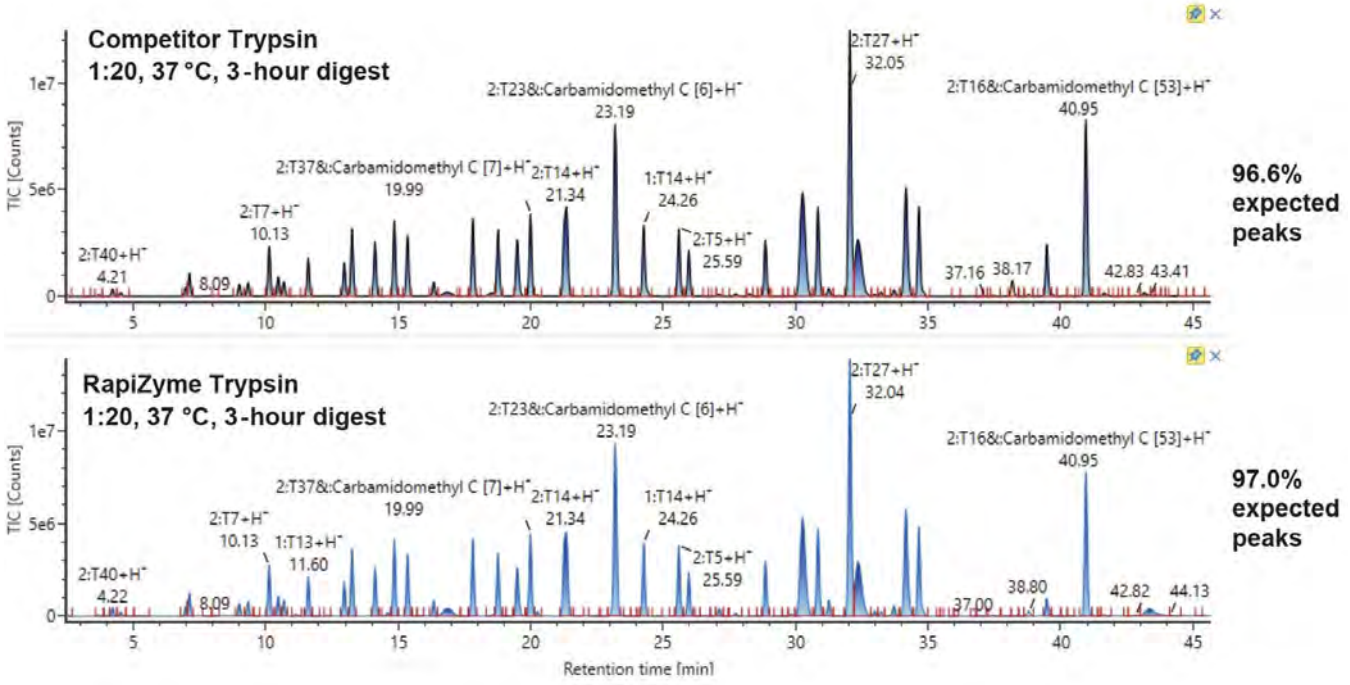


Figure 2. TIC chromatograms for 3-hour 1:20 enzyme:protein ratio digestions of Remicade with another industry-leading trypsin (black trace) and RapiZyme trypsin (blue trace), showing equivalent results.

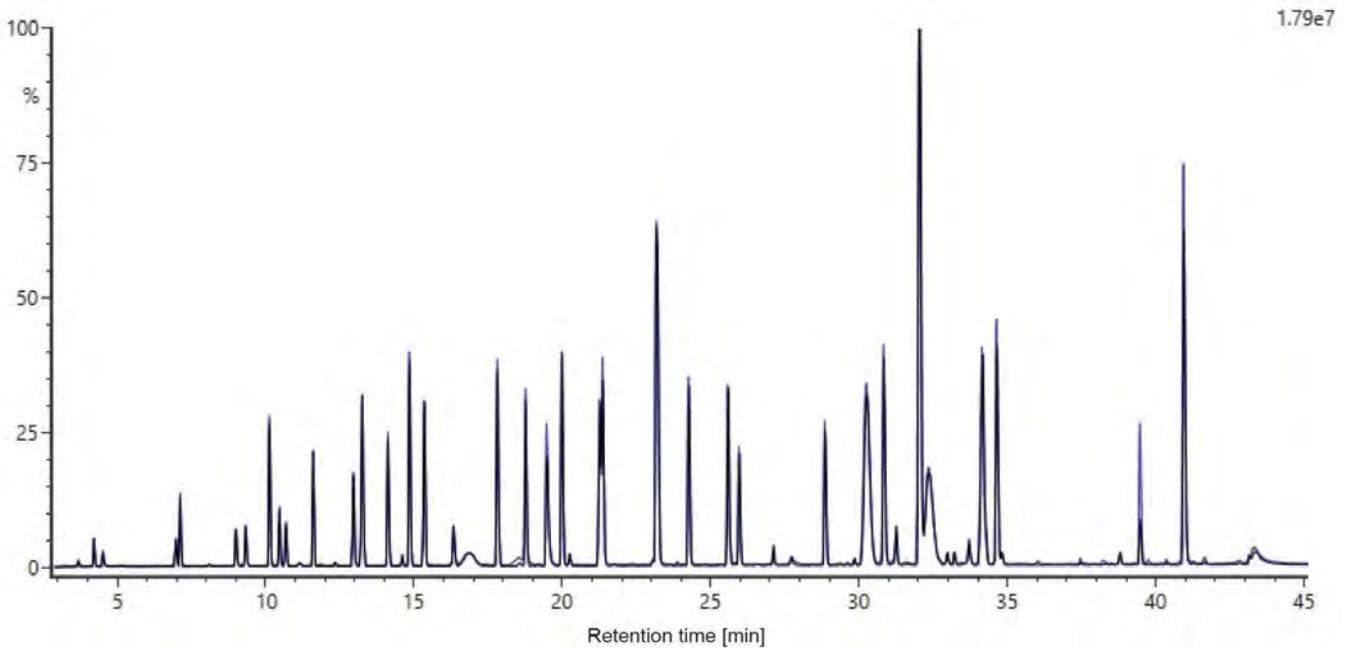


Figure 3. TIC chromatograms for a 3-hour (black trace) 1:20 ratio digestion of Remicade with RapiZyme trypsin, achieving the same level of digestion in only 1 hour (blue trace) with same conditions.

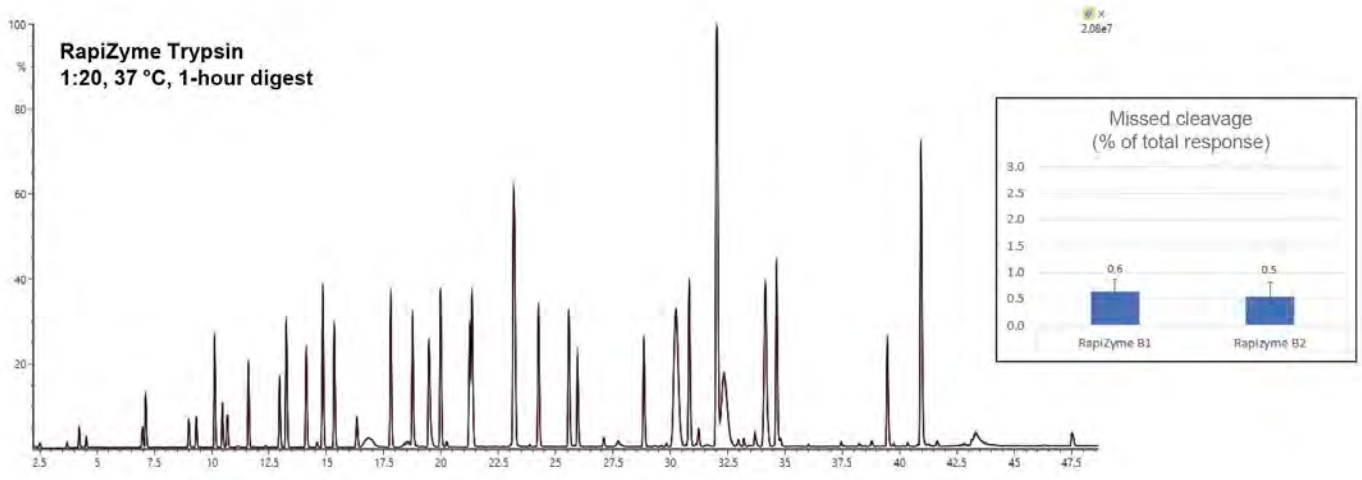


Figure 4. TIC overlay for 3 digestion replicates each for two different batches of RapiZyme trypsin as obtained with 1:20 enzyme:protein digestions. Inset shows a bar graph comparison of % missed cleavage for each batch of RapiZyme & trypsin (replicates n=3).

to another leading competitor trypsin, at an E:P ratio of 1:20, incubated for three hours at 37 °C. Detailed analyses were then performed by LC-MS. Comparable performance was observed (Figure 2, TIC traces shown) for RapiZyme trypsin and the competitor trypsin when three hours of incubation were applied. Each give >93% sequence coverage, and more than 95% of the integrated TIC area is comprised of Remicade peptides with expected tryptic cleavages. No significant difference in deamidation or oxidation levels was observed upon comparing the digestions.

Since the three hour digestion with a 1:20 E:P ratio gave near complete digestion of the mAb, we decided to test a shorter incubation time with the same conditions. The overlay in Figure 3 demonstrates excellent comparability for the one hour (blue trace) and three hour (black trace) digestions of Remicade, indicating that it is possible to achieve equivalent results in only one hour when RapiZyme trypsin is applied.

In peptide mapping sample preparation, reproducibility is of utmost importance. The repeatability and batch to batch reproducibility of the one hour RapiZyme trypsin digest of Remicade was further evaluated using two different batches of RapiZyme trypsin, each represented with three digestion replicates for each batch. Figure 4 displays the TIC overlay for these six injections, and it can be seen that there is excellent reproducibility, similar levels of digestion completeness and TIC patterns for the expected peptides. The total amount of missed cleavage for the study was 0.55% ± 0.3%. Overall, 97.5% ± 0.3% of the TIC area is made up of fully tryptic peptides derived from the Remicade light and heavy chain sequences.

Can We Go Even Faster?

In biopharmaceutical development, there is an ongoing search for ways to improve both workflow efficiency and sample turnaround time. At the same time, there are efforts made to minimize artifactual modifications that arise from longer digestion times.[3–4] The novelty of RapiZyme trypsin lies specifically in its stability, autolysis resistance, and high activity. This allows

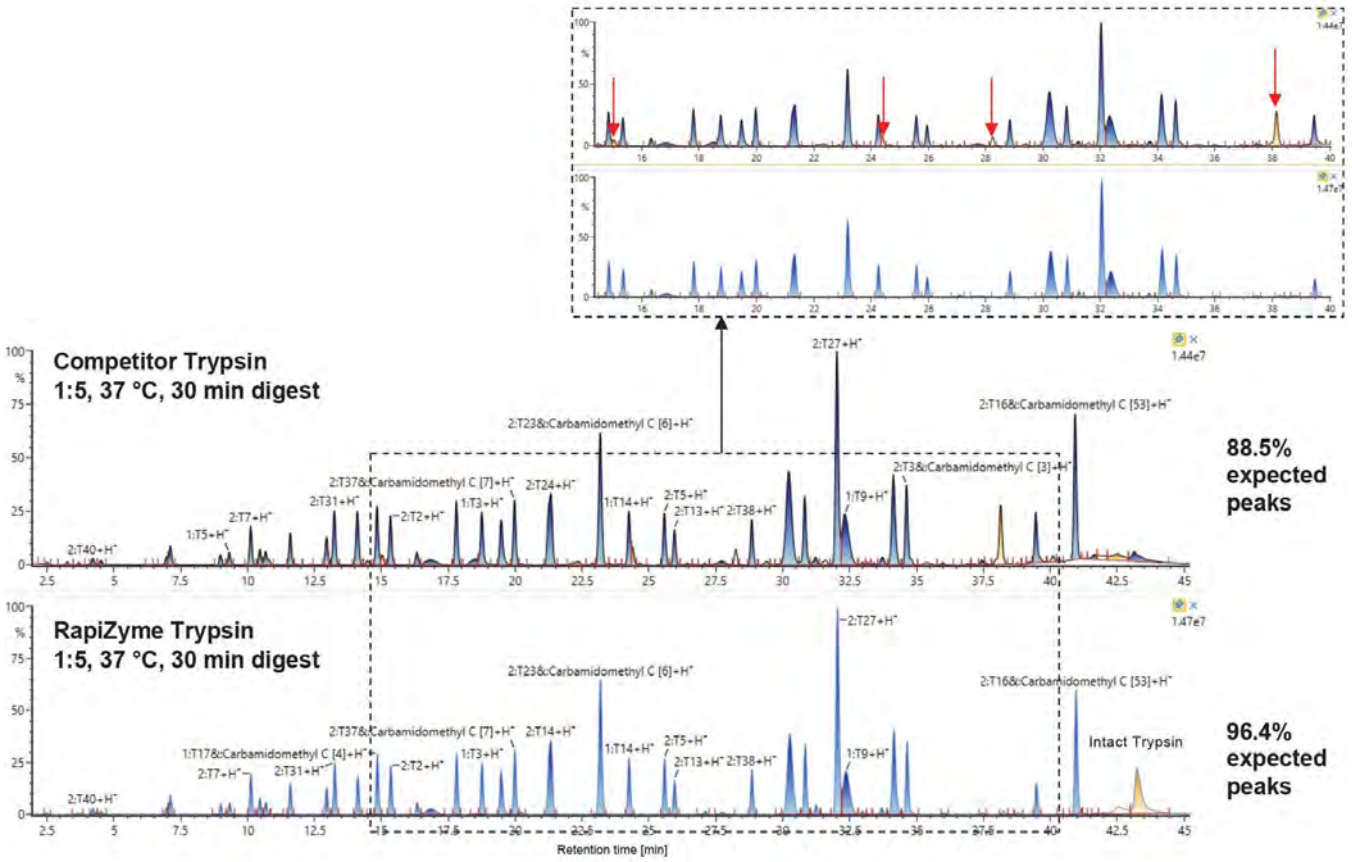


Figure 5. Comparison of 1:5 digests, another industry-leading competitor (top panel) vs RapiZyme trypsin (bottom panel), with zoomed section of retention time window 14 to 40 min. Red arrows highlight trypsin autolysis and unknown peaks.

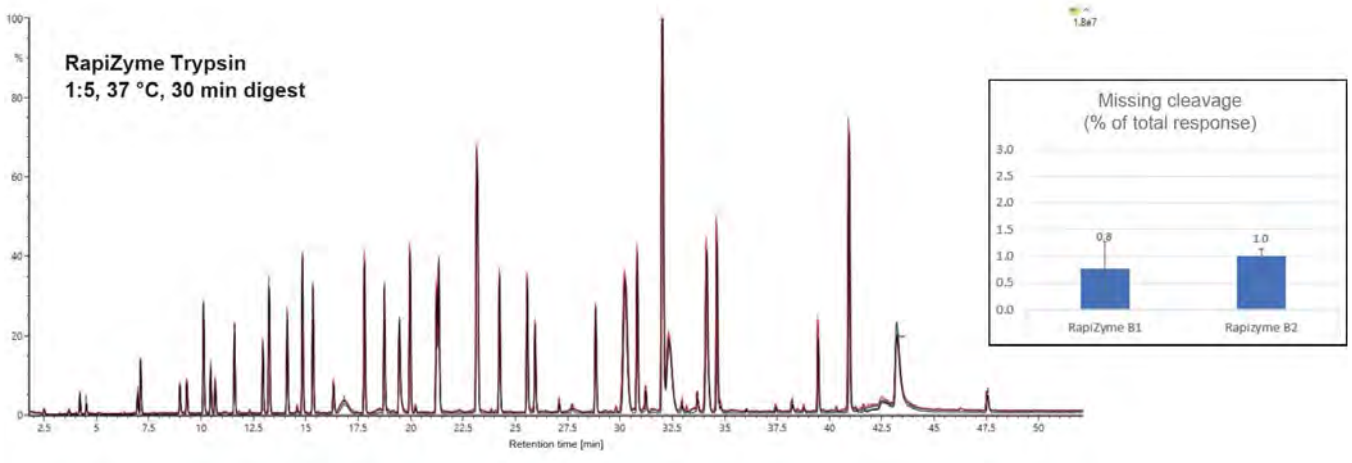


Figure 6. TIC overlay for 3 digestion replicates each for two batches of RapiZyme trypsin as obtained with 1:5 enzyme:protein digestions. Inset shows a bar graph comparison of % missed cleavage for each batch of RapiZyme trypsin (replicates n=3).

more flexibility to use higher enzyme-to-protein ratios and the chance to explore shorter digestion times along with the use of a wider range of temperatures. For Remicade, we opted to test a 1:5 ratio for 30-minutes at a standard incubation temperature of 37 °C. The resulting labeled TIC chromatograms for RapiZyme trypsin vs another industry-leading trypsin are shown in Figure 5. There is a substantial difference in the quality of the digestions, mostly due to peptides generated from trypsin autolysis during the digestion when using the competitor enzyme at a higher E:P ratio. The zoomed section in Figure 5 highlights a section with a significant amount of autolysis species (indicated with red arrows). RapiZyme trypsin did not show any appreciable signal for interference peaks in this retention window, nor in any others where Remicade peptides elute. RapiZyme trypsin provided a fast, clean digestion with >93% sequence coverage, <1% missed cleavage, and <0.1% trypsin autolysis. (Relative abundance (%) of missed and non-specific cleavage are calculated via MS response of these species compared to the total MS response for all Remicade-derived peptides, in a manner consistent with previously published work.)[2]

The same repeatability and batch-to-batch reproducibility exercise from above was applied to this accelerated digestion condition. Figure 6 shows the TIC overlay of six samples corresponding to two batches of RapiZyme trypsin, each represented with three digestion replicates. Again, excellent digestion reproducibility and overall completeness of digestion were observed. Overall, 96.3% ± 0.6% of the TIC area is made up of fully tryptic peptides derived from Remicade.

What About Improving Overnight Digestion?

While many scientists desire faster digestions workflows, there are others who prefer the flexibility of setting up and returning to an overnight digestion. There are a few considerations to take into account for overnight digestions. First, RapiZyme trypsin is quite active, so the E:P ratio and temperature should be optimized to control any non-specific cleavage (over-digestion) that may arise. The other consideration is PTMs, such as deamidation, that occur due to digestion conditions, namely neutral-to-high pH and elevated temperatures.[3] Unfortunately, this is where most trypsin enzymes retain their highest activity. In order to mitigate these concerns in our overnight digestion evaluation with RapiZyme trypsin, we chose to use a low 1:100 enzyme ratio, ambient temperature conditions (20–25 °C), and a pH of 6.5 buffer. These parameters provide a clean complete overnight digestion, as shown in the TIC in Figure 7A. We observe >90% sequence coverage with a < 1% level for missed cleavages, non-specific cleavages, and unknown peaks combined. Very low levels of overall deamidation and oxidation are observed. To demonstrate the effects of temperature and pH with overnight incubation, an additional digestion of Remicade was performed with the 1:100 E:P ratio, overnight time interval but with a pH 7.5 buffer and 37 °C incubation. Select deamidation-prone peptides were compared between 1) the accelerated 30-minute digestion described previously, 2) the overnight pH 6.5 ambient temperature digestion, 3) and the overnight pH 7.5, 37 °C digestion. The extracted ion chromatograms (Figure 7B) for heavy chain peptide T27 (VVSVLTVLHQDWLNGK) show equivalently low

levels of deamidation for the 30-minute and pH 6.5 optimized overnight digestions. Meanwhile, > 20% deamidation was observed for this peptide with the pH 7.5, overnight 37 °C condition. This verifies that the optimized overnight protocol does not introduce deamidation artifacts. Overall, the 1:100 enzyme ratio pairs with a pH 6.5 buffer and room temperature overnight incubation to provide a third alternative for generating clean and complete digestion with RapiZyme trypsin.

Can We Skip The Desalting Step?

All of the protocols discussed up to this point used samples in which a desalting cartridge is employed to remove guanidine hydrochloride denaturant and reduction/alkylation reagents prior to trypsin digestion. Most trypsins are greatly hindered by common protein denaturants like guanidine hydrochloride and urea.[2] However, many analysts would desire a protein digestion protocol that does not include the desalting step, for a variety of reasons. An inherent risk in using desalting cartridges is the variability in protein recovery after buffer exchange. Most commercially available devices report an average recovery of 70–90%, and it can be highly sample dependent.[8] Eliminating the desalting step promotes more consistency among sample concentrations and digestion conditions. It is also an extra step that adds more time and effort to the sample preparation workflow. Given that RapiZyme trypsin has proven to be reproducible, autolysis resistant, and efficient when applied to the desalted samples, it was worth exploring its possible application to a one pot digestion protocol that skips the traditional desalting step. To facilitate the digestion, a dilution step was incorporated to bring the final guanidine hydrochloride concentration to 0.6 M after the Remicade sample was reduced and alkylated. RapiZyme trypsin was then added to the sample to give a 1:5 enzyme ratio and the digestion was incubated for 2 hours at 37 °C. The results from this work, as compared to another industry-leading competitor trypsin, are presented in Figure 8. Overall TIC area for the RapiZyme sample is 96% comprised of full tryptic Remicade peptides, whereas the competitor was only 82% (Figure 8A).

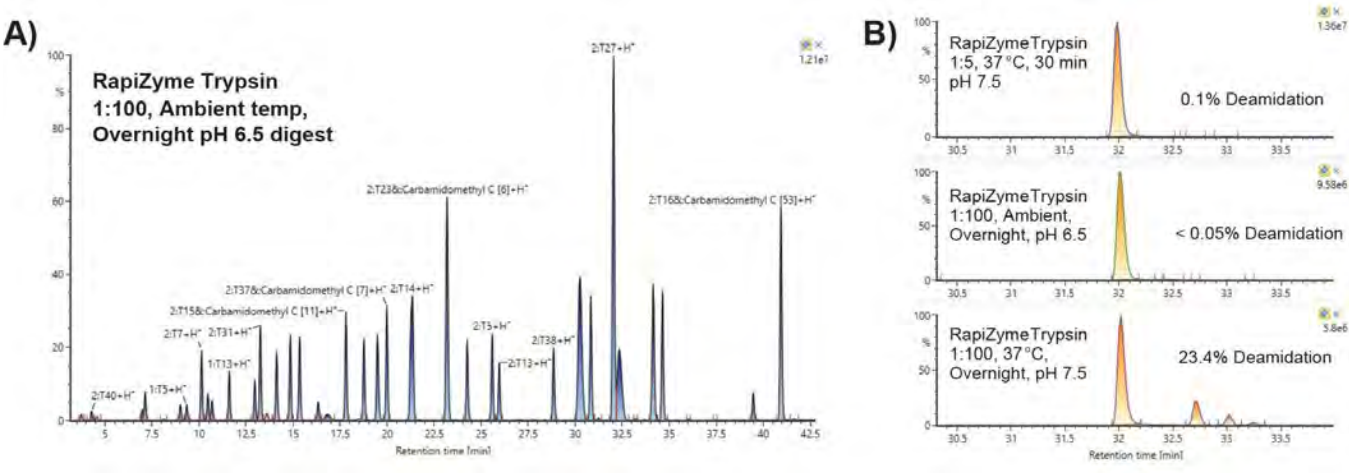


Figure 7. A) Labeled TIC for Remicade 1:100 Overnight pH 6.5 digestion. B) Extracted ion chromatograms for deamidation-prone peptide HC T27 (VVSVLTVLHQDWLNGK), m/z 603.6 (3+) and 904.4 (2+), for 30 min and overnight incubations with a pH 6.5 versus 7.5 buffer.

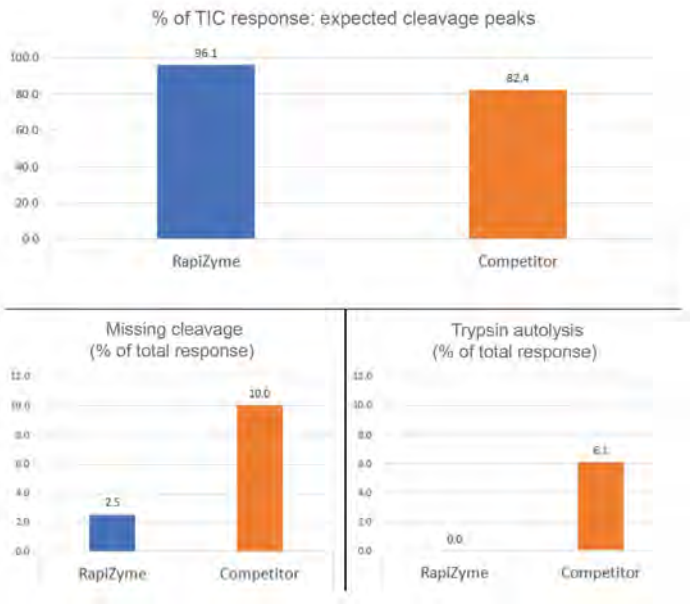


Figure 8. One-Pot (non-desalted) diluted protocol results. Panel A) shows a comparison for % TIC response that is made up of expected cleavage peaks in the 1 Pot digestions of Remicade with RapiZyme trypsin and another industry-leading competitor trypsin. "Impurities" include missed and non-specific cleavages, trypsin autolysis, and unknown peaks. The most significant differences stem from missed cleavage (Panel B) and trypsin autolysis (Panel C).

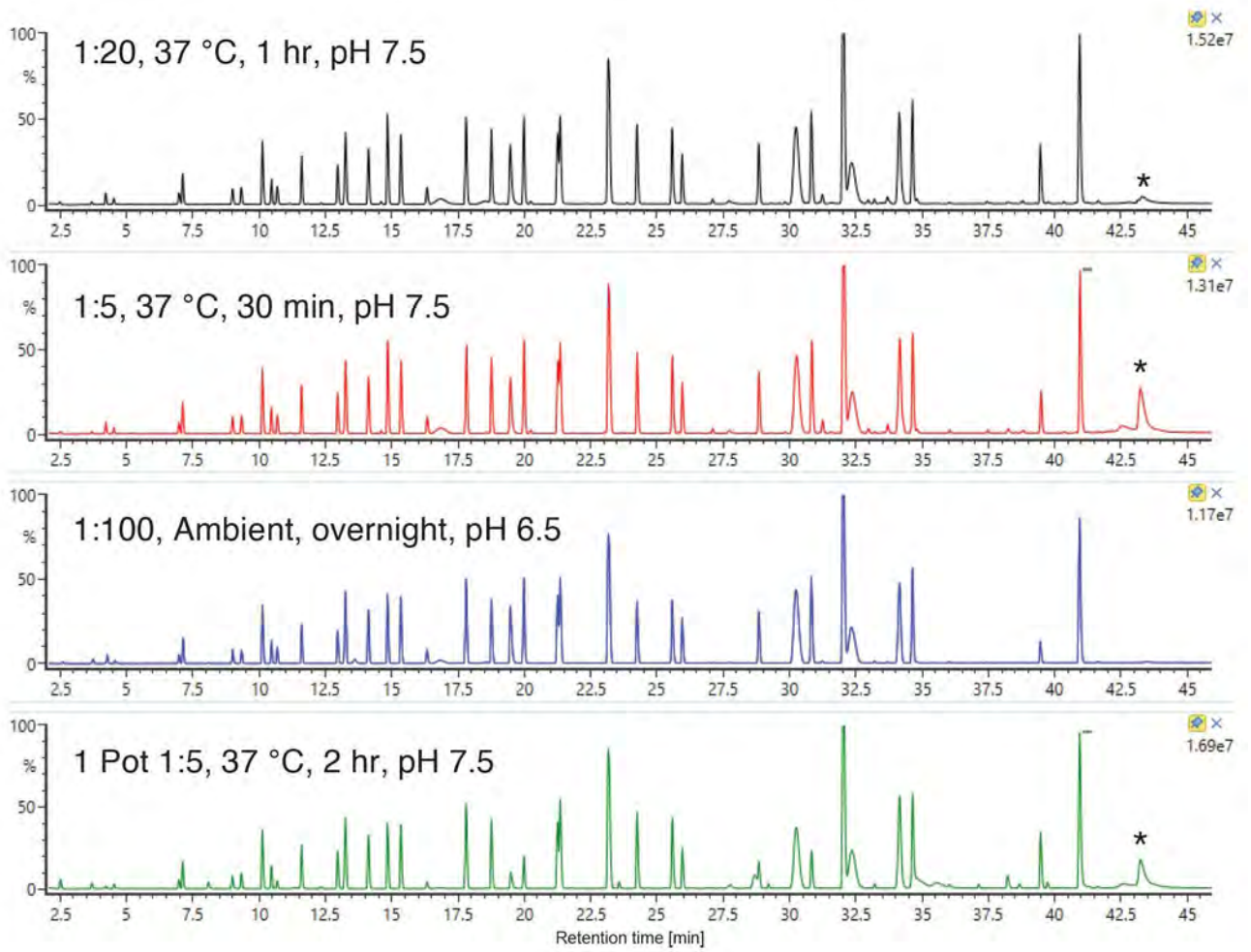


Figure 9. Summary of digestion conditions—Stacked TIC chromatograms showing high level of similarity between all four digestion conditions performed for Remicade (* indicates intact trypsin peak).

This disparity stems primarily from differences in missed cleavages and trypsin autolysis species (Figure 8B & 8C, respectively). The competitor trypsin contained ~10% of the total TIC peak area as missed cleavage, and ~6% as autolysis peaks. This indicates that RapiZyme trypsin can provide a near complete digestion even in the presence of significant concentrations of guanidine hydrochloride. This may offer a viable alternative for those who desire to avoid desalting.

Conclusion

Peptide mapping is, and will continue to be, one of the most important data-rich assays that can be applied for biopharmaceutical characterization and monitoring. As such, the sample preparation and methods for this assay must be robust and reliable. That said, many labs have a variety of requirements and standard protocols they wish to follow. The method development exercise outlined in this application note shows that RapiZyme trypsin, with its unique autolysis resistance and digestion efficiency, can be a means to more quickly and more creatively prepare high quality peptide mapping samples. RapiZyme trypsin has proven to be useful in traditional digestions, in rapid 30-minute and flexible overnight digestions, and even in simple one-pot protocols. RapiZyme trypsin provided peptide maps with high levels of similarity and data quality for Remicade peptide mapping with each of the newly envisioned protocols (Figure 9).

References

1. Ranbaduge R, Yu YQ. A Streamlined Compliant Ready Workflow for Peptide-Based Multi-Attribute Method (MAM). Waters Application Note, 720007094. 2020 Dec.
2. Mouchahoir T, Schiel JE. Development of an LC-MS/MS peptide mapping protocol for the NIST mAb. *Anal and Bioanal Chem*. 2018; 410: 2111–2126.
3. Ren D, Pipes GD, Liu D, Shih L, Nichols A, Treuheit MJ, Brems DN, Bondarenko PV. An Improved Trypsin Digestion Method Minimizes Digestion-Induced Modifications on Proteins. *Anal Biochem*. 2009; 392: 12–21.
4. Millian-Martin S, Jakes C, Carillo S, Buchanan T, Guender M, Kristensen DB, Sloth TM, Oregaard M, Cook K, Bones J. Inter-laboratory Study of an Optimized Peptide Mapping Workflow Using Automated Trypsin Digestion for Monitoring Monoclonal Antibody Product Quality Attributes. *Anal and Bioanal Chem*. 2020; 412: 6833–6848.
5. Evans AR, Hebert AS, Mulholland J, Lewis MJ, Hu P. ID-MAM: A Validated Identity and Multi-Attribute Monitoring Method for Commercial Release and Stability Testing of a Bispecific Antibody. *Anal Chem*. 2021; 93 (26): 9166–9173.
6. Song YE, Dubois H, Hoffman M, Eri SD, Fromentin Y, Wiesner J, Pfenninger A, Clavier S, Pieper A, Duhau L, Roth U. Automated Mass Spectrometry Multi-Attribute Method Analyses for Process Development and Characterization of mAbs. *J Chrom B.* 2021; 1166: 122540.
7. Hao z, Moore B, Ren C, Sadek M, Macchi F, Yang L, Harris J, Yee L, Liu E, Tran V, Ninonuevo M, Chen Y, Yu C. Multi-Attribute Method Performance Profile for Quality Control of Monoclonal Antibody Therapeutics. *J Pharm Biomed Analysis*. 2021; 205: 114330.
8. Instruction Manual for PD-10 Desalting Columns. Cytiva #52130800 BD. Accessed via www.cytivalifesciences.com on 12 Dec 2022.

Fast and Robust LC-UV-MS Based Peptide Mapping Using RapiZyme™ Trypsin and IonHance™ DFA

Abraham S. Finny Nick Zampa Balasubrahmanyam Addepalli Matthew A. Lauber
Waters Corporation

Abstract

Laborious and time-consuming peptide mapping protocols are made faster and more robust by using a novel RapiZyme Trypsin and IonHance difluoroacetic acid (DFA). High activity and enhanced autolysis resistance enabled the RapiZyme Trypsin to be used at higher amounts leading to reduced digestion time (~1 hour) in contrast to the traditional enzyme-to-protein ratios and long digestion time (≥3 hours) employed in protein sequencing methodologies. The use of IonHance DFA as a mobile phase additive in the peptide mapping workflows made high-quality mass spectral (MS) and UV data acquisition possible without adduct-induced interferences. Using Waters MassPREP™ peptide standard mixture and NISTmAb as protein digest standards, we demonstrate the direct alignment of high-quality UV and MS data while maintaining the high signal intensity of sequence-informative fragment ions in the MS^e-based fragmentation of adduct-free target peptide molecular ions. Thus, after annotating peptide peaks to specific retention times in the initial experiments, UV only runs can be considered for subsequent quality control (QC) analyses. Such improved methodologies are expected to accelerate the testing of pharmaceutical proteins as a result of the ease and efficiency with which high quality can now be obtained.

Benefits

- The use of RapiZyme trypsin enables 1:5 enzyme-to-protein ratio digestions, thereby reducing digestion times
- Total sample preparation time is under 3 hours, significantly less than many conventional peptide mapping workflows
- Versus formic acid, IonHance DFA results in enhanced UV sensitivity in a sequence-independent fashion allowing the monitoring of low-abundant peptides
- Ion pairing properties of IonHance DFA further improves the reproducibility of peptide retention times while maintaining a sensitive response without the associated adducts

Introduction

Peptide mapping is a technique used in proteomics & biopharmaceutical characterization to identify and characterize proteins. A single chain of protein is also referred to as a polypeptide. A polypeptide is distinguished by its sequence of amino acids connected through the peptide bonds. Proteomic studies involve

breaking down proteins into smaller peptides through the cleavage of peptide bonds by a protease such as trypsin, followed by their separation and mass analysis by liquid chromatography-mass spectrometry (LC-MS). The resulting peptide map can be used to identify the protein and determine its primary structure. Robust peptide mapping is necessary for several reasons. First, it can be used to confirm the identity of a protein, which is important for quality control and validation purposes. Second, peptide mapping can be used to determine the primary structure of a protein, which can be essential to early research programs. Finally, peptide mapping can detect post-translational modifications in the amino acid sequence, which can affect protein function and stability.

This application note focuses on the use of RapiZyme Trypsin and IonHance DFA for peptide mapping and discusses the unique advantages of this combination to facilitate a quick peptide mapping workflow, simultaneous optimization of UV and MS responses, and adduct-free mass spectra.

Experimental

Sample Preparation

NIST mAb samples [10 µL at 10 µg/µL] were denatured with 90 µL of 6 M guanidine hydrochloride (GuHCl) [5.4 M final concentration] solution and reduced with 5 mM dithiothreitol (DTT) [2 µL of 250 mM] for 30 minutes at ambient conditions. This was followed by alkylation with 10 mM iodoacetamide (IAM) [3 µL of 350 mM] for another 30 minutes at ambient conditions.

Desalt Protocol

A digestion buffer consisting of 10 mM calcium chloride ($CaCl_2$) in 0.1 M tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) was first prepared from Waters Tris $CaCl_2$ Buffer Salts pH 7.5, 4/pk (p/n: 186010111). A gravity flow size-exclusion chromatography (SEC)-based desalt cartridge was conditioned by loading 400 µL of the digestion buffer three times and discarding the eluent each time. 100 µL of the denatured NIST mAb sample was then loaded onto the column and flowthrough discarded. The column was washed with 100 µL of digestion buffer, discarding the flowthrough. After ensuring that the cartridges were free of residual drops, the sample collection container was placed under the cartridge, and the desalted protein was collected with 300 µL of the digestion buffer.

Protein Estimation

To ensure that the correct amount of the denatured and alkylated NIST mAb was used for the digestion, protein estimation was performed after the desalting step. Precise concentration readings were obtained by A280 measurements using a UV-Vis plate reader. Alternatively, protein estimation can also be achieved through UV-Vis measurements of the droplets. This optional step ensures the use of the correct protein amount for digestion so that the enzyme-to-protein ratio values are consistently maintained for reproducible signal behavior across multiple digests.

Protein Digestion

20 µg (in 200 µL) of the denatured, reduced, and alkylated NIST mAb was taken in a 300 µL PCR (polypropylene) tube, and 4 µg (in 4 µL) of RapiZyme

LC Conditions

LC system:	ACQUITY™ UPLC™ I-Class PLUS
Detection:	ACQUITY TUV/PDA
Wavelength:	219 nm
Vials:	Clear Glass 12 x 32 mm Screw Neck Qsert Vial, 300 µL Volume (p/n: 186002804)
Column(s):	ACQUITY Premier Peptide CSH™ C_{18} Column, 130 Å, 1.7 µm, 2.1 x 150 mm (p/n: 186009489)
Column temp.:	65 °C
Sample temp.:	6 °C
Injection volume:	50 µL
Flow rate:	0.25 mL/min
Mobile phase A:	0.1% (v/v) IonHance DFA (p/n: 186009201) in LCMS-grade Water
Mobile phase B:	0.07% (v/v) IonHance DFA in LCMS grade Acetonitrile
Gradient:	Please refer the table below.

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.250	99.0	1.0	Initial
5.00	0.250	99.0	1.0	6
65.00	0.250	60.0	40.0	6
68.00	0.250	30.0	70.0	6
70.00	0.250	30.0	70.0	6
71.00	0.250	99.0	1.0	6
85.00	0.250	99.0	1.0	6

Data Management

Chromatography software:	ACQUITY RDa
MS software:	UNIFI™ v 3.0.0.15, Empower™ 3 (For UV only analyses)
Informatics:	UNIFI v 3.0.0.15

MS Conditions

MS system:	ACQUITY RDa
Ionization mode:	Full scan with fragmentation
Acquisition range:	Low (50–2000 *m/z*)
Capillary voltage:	1.20 kV
Cone voltage:	20 V
Fragmentation cone voltage:	60 V to 120 V
Polarity:	Positive
Scan rate:	2 Hz
Desolvation temp.:	350 °C

MassPREP peptide mixture (p/n: 186002337)	
Peptide	**Sequence**
P1	RGDSPASSKP
P2	DRVYIHP
P3	RPPGFSPFR
P4	DRVYIHPF
P5	DRVYIHPFHL
P6	DRVYIHPFHLLVYS
P7	WLTGPQLADLYHSLMK
P8	YPIVSIEDPFAEDDWEAWSHFFK
P9	GIGAVLKVLTTGLPALISWIKRKRQQ

Peptide sequences of the nine peptides in the MassPREP Peptide Mixture

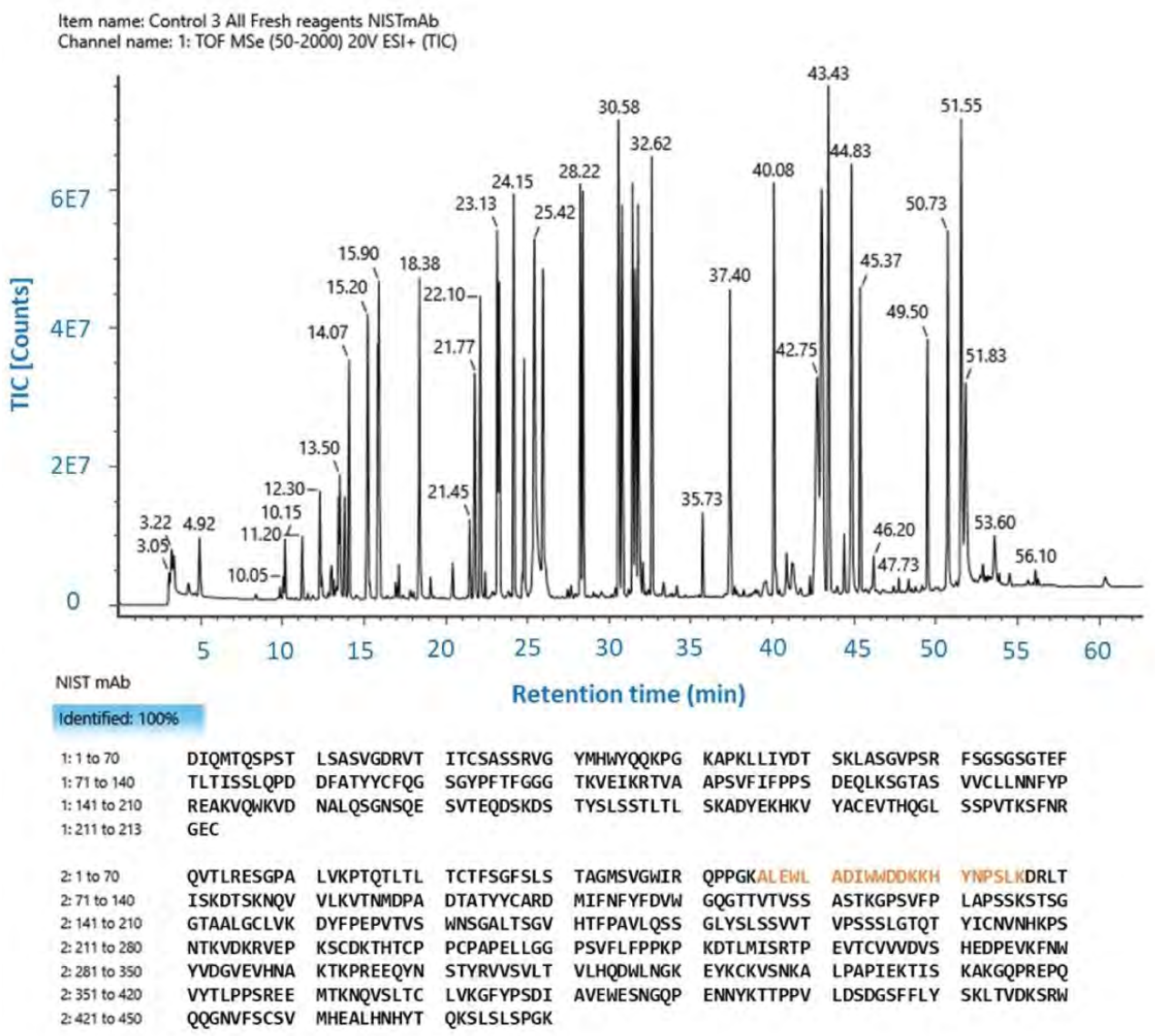


Figure 1. Representative total ion chromatogram (TIC) chromatogram and the observed sequence coverage of the NISTmAb sequence are shown. About 5 µg of protein digest was subjected to LC-UV-MS analysis using a BioAccord LC-MS system as described in the methods. The MSe data was analyzed by the UNIFI-based peptide mapping data analysis method to identify the high-confidence amino acid sequences of tryptic peptides and compute the sequence coverage.

Trypsin (p/n: 186010108) was added to perform the digestion at a 1:5 (enzyme: protein) ratio. This solution was incubated at 37 °C for 1 hour on a revolving PCR block @ 300 RPM. After digestion, the reaction was quenched by acidifying the samples with 20 µL of 1% formic acid (0.1% final concentration). The samples were vortexed and quantitatively transferred to Qsert vials for LC-MS analyses.

Results and Discussion

The current study intended to demonstrate the beneficial effects of RapiZyme Trypsin and IonHance DFA for use in quick and robust LC-UV-MS peptide mapping workflows. Using NIST mAb as an example biopharmaceutical protein of interest, we evaluated the efficacy of RapiZyme Trypsin at a 1:5

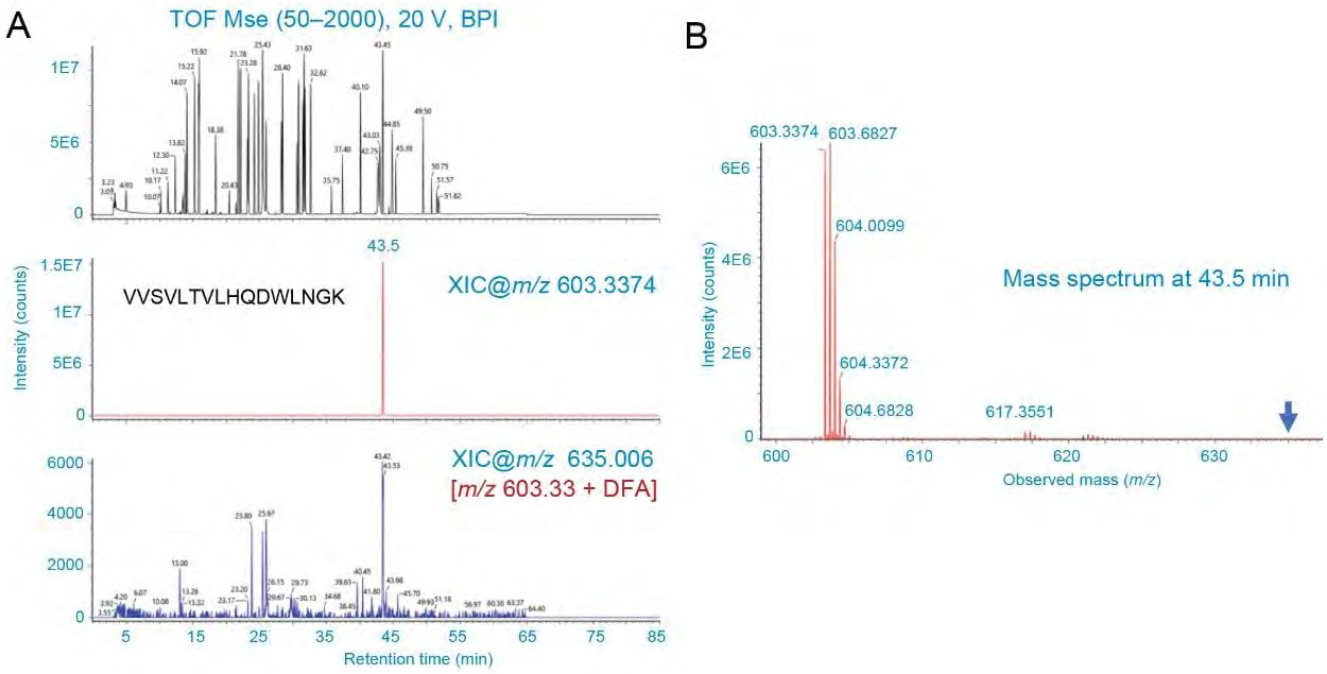


Figure 2. Analysis of LC-MS spectra for DFA adducts.
(A) The top panel depicts the base peak ion chromatogram (BPI) of the NISTmAb protein digested with RapiZyme Trysin. The peptide peak at 43.5 min exhibited at highest ion abundance as seen in both BPI and extracted ion chromatogram (XIC) for m/z 603.3374 (middle panel) that corresponds to the peptide VVSVLTVLHQDWLNGK. If adducts are formed, this should be noticeable with this most abundant peptide signal. But the XIC for m/z 635.006 (bottom panel) corresponding to the DFA adduct (+95 Da after accounting for proton replacement) did not yield a clear peak as the signal was at baseline level (see the intensity for the bottom panel).
(B) The mass spectrum corresponding to 43–43.5 min did not show the presence of ions corresponding to the adducts (see the arrow).

enzyme-protein ratio and 1 hour digestion time. The resulting peptides were subsequently subjected to LC-UV-MS analysis using IonHance DFA as a mobile phase additive in liquid chromatography.

This analysis revealed 100% amino acid sequence coverage of both the heavy and light chains (Figure 1). Like is expected for a formic acid mobile phase, the DFA modified mobile phases did not yield any ion pairing adducts (Figure 2) nor were there seen to be any adverse effects on the overall quality of the mass spectral data, including the acquisition of sequence-informative product ions. Further, the UV chromatograms exhibited enhanced UV signals with minimal baseline drift. The reproducibility of the protocol was demonstrated by the consistent UV and BPI chromatograms of separate, independently prepared protein digests (Figure 3–4).

The first advantage of this protocol is the quick sample preparation process which is under 3 hours. This is partly due to the RapiZyme Trypsin's ability to facilitate high enzyme:protein ratio digestions. The second advantage is that chromatograms showed high UV signal intensities. This can be attributed to differences in the refractive index properties of DFA versus formic acid

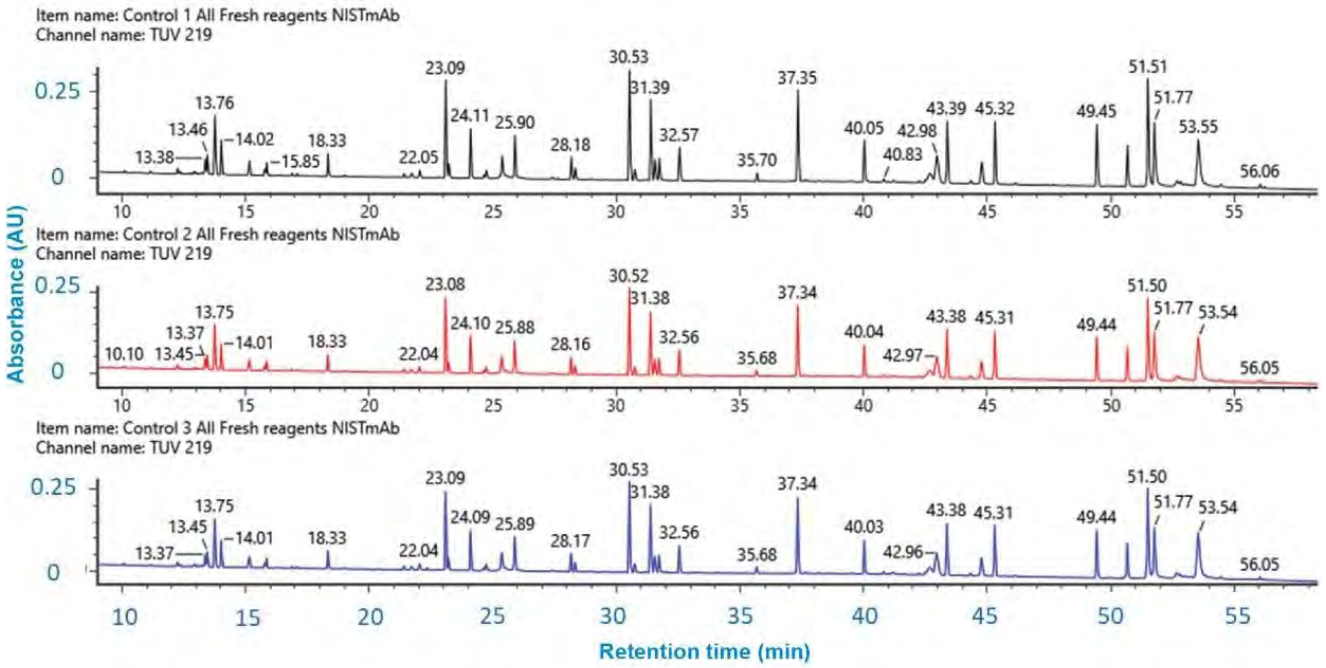


Figure 3. Reproducibility of UV chromatograms for three separate, independently prepared digest replicates of NISTmAb. The UV signal at 219 nm remained constant for all three digests indicating high reproducibility.

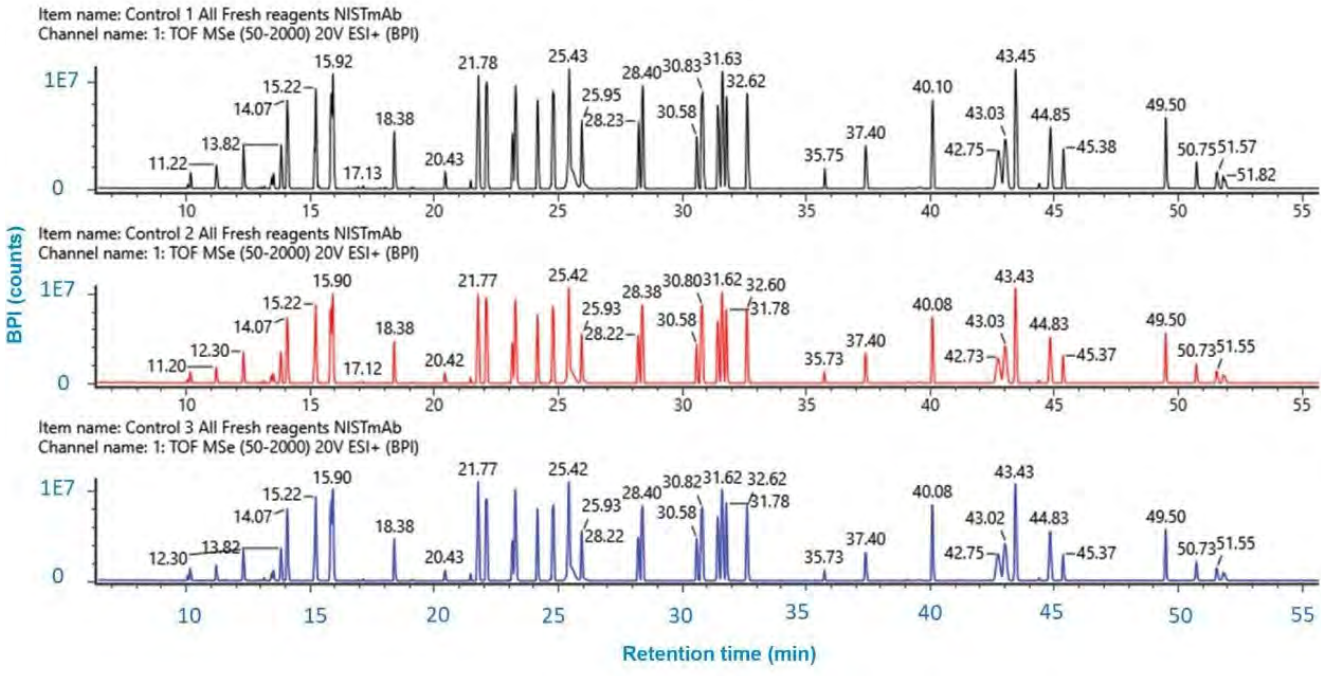


Figure 4. Reproducibility of BPI chromatograms for three separate, independently prepared digest replicates of NISTmAb. The UV signal at 219 nm remained constant for all three digests indicating high reproducibility.

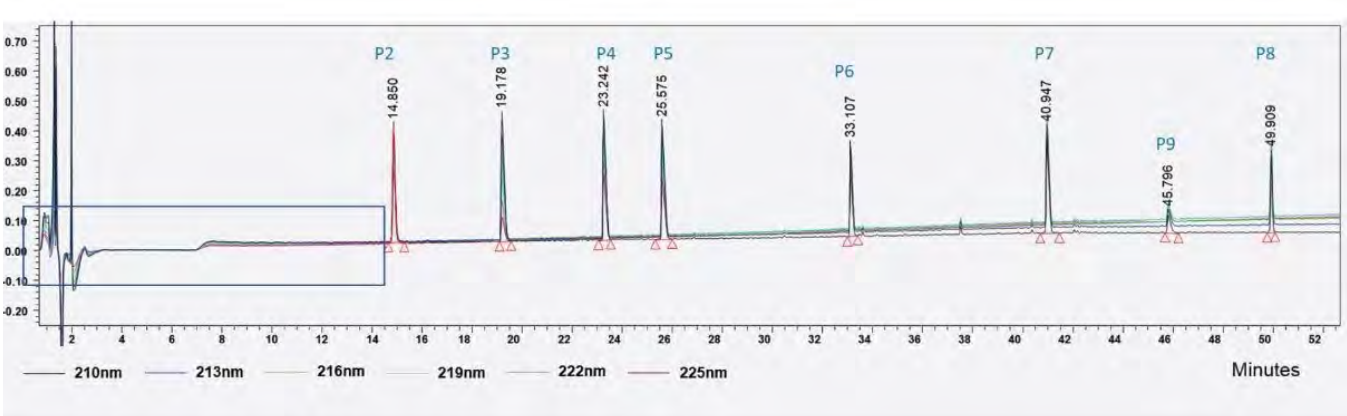


Figure 5. LC-UV-based detection of peptides from MassPREP Peptide Mixture (P1–P9) using formic acid-based mobile phase. The presence of P1 cannot be deciphered unambiguously from this profile (see the rectangular area on the chromatogram). Note the change in the elution pattern of P9 compared to P8.

as well as the improved peak shapes afforded by DFA ion pairing. The third is IonHance DFA's role in enabling adduct-free mass spectra. Finally, the excellent reproducibility of the peptide mapping was demonstrated by comparing the UV and BPI chromatograms between independent sample preparations.

Why do we need quick peptide mapping workflows?

Quick peptide mapping workflows can provide timely results to laboratories and companies working to research, develop and release protein drugs. Longer digestion and sample preparation times could introduce or allow the formation of sample artifacts, adversely impacting the analyses quality. Quick peptide mapping workflows can also enable the automation of high-throughput analysis in large-scale studies, making the QC analyses faster and less error-prone. A quicker turn around of results means quicker decisions, and fewer artifacts could help ensure that the right results are reported in the first pass of project work.

Why use IonHance DFA to enhance UV?

Formic acid is usually preferred over trifluoroacetic acid (TFA) in most LC-MS-based peptide mapping analyses because of the sensitive MS response and because adduct-free mass spectra are obtained. However, some peptides are not easily detectable with formic acid-based mobile phase (an example shown in Figure 5) because it does not have the ion pairing characteristics of TFA to improve chromatographic retention.[1] This was evident by the behavior of MassPREP Peptide Mixture (p/n: 186002337) when subjected to UV-based detection. Peptide 1 was not detectable under these conditions at any of the wavelengths (210–225 nm) tested. It is not clear whether this peptide was pushed into the void volume partially or completely, leading to a loss of signal.

IonHance DFA, on the other hand, exhibits ion pairing characteristics (more like TFA); therefore, better retention and UV sensitivity may be expected in a peptide-independent fashion. Working with this insight, we sought to achieving the highest quality UV chromatograms possible. In order to identify the most suitable UV wavelength

for peptide measurements when using IonHance DFA, the MassPREP Peptide Mixture was subjected to LC-UV analysis at detection wavelengths ranging from 210 to 225 nm. The nine peptides expected in the MassPrep peptide mixture could be detected in all situations despite the UV-baseline exhibiting wavelength-dependent drift (Figure 6). Although the maximum signal was

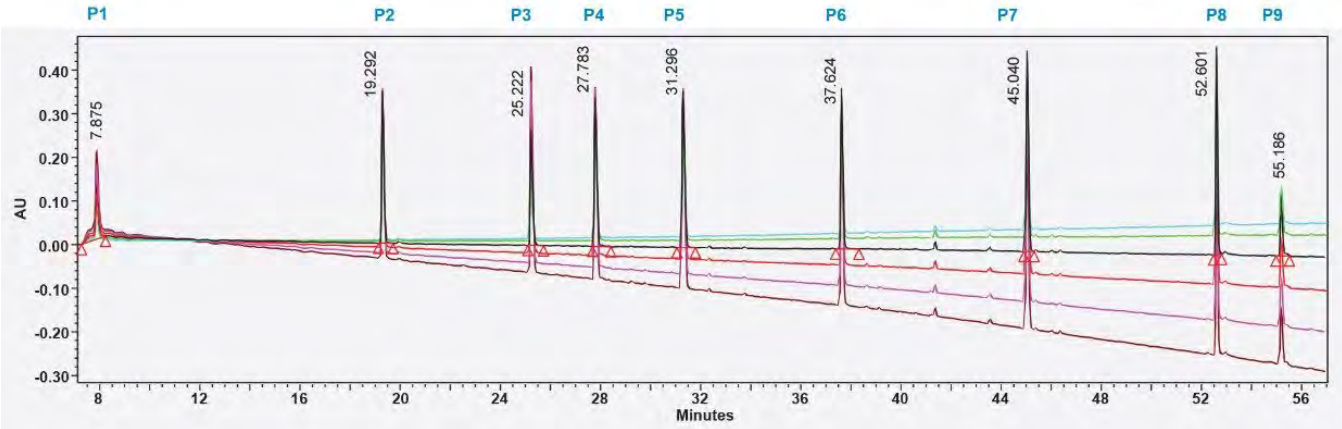
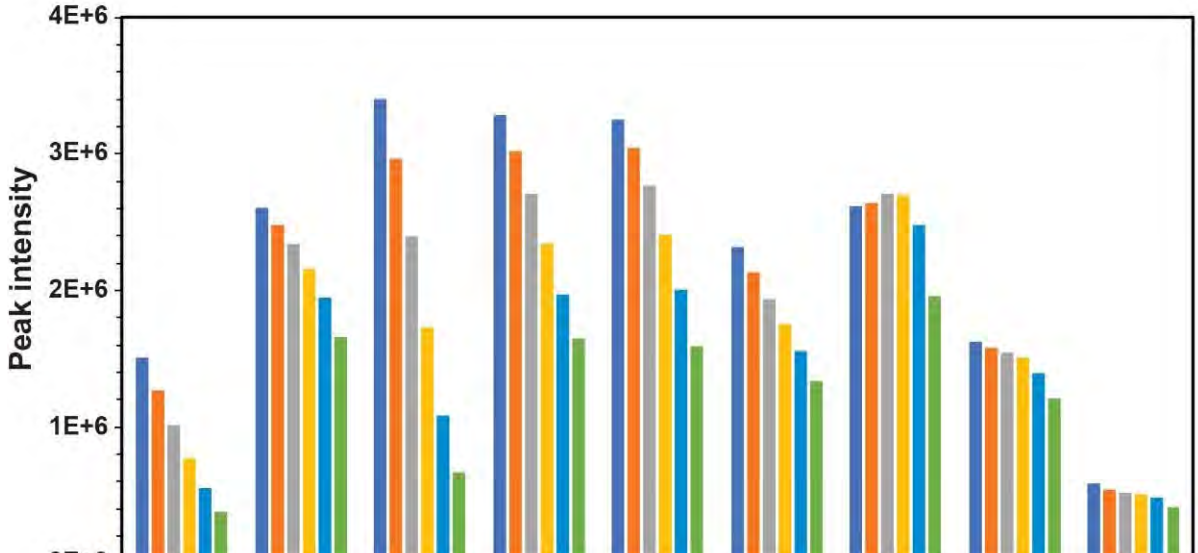


Figure 6. LC-UV-based detection of peptides from MassPREP Peptide Mixture (P1-P9) using IonHance DFA-based mobile phase. The presence of P1 can easily be deciphered unambiguously from this profile. Note the change in elution order and improved signal for P9 compared to the formic acid-based mobile phase.



	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5	Peptide 6	Peptide 7	Peptide 8	Peptide 9
210 nm	1509295	2601550	3399614	3280815	3253899	2319734	2621384	1623347	589043
213 nm	1261786	2480670	2965385	3016228	3038347	2128512	2641310	1573073	535516
216 nm	1009117	2335388	2393378	2706700	2761516	1932724	2713184	1549192	518529
219 nm	769567	2160368	1731266	2341601	2411732	1753848	2699898	1511754	510166
222 nm	557456	1947974	1084541	1975226	2002871	1553552	2476548	1397948	480140
225 nm	383325	1663405	669628	1644520	1595789	1332671	1954614	1204472	409655

Figure 7. Peak area comparison of all the peptides detected by IonHance DFA-based mobile phase at UV lengths ranging from 210–225 nm.

noticed at 210 nm the baseline drifted downward, which may interfere with the quantitation. On the other hand, the baseline at 219 nm was comparatively steady, and the peptide signal did not suffer significant loss at this wavelength (Figure 7). Slightly lowering the concentration of DFA in mobile phase B resulted in further improvement and reproducible baseline behavior. Importantly, all the expected peptides, including peptide 1 were detected without any ambiguity. Thus, 219 nm was identified as the ideal wavelength to obtain a level baselines and peptide-independent UV signal acquisitions.

Strong, reliable ultraviolet (UV) signal can provide several advantages in peptide mapping.

- Improved sensitivity: Sequence-independent UV-based recognition allows efficient detection of low-abundance peptides.[2]
- Increased accuracy: Provides distinct and well-defined peaks in the chromatogram.[3]
- Enhanced resolution: Fewer co-elutions in the chromatogram, improving the analyses of complex peptide mixtures.[2]
- Enhanced reproducibility: Provides more consistent and reliable results.[3]

Also, in QC environments where users might not yet have LC-MS techniques, high-quality UV chromatograms can enable the analysts to obtain the required peptide mapping information without repeated MS data acquisition. This is because the initial LC-UV-MS analysis at the analytical level would help annotate the peptide peaks in the LC profiles, which can easily be transferred to QC situations without any intermediary data loss or ambiguity.

Conclusion

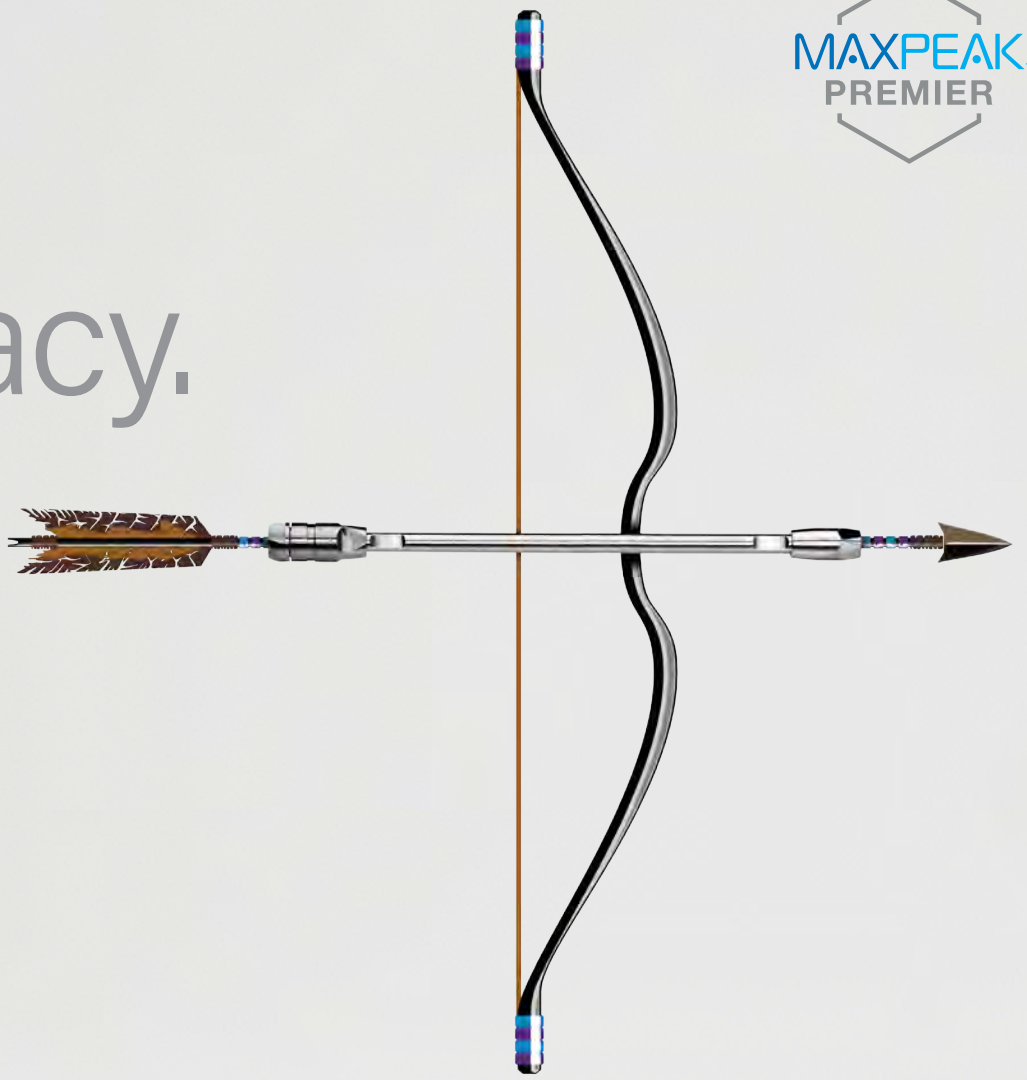
Peptide mapping workflows are typically complex and time-consuming. Hence, there is a need for simple, reproducible, and robust workflows that can facilitate reliable QC analyses and monitoring, as in the case of the biopharmaceutical industry. Sample preparation is a crucial step in these workflows and is usually time-consuming; however, RapiZyme Trypsin can enable users to reduce their sample preparation times, as demonstrated in this application note. Furthermore, the use of IonHance DFA as a mobile phase additive allows a robust LC-UV-MS peptide mapping experience through high-quality and reproducible data acquisition. This type of method show promise for becoming a platform method and enabling the timely assessment of protein therapeutical products in highly regulated environments.

References

1. J. M. Nguyen, J. Smith, S. Rzewuski, C. Legido-Quigley, and M. A. Lauber, 'High Sensitivity LC-MS Profiling of Antibody-Drug Conjugates With Difluoroacetic Acid Ion Pairing', MAbs, vol. 11, no. 8, pp. 1358–1366, Nov. 2019, doi: 10.1080/19420862.2019.1658492/SUPPL_FILE/KMAB_A_1658492_SM8763.DOCX.
2. M. L. Gross, G. Chen, and B. N. Pramanik, 'Protein and Peptide Mass Spectrometry in Drug Discovery', p. 464, 2012.
3. T. Mouchahoir and J. E. Schiel, 'Development of an LC-MS/MS Peptide Mapping Protocol for the NISTmAb', *Anal Bioanal Chem*, vol. 410, no. 8, pp. 2111–2126, Mar. 2018, doi: 10.1007/S00216-018-0848-6/FIGURES/9.

MAXPEAK™ PREMIER

MAX accuracy.



Hit your targeted separation goals with MaxPeak™ Premier Columns.

Trusted columns that eliminate non-specific adsorption to improve data accuracy, reduce variability, and boost confidence in your separations.

Eliminate doubt with consistent performance every time.

Find out more at
waters.com/ToTheMax

Waters™

February 24-12402 720008278EN Rev A