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Peptide mapping of challenging monoclonal antibodies

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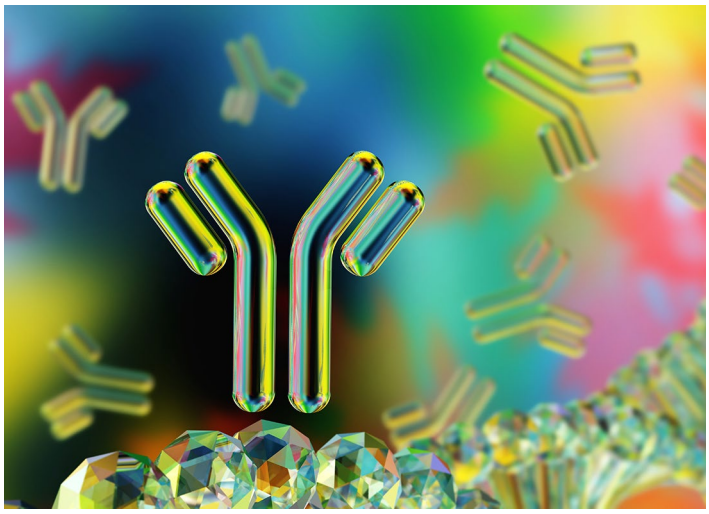

Goal

- Here we focused on finding a solution to address the challenges of biotherapeutic peptide analysis that result from the analyzed protein's structural complexity.
- Analytical strategies were evaluated to produce quantifiable peptides covering key CDR regions of certain mAbs that are otherwise not detectable with traditional tryptic digestion strategies due to the production of hydrophobic peptides or missed cleavages.
- We compared peptide mapping data acquired using Thermo Scientific™ SMART Digest™ Trypsin kits and Thermo Scientific™ SMART Digest™ Pepsin kits in Magnetic Bulk Resin options. The study focused on protein sequence coverage and identification of PTMs.

Application benefits:

- Combining the Thermo Scientific SMART Digest magnetic kits with the Thermo Scientific™ KingFisher™ Duo Prime purification system offers an automated approach to optimize protein digestion methods.
- Improved analytical strategies for peptide mapping provide higher confidence while not missing information on peptides potentially critical to the product quality of monoclonal antibodies.
- Alternative solutions are found for detecting and consequently allowing reliable quantitation of challenging hydrophobic peptides.

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Introduction

Analysis of biotherapeutic proteins can be challenging due to their structural complexity and heterogeneity. Minor PTMs of the protein can give rise to changes in the efficacy or immunogenicity of the drug. Consequently, such modifications are defined as critical quality attributes (CQAs), which must be characterized and monitored throughout production and storage. A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. CQAs are generally associated with the drug substance, excipients, intermediates (in-process materials), and drug product.[1]

Characterization of the primary structure by peptide mapping is a mandatory step requested by regulators to ensure drug safety and to allow adequate monitoring of many CQAs of a protein therapeutic. Therefore, peptide mapping methods must be capable of assessing low levels of critically relevant PTMs. In some molecules, due to the hydrophobic properties of areas of the protein sequence, commonly used proteases (such as trypsin and chymotrypsin) do not deliver detectable peptides for LC-MS analysis. Here, alternative sample preparation strategies may be needed, or alternative proteases, cleaving after different amino acids, to be used, assuring the production of detectable proteolytic peptides for all regions of the protein sequence.

A complementarity-determining region (CDR) is a variable area of an immunoglobulin (antibody) sequence where the molecule binds to its specific antigen. Antibodies have three CDRs on each light and heavy chain: CDR1, CDR2, and CDR3. Investigation of the CDR region is important, as PTMs in this region may negatively impact target binding.[2]

We observe that some mAb CDRs are not adequately covered with peptide mapping using the commonly employed enzyme trypsin. We evaluated a variety of enzymes when preparing samples for use in peptide mapping studies to investigate PTMs that may be induced during protein production, such as deamidation and oxidation. Several enzymes were tested in previous studies to evaluate if they can be used for mAbs with hydrophobic regions, which proved challenging to analyze with traditional tryptic digestion strategies.

We demonstrate the use of automated sample preparation combined with enzymes immobilized on magnetic beads allows for minimal user intervention and highly reproducible peptide generation.

Experimental

An analytical method using automated sample preparation supported by SMART Digest kits, combined with LC-MS/MS-based peptide mapping, can be leveraged to support characterization and at later stages process development of biopharmaceuticals, due to its ability to identify and quantify key PTMs. For all peptide mapping analyses, prepared peptides were analyzed using the Thermo Scientific™ Vanquish™ Horizon UHPLC system and Thermo Scientific™ Acclaim™ VANQUISH™ C18 column. Mass spectrometric detection was performed using a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ high-resolution accurate mass (HRAM) mass spectrometer.

Instrumentation and software

- Vanquish Horizon UHPLC system consisting of:
  - Vanquish System Base (P/N VH-S01-A)
  - Vanquish Binary Pump H (P/N VH-P10-A)
  - Split Sampler HT (P/N VH-A10-A)
  - Vanquish Column Compartment H (P/N VH-C10-A-02)
  - Active Pre-heater (P/N 6732.0110)
- Orbitrap Fusion Tribrid Mass Spectrometer (P/N FSN02-10002)
- KingFisher Duo Prime Purification System (P/N 5400110)
- Thermo Scientific™ BindIt™ Software 4.0 (P/N 5189009)

Consumables

- SMART Digest Trypsin Kit, Magnetic Bulk Resin option (P/N 60109-101-MB)
- SMART Digest Pepsin Kit, Magnetic Bulk Resin option (P/N 60109-110-MB)
- KingFisher deep-well, 96 well plate (P/N 95040450)
- KingFisher Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Bond-Breaker™ TCEP Solution (P/N 77720)

- Acclaim VANQUISH C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Fisher Chemical™ Water, Optima™ LCMS grade (P/N 0505904)
- Fisher Chemical™ Acetonitrile, Optima™ LCMS grade (P/N 0489553)
- BioChromato™ RAPID Slit Seal (P/N RSS-S96-80122)

Sample preparation - automated protein digestion

The digestion was automated using KingFisher 96 deep-well plates with the KingFisher Duo Prime purification system, controlled by Thermo Scientific™ BindIt™ 4.0 software using protocols defined elsewhere[3]. Digestion was performed using Magnetic SMART Digestion kits.

- 100 µL of SMART Digest buffer was added to the resin lane (row D) of a KingFisher 96 deep-well plate (Figure 1 and Table 1).

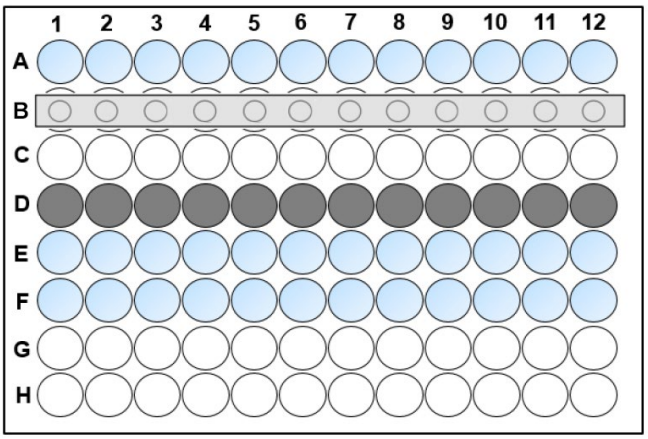

Figure 1. Layout of KingFisher deep-well, 96 well plate

Table 1. Sample and solvent conditions for KingFisher deep-well, 96 well plate

Row	Description	Contains	Volume per well
A	Sample lane	SMART Digest buffer	200 µL minus the sample volume (max 50 µL)
		TCEP (0.5 M)	2 µL
		Sample	200 µg (maximum 50 µL dependent on sample conc.)
B	Tip lane	12-tip comb	-
C	Blank (Empty)		
D	Resin lane	SMART Digest buffer	100 µL
		Magnetic Bead Bulk Resin Slurry	15 µL
E	Wash lane	SMART Digest buffer	200 µL

- The SMART Digest magnetic resin slurry was mixed thoroughly and 15 µL of slurry was added to the resin lane (row D).
- 200 µL of SMART Digest buffer was added to the wash lane (row E).
- SMART Digest buffer. Between 150 and 200 µL of SMART Digest buffer was added to the sample lane (row A). The final total volume of SMART Digest buffer and sample combined must be 200 µL.
- 2 µL of 0.5 M TCEP was added to the sample lane (row A).
- Finally, 200 µg of the sample (maximum volume of 50 µL, depending on concentration) was added to the sample lane (row A).
- The KingFisher 96 deep-well plate was then transferred to the KingFisher Duo Prime system and the digestion program was started.
  - Digestion was performed for 30 min at 75 °C using medium mixing speed.
  - After digestion, the magnetic beads were collected and transferred to the waste lane (lane F) and the sample lane (row A) was actively cooled to 5 °C.
- Post digestion
  - The KingFisher 96 deep-well plate was removed from the KingFisher Duo Prime system.
  - 10 µL of 20% TFA and 70 µL of 8 M GuHCl (to prevent loss of hydrophobic peptides) were manually added to each sample in the sample lane (row A).
  - A RAPID Slit Seal cover was placed on the 96 deep-well plates, which were mixed using a thermomixer and transferred directly to the Vanquish autosampler for analysis.

Chromatographic parameters for peptide mapping analysis

Table 2. LC parameters

Parameter	Value
Column	Acclaim VANQUISH C18, 2.2 µm, 2.1, 250 mm
Mobile phase A	0.1% difluoroacetic acid (DFA) in water
Mobile phase B	0.1% DFA in acetonitrile
Flow rate	0.3 mL/min
Column temperature	25 °C, still air
Sample load	10 µg
Gradient	See Table 3 for details

Table 3. Mobile phase gradient for peptide mapping assessment

Time	Flow (mL/min)	%B	Curve
0.0	0.300	2.0	5
45.0	0.300	45.0	5
46.0	0.300	90.0	5
50.0	0.300	90.0	5
51.0	0.300	2.0	5
53.0	0.300	2.0	5
54.5	0.300	9.0	5
56.0	0.300	90.0	5
56.5	0.300	2.0	5

MS parameters

Table 4. MS source and analyzer conditions

MS source parameters	Settings
Ion source/probe	Thermo Scientific™ Ion Max ion source with HESI-II probe
Sheath gas	45 arb units
Auxiliary gas	10 arb units
Ion transfer tube temperature	325 °C
Vaporizer temperature	350 °C
Source voltage	3.5 kV

Table 5. MS method parameters utilized for peptide mapping analysis

Parameter	Setting
General	
Application mode	Peptide
Runtime	60 min
Polarity	Positive
Full MS	
Full mass range	*m/z* 375–2000
Resolution settings of OT	120,000
Isolation	Quadrupole
Max injection time	50 ms
In-source CID	Disabled
Microscans	1
AGC target	Standard
MS^2 scan type 1	
Scan type 1	$ddMS^2$ EThcD
Activation type	ETD
SA collision energy type	EThcD
Included charge states	2–10
Isolation mode	Quadrupole
Isolation window	1.6
Collision energy (%)	25
Detector type	Ion trap
Microscans	1
AGC target	Standard
Maximum injection time	Dynamic
MS^2 scan type 2	
Scan type 2	$ddMS^2$ HCD
Included charge states	2–8
Isolation mode	Quadrupole
Isolation window	1.6
Activation type	HCD
Collision energy mode	Stepped
HCD (%)	15, 30, 45
Detector type	Ion trap
Microscans	1
AGC target	Standard
Maximum injection time	Dynamic
MS^2 scan type 3	
Scan type 3	$ddMS^2$ HCD
Intensity treshold	5.0e5
Included charge states	1
Isolation mode	Quadrupole
Isolation window	1.6
Activation type	HCD
Collision energy mode	Stepped
HCD (%)	15, 30, 45
Detector type	Ion trap
Microscans	1
AGC target	Standard
Maximum injection time	Dynamic

Data analysis

Data processing and reporting was performed using Byos™ software (Protein Metrics Inc.); settings are shown for trypsin and pepsin in Figure 2.

Results and discussion

Assuring complete peptide mapping coverage for the CDR region

Complete peptide map coverage is not always possible when antibody sequences contain several hydrophobic amino acids, which result in the production of highly hydrophobic peptides.

Analytically challenging mAbs can contain regions with aromatic amino acids, such as those shown in Figure 3. Such regions are poorly proteolytically digested with trypsin. Alternative enzymes were evaluated to produce peptides more amenable to LC-MS analysis for this region. Previous peptide mapping experiments of mAb1 using trypsin for proteolytic digestion demonstrated that its hydrophobic regions were not sufficiently covered with detectable peptides (Figure 4).

Trypsin - analysis settings

Instrument Parameters	
Precursor Mass Tolerance	3.00 ppm
Fragmentation Type	Both: HCD & EThcD
Fragment Mass Tolerance 1	0.30 Da
Fragment Mass Tolerance 2	0.30 Da
Recalibration (from Preview)	None
Recalibration (lock mass)	None
Digestion	
Cleavage Site(s)	RK
Cleavage Side	C-terminal
Digestion Specificity	Fully specific (fastest)
Missed Cleavages	2

Pepsin - analysis settings

Instrument Parameters	
Precursor Mass Tolerance	3.00 ppm
Fragmentation Type	Both: HCD & EThcD
Fragment Mass Tolerance 1	0.30 Da
Fragment Mass Tolerance 2	0.30 Da
Recalibration (from Preview)	None
Recalibration (lock mass)	None
Digestion	
Cleavage Site(s)	
Cleavage Side	C-terminal
Digestion Specificity	Non specific (slowest)
Missed Cleavages	-1

Figure 2. Peptide mapping settings used in Byos software

Figure 3. Examples of Symphogen mAbs with regions that are not covered with a trypsin digestion platform. HPLC index calculated in GPMAW software according to published methods.[4]

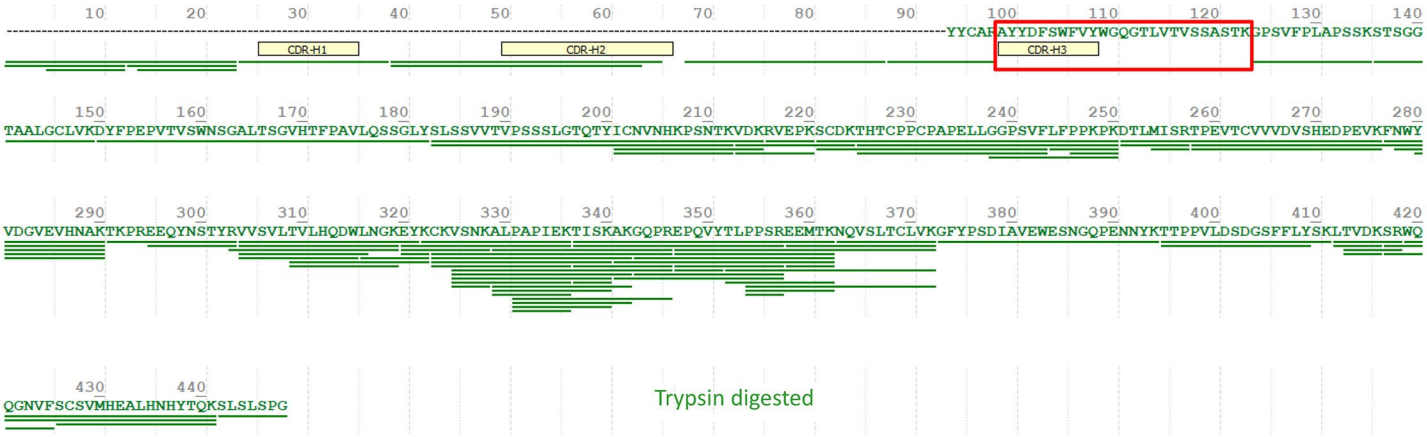

Figure 4. Results of tryptic digestion of mAb1 and identified wildtype peptides. As shown for mAb1 and highlighted with the red cluster, no tryptic peptides have been detected covering that specific region. These regions are often characterized by the presence of multiple hydrophobic amino acids.

Looking closer at the results in Figure 4, for the area showing no peptide coverage, it was postulated that the lack of the peptide for the highlighted region could not be due to poor trypsin activity because good coverage was obtained for peptides flanking this region with strong peptide signals, as shown in Figure 5.

Attempts were made to improve solubilization of the missing peptide (AYYDFSWFVYWGQGTLVTVSSASTK) by adding GuHCl and other additives during and after the digestion procedure. This did not result in improved peptide coverage, Figure 5.

Investigating alternative enzymes

To provide better sequence coverage, a protease cleaving after aromatic amino acids was evaluated; the hypothesis was it would better enable detection of a peptide from the missing region by cleaving within the hydrophobic part of the sequence.

Pepsin is an endopeptidase that has broad specificity with a preference for peptides containing linkages with aromatic or carboxylic L-amino acids. Pepsin is available in the SMART Digest Kit format and was evaluated at Symphogen on their already optimized sample preparation platform set-up for automation. While trypsin cleaves after lysine and arginine (K and R), and its optimal activity (when using SMART Digest Kits at elevated temperature) is at pH 6.5, pepsin cleaves after phenylalanine, tryptophan, tyrosine (F/W/Y) with cleavage at slower rates after leucine, methionine and cysteine (L/M/C) and is active at pH 2.1.

For mAb1, pepsin produced several peptides in the region that were retained on the column during reversed-phase separation and detectable, where tryptic peptides were not. Differences in detected peptides after trypsin and pepsin is demonstrated in Figure 6.

High-intensity peptides were detected for the challenging region and confident identification of these peptides was obtained from HRAM MS (Figure 7) and MS/MS (data not shown).

Unlike long-chain hydrophobic tryptic peptides from the challenging region, smaller peptides were created by pepsin and were retained well on the reversed-phase column. This is due to the presence of hydrophobic amino acids, rather than the charged amino acids found at the end of tryptic peptides.

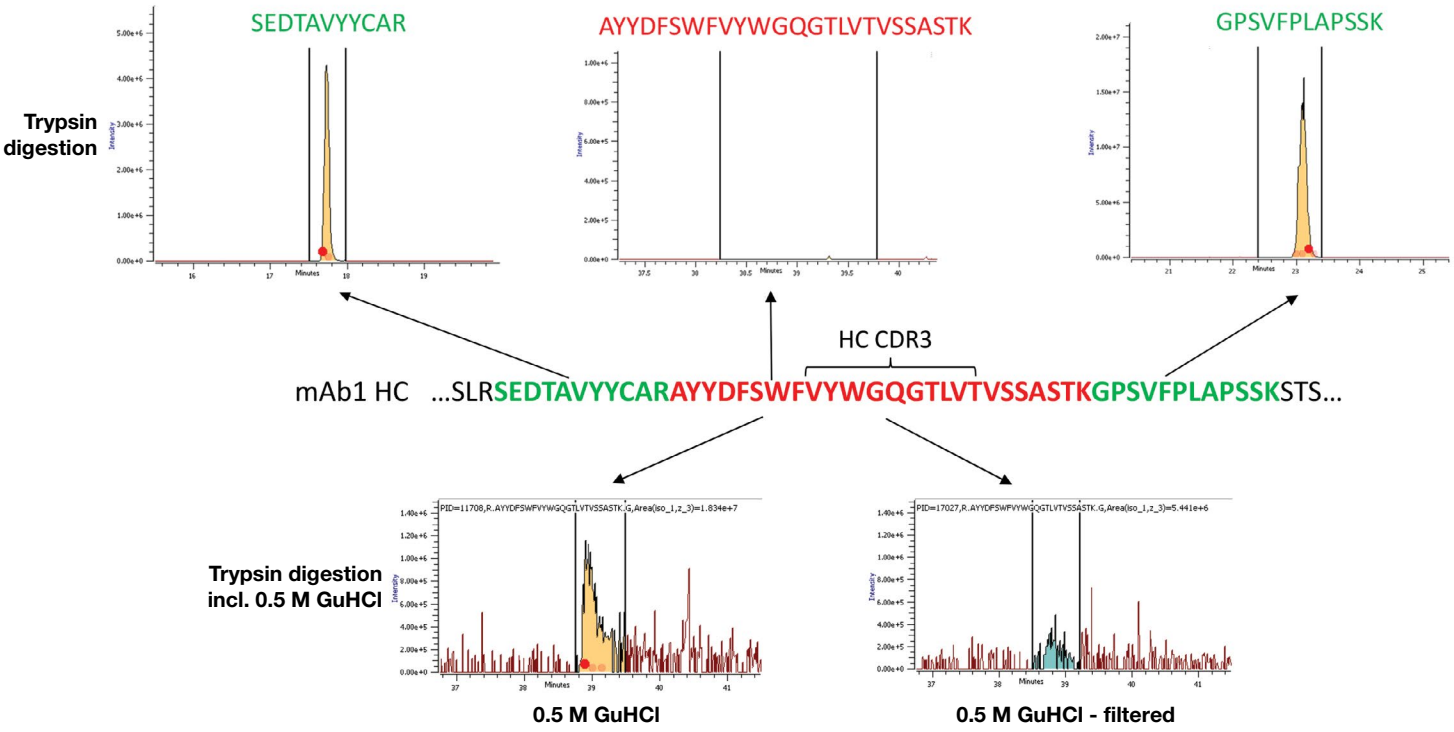

Figure 5. Investigation of generated peptides of mAb1 by trypsin. Flanking peptides confirm good trypsin activity. Additives (urea, GuHCl, DMSO, isopropanol, acetonitrile) during and after digestion did not increase the detection of the missing peptide.

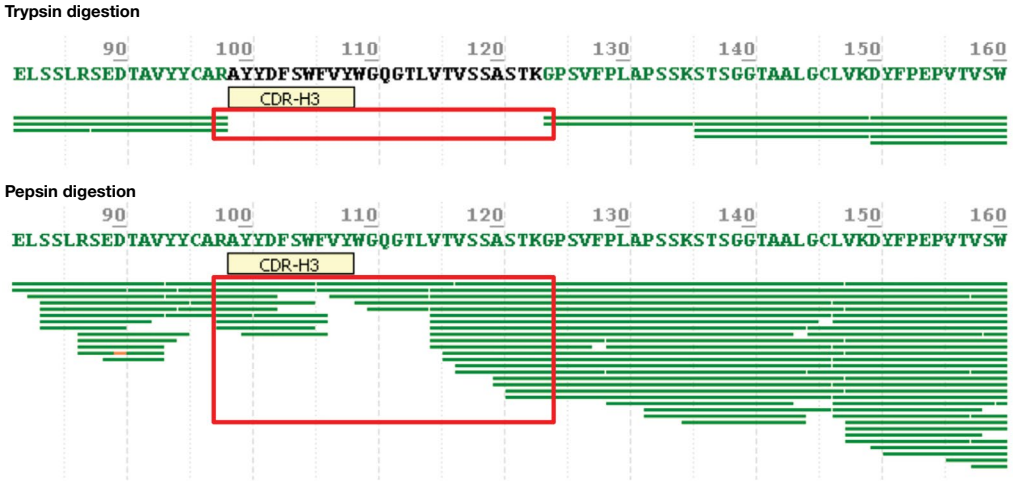

Figure 6. Results of peptide coverage for CDR3 region of mAb1. After trypsin digestion no peptides were detected in the highlighted region (top), while after pepsin digestion multiple peptides were detected at varying lengths in the highlighted region (bottom).

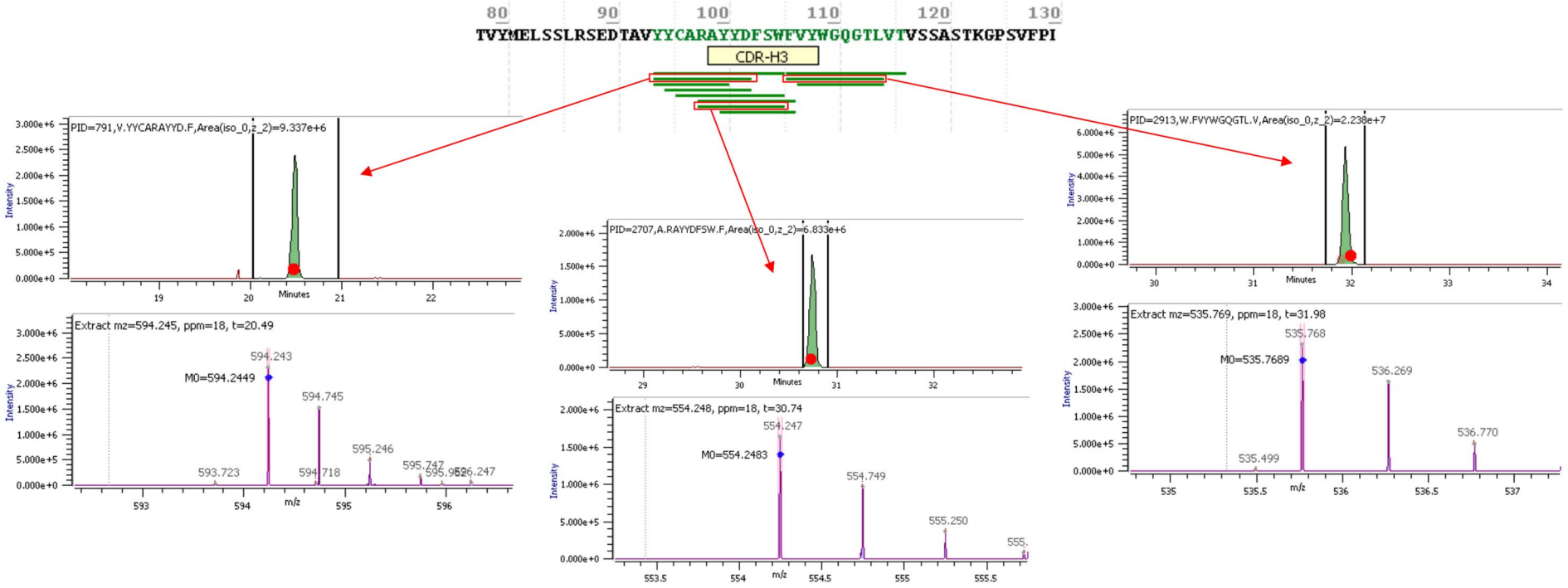

Figure 7. Pepsin digestion - validated peptide hits from difficult region. Good chromatographic separation and peak retention was observed with intensive peaks and reliable MS data for the peptides covering the difficult region of mAb1.

Comparability of results - trypsin versus pepsin

A qualitative and quantitative evaluation of pepsin versus trypsin digests were performed to ensure high confidence results are delivered by the new enzyme. For this evaluation, a previously characterized mAb (mAbX) was chosen that had produced well detectable peptides both after digestion with trypsin and pepsin. The chosen mAb has an oxidation affecting a tryptophan (W) located in the CDR3 region. Results were compared following digestion with both proteases, with specific focus on quantifying levels of oxidation.

In previous experiments, mAbX was determined to be prone to an oxolactone modification affecting W33 of heavy chain. The relative levels of oxidation were assessed in a series of degradation time course study samples. The samples were digested with both trypsin and pepsin, and the resulting peptides comprising the W33 amino acid determined and oxidation levels were compared (Figure 8).

Both proteases resulted in similar trends for the observed quantities of the oxidation for the time series, with some relative value differences. The trypsin digestion method reported 12% oxidation, while the pepsin digestion method reported approximately 8% oxidation for the sample at the longest degradation time and so the highest oxidation levels.

Trypsin is a common protease used by most biopharmaceutical analytical laboratories due to its high specificity cleavage. However, trypsin's activity is limited to a small and relatively high pH window, and in some specific cases does not provide complete sequence coverage.

We investigated the use of alternative proteases to address these challenges and highlighted the importance of obtaining sequence coverage in the CDR and the hinge region of some challenging monoclonal antibodies (mAbs). Simple to use laboratory automation was utilized to further improve the reproducibility of the digestion procedure, along with minimized manual steps to ensure method transferability and save significant manual handling time.

Pepsin as an alternative enzyme to trypsin provided good sequence coverage for regions of some mAbs that were not detected with trypsin digestion. Furthermore, similar trends were observed in oxidation levels detected by peptide mapping with trypsin and pepsin digestion.

Method transfer from one sample preparation strategy to another always needs to be carefully evaluated as information obtained for the quantitative levels of PTMs may be critical when making decisions along with the development of therapeutic mAb products.

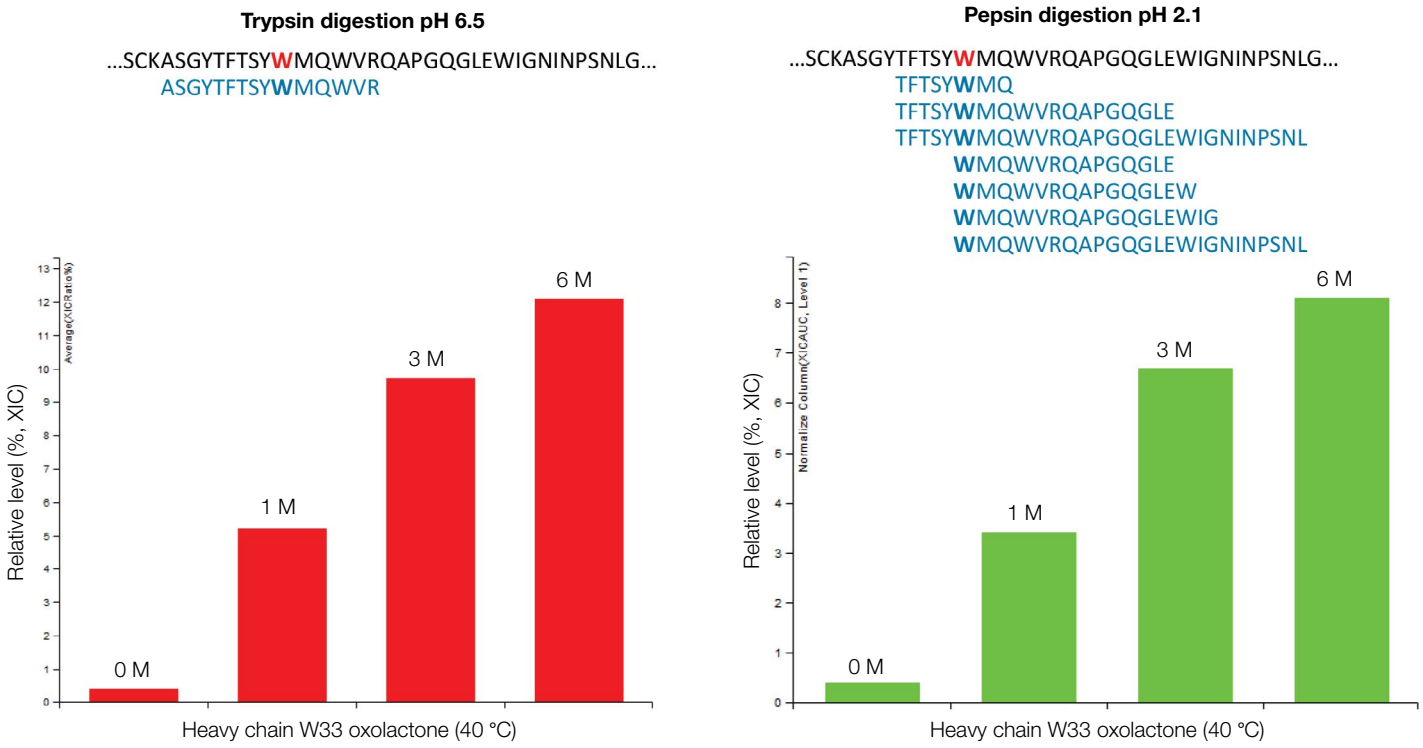

Figure 8. Comparison of cumulative quantitative results of peptides detected after trypsin and pepsin digestion for CDR regions of mAbX. Similar oxidation levels were obtained for all samples (frozen reference samples (0 M), after 1 month (1 M), 3 months (3 M), and 6 months (6 M) forced degradation); however, significantly fewer peptides needed to be monitored for the trypsin workflow.

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Conclusion

Here we presented the benefits of using the SMART Digest Pepsin Kits in combination with the KingFisher Duo Prime system for sample preparation of monoclonal antibodies with challenging hydrophobic regions for studies confirming sequence coverage by peptide mapping.

- Using pepsin as an alternative enzyme in SMART Digest format provided well detectable and quantifiable peptides across regions that proved challenging to analyze using trypsin digests.
- Comparable PTM levels were observed with trypsin and pepsin digestion.
- Reproducible automated sample preparation was assured by limiting user intervention and delivering reproducible digests.
- Reliable and robust chromatographic separation was consistently achieved using a Vanquish Horizon UHPLC in combination with an Acclaim VANQUISH C18 (2.1 × 250 mm) UHPLC column.
- Pepsin is less specific than trypsin and pepsin digests contain a higher number of peptides and are more complex to interpret. However, data complexity can be effectively addressed using modern software tools, such as Byos software.

References

1. U.S. FDA Guidance for Industry Q8(R2) Pharmaceutical Development, https://www.fda.gov/media/71535/download
2. Haberger, M. et al. Assessment of chemical modifications of sites in the CDRs of recombinant antibodies, *mAbs* **2014,** *6*:2, 327–339, https://www.tandfonline.com/doi/full/10.4161/mabs.27876
3. Millán-Martín, S.; Jakes, C.; Oliviero, G.; Carillo, S.; Bones J. An automated high-throughput workflow for peptide mapping to monitor post-translational modifications (PTMs) of monoclonal antibodies, Thermo Scientific Application Note 21835 (2018), https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-21835-lc-ms-peptide-mapping-ptm-mab-an21835-en.pdf
4. Sakamoto, Y.; Kawakami, N.; Sasagawa, T. Prediction of peptide retention times, *J. Chrom.* **1988,** *442,* 69–79.

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