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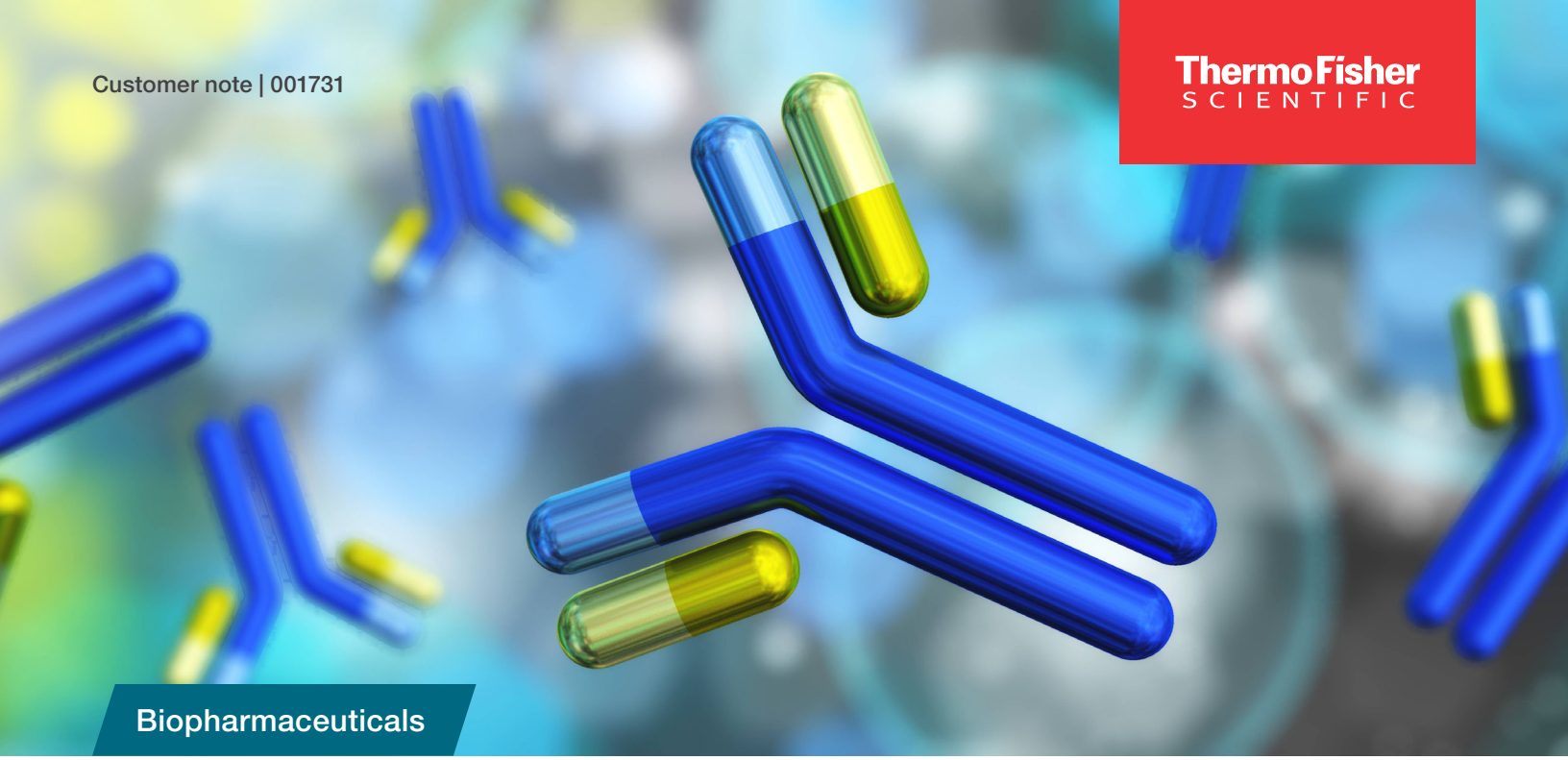

Biopharmaceuticals

Development of a systematic approach for improving tryptic digestion, recovery and chromatographic performance for mAb analysis

Addressing missed cleavages and optimizing chromatographic performance of hydrophobic peptides in peptide mapping assays

Authors

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Keywords

Peptide mapping, missed cleavages, peak shape, tailing, carry-over, protein digest, high-resolution accurate mass (HRAM), Orbitrap, hydrophobic peptides

Application benefits

- Reduction of missed cleavages in peptide mapping analysis
- Simpler data interpretation due to more consistent cleavage
- Improvement in chromatographic peak shape and carry-over of hydrophobic peptides
- Systematic approach to method optimization

Goal

Reduction of missed cleavages during tryptic digestion of monoclonal antibodies (mAb) using an automated modified two-step Thermo Scientific™ SMART Digest™ kit protocol, assessed against a conventional protein digest protocol[1] and a one-step SMART Digest kit protocol. Improvement of chromatographic conditions to reduce peak tailing and carry-over seen of hydrophobic peptides on traditional C18 column chemistries.

Introduction

Peptide mapping experiments are a critical tool to verify the primary structure and mapping/quantitation of post-translational modifications (PTM) or product quality attributes in biopharmaceutical development. A key sample preparation step in the process is proteolytic digestion, often involving multiple manual steps prone to variability and manual errors. However, this step can be fully automated using the Thermo Scientific™ KingFisher™ Duo Prime purification system and SMART Digest kit; a temperature stable enzyme immobilized onto magnetic beads. Not only does this reduce manual labor but allows the digestion time and enzyme amounts to be strictly controlled, providing very robust results. High-temperature digestion can be performed using this combination, further reducing the steps and need for chemical denaturation of the target mAb. Here, we explore an optimized protocol for automated digestion which reduces the number of missed cleavages compared to both traditional in solution methods and published automation methods, whilst maintaining the simplicity of the sample preparation.

In addition, peptide mapping experiments rely on good chromatographic performance, however tryptic digestion can produce hydrophobic peptides which suffer from poor peak shape (tailing) and carry-over on C18 column chemistries used by the industry. Here, we explore alternative, less retentive chemistries (C4) to improve both peak shape (less tailing) and reduce chromatographic carry-over between runs.

Experimental

A total of eight mAbs were analyzed in the current study. These were the NISTmAb, USP mAb 001, USP mAb 002, USP mAb 003, and three internal projects (Project 3 is a mixture of two mAbs).

Difluoroacetic acid (DFA), 0.5 M tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 8 M guanidine-HCl (GuHCl), DL-dithiothreitol, iodoacetamide, and Ultrapure 1 M Tris-HCl pH 8.0 were purchased from a reputable source.

Reagents and consumables

- Thermo Scientific™ UHPLC-MS water (P/N W81 1L)
- Thermo Scientific™ UHPLC-MS acetonitrile (P/N A9561 1L)
- Thermo Scientific™ Accucore™ 150-C4 LC column, 2.1 × 150 mm, 2.6 µm (P/N 16526-152130)
- Thermo Scientific™ Accucore™ C18 HPLC column, 2.1 × 150 mm, 2.6 µm (P/N 17126-152130)
- Thermo Scientific™ Hypersil GOLD™ C4 HPLC column, 2.1 × 150 mm, 2.6 µm (P/N 25502-152130)
- Thermo Scientific™ Hypersil GOLD™ C18 Selectivity HPLC Column, 2.1 × 150 mm, 2.6 µm (P/N 25003-152130)
- SMART Digest Trypsin kit, magnetic bulk resin (P/N 60109-101-MB)

Instrumentation

Chromatographic separation was performed using a Thermo Scientific™ Vanquish™ Horizon UHPLC system, consisting of:

- Vanquish System Base (P/N VF-S01-A-02)
- Vanquish Binary Pump H (P/N VH-P10-A-02)
- Vanquish Split Sampler FT (P/N VF-A10-A-02)
- Vanquish Column Compartment H (P/N VH-C10-A-03)
- KingFisher Duo Prime purification system was used for automated sample digestion (P/N 5400110)

The LC was connected to a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer equipped with a Thermo Scientific™ Ion MAX™ ion source and the Thermo Scientific™ HESI-II-probe.

Sample preparation

Digestion protocols

Three digestion protocols were assessed as part of this study.

Method 1—optimized two-step SMART digestion protocol (updated version of method described in ref. 2)

This method involves a short high temperature digestion period at 75°C for 15 min, followed by a lower temperature step at 40°C for 30 min. Fresh SMART Digest trypsin resin is used in each digestion step (i.e., the 96 well plate contains two rows with SMART Digest trypsin resin).

Samples were digested in a KingFisher Duo Prime robot using SMART Digest trypsin resin. Samples were mixed with SMART digestion buffer (pH 6.5) and TCEP in a KingFisher 96 deep-well plate. Final sample and TCEP concentration were 1 mg/mL and 5 mM, respectively. Final digestion volume was 100 µL. The KingFisher Duo digest program consisted of the following steps:

1. Collect 1st SMART Digest trypsin resin (row C) and wash in SMART Digest buffer (row E)
2. Digest for 15 min at 75°C (row A)
3. Collect 1st SMART Digest trypsin resin from row A after digestion, wash in 8 M GuHCl (row G), collect and discard in waste lane (row F)
4. Collect 2nd SMART Digest trypsin resin (row D) and wash in SMART Digest buffer (row D)
5. Digest for 30 min at 40°C (row A)
6. Collect 2nd SMART Digest trypsin resin from row A after digestion, wash in 8 M GuHCl (row G), collect and discard in waste lane (row F)
7. KingFisher Duo digest program complete
8. Transfer GuHCl wash solution (row F) by pipette to sample (row A) and mix by vortex.

After completion of the KingFisher digest program, GuHCl in row F was manually transferred to the sample row (row A). The wash step in 8 M GuHCl ensured that all surfaces the sample was exposed to (row A sample wells, KingFisher digest comb, SMART Digest trypsin resin) got exposed to GuHCl, thereby keeping potentially surface-sticking peptides in solution (see Results and Discussion). Finally, 5 µL of 20% TFA were added to the sample row (row A). The 96 microwell plate was sealed, mixed briefly, and stored at –80°C until the time of analysis. No alkylation step was necessary.

Method 2—original one-step SMART digestion protocol

This method involves a single KingFisher Duo digestion step at 75°C for 30 min.[2] No alkylation step was necessary.

Method 3—conventional MAM digestion protocol

Sample digestion was performed as described by Jakes *et al.*[1] with the following exceptions:

a) alkylation was performed using iodoacetamide;

b) buffer exchange prior to digestion was performed using Thermo Scientific™ Zeba™ descriptor 0.5 mL 7K MWCO Spin desalting columns;

c) after digestion samples were stored at –80°C until the time of analysis.

Liquid chromatography—mass spectrometry

The LC-MS analysis was carried out using the following conditions:

Table 1. LC conditions

Parameter	Value
Mobile phase	A: 0.1% DFA in water B: 0.1% DFA in 95:5 (Acetonitrile/water)
Flow rate (solid core columns)	0.5 mL/min
Flow rate (fully porous columns)	0.4 mL/min
Autosampler temperature	5°C
Injection volume	4.2 µL (2 µg protein load)
Needle wash solvent	20% Ethanol
Column temperature	25°C (still air mode)
Divert valve timing	Flow to waste from 0 to 1.2 min

Table 2. UHPLC gradient conditions

Time (min)	Mobile phase B (%)
0.0	2
1.0	2
52	45
53	90
56	90
57	2
60	2
61	90
64	90
65	2
70	2

LC-MS/MS was performed using data dependent acquisition (DDA) on a Thermo Scientific™ Orbitrap Fusion Tribrid mass spectrometer. The HESI source conditions and relevant MS method parameters are detailed in Tables 4 and 5.

Table 3. MS HESI source conditions

Parameter	Accucore C4/C18 columns 2.1 × 150 mm, 2.6 µm	Hypersil GOLD C4/C18 columns 2.1 × 150 mm, 1.9 µm
Flow rate	0.5 mL/min	0.4 mL/min
Pos ion voltage	3,500 V	3,500 V
Sheath gas	50 arb	45 arb
Aux gas	15 arb	10 arb
Sweep gas	2 arb	1 arb
Ion transfer tube temperature	350°C	300°C
Vaporizer temperature	400°C	350°C

Table 4. MS method parameters

Parameter	Value
Ion-source settings	
Source type	H-ESI
Positive ion	3,500 V
Sheath gas	45 arb
Aux gas	10 arb
Sweep gas	1 arb
Ion transfer tube temperature	300°C
Vaporizer temperature	350°C
MS global settings	
Expected LC peak width(s)	10
Default charge state	1
Internal mass calibration	Off
Experiment #1	
Start time	0 min
Stop time	70 min
Resolution	*m/z* 120,000
Scan range	*m/z* 350–2,000
RF lens	60%
AGC target	Standard
Max injection time	Auto
Polarity	+ive
Intensity threshold	1.0e5
Dynamic exclusion	
Exclude after n times	1
Exclusion duration	7
Exclude isotopes	True

Parameter	Value
Data dependent	
N of dependent scans	5
Scan event type 1	
Include charge states	2–10
Include undetermined charge states	False
$ddMS^2$ IT EThcD	
Isolation window	1.2 *m/z*
SA collision energy type	EThcD
SA collision energy	25%
Detector type	Ion trap
AGC target	Custom
Normalized AGC target	200%
Max injection time	Auto
Scan event type 2	
Include charge states	1–2
Include undetermined charge states	False
Intensity threshold	1.0e6
$ddMS^2$ IT HCD	
Isolation window	1.2 *m/z*
SA collision energy type	HCD
SA collision energy	30%
Detector type	Ion trap
AGC target	Custom
Normalized AGC target	200%
Max injection time	Auto

Instrument control and data analysis

All data were acquired using Thermo Scientific™ Xcalibur™ software version 4.4 and subsequently imported into Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.2.10 ES. LC-MS/MS data stored in Chromeleon CDS were automatically synchronized to the Protein Metrics Byosphere™ enterprise software. All MS data processing was performed in the Byosphere enterprise.

Results and Discussion

Optimized two-step SMART digest protocol

Comparison of the three digestion methods shows a significant reduction of missed cleavages when utilizing the two-step SMART digest protocol compared to standard SMART digest protocol and in-solution digest (Figure 1). By utilizing high temperature in step one for a short period of time, the proteins are sufficiently denatured to allow for the enzymatic digestion to begin. Step 2 allows for complete digestion to occur at a lower temperature, reducing the missed cleavages.

Signal intensities are comparable across the three protocols, however the two-step procedure yielded less peaks overall due to significantly less missed cleavages, providing a ‘cleaner’ chromatogram.

The peptide count for the optimized two-step method is lower due to the reduction of missed cleavages; the percentage of peptides with zero missed cleavages (teal bar) is on average 60, 32, and 34 for the two-step SMART digest, the one-step SMART digest, and the conventional MAM digest, respectively (Figure 2).

Fewer missed cleavages simplifies quantitative reporting of PTMs by yielding a single prominent peptide rather than multiple peptides.

Furthermore, the two-step approach (as well as the original SMART digest protocol) is fully automated and uses off-the-shelf reagents, making the whole sample preparation process simple and robust.

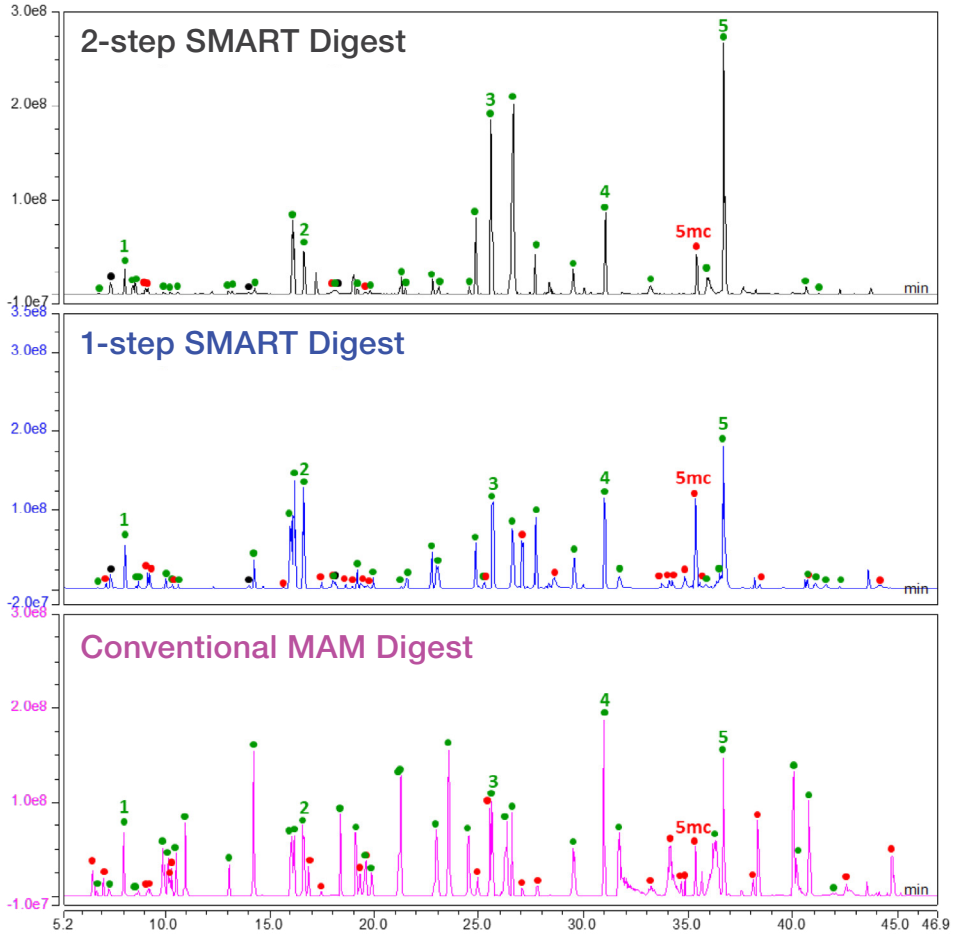

Figure 1. Base peak chromatograms (BPCs) of NISTmAb digested with the two-step SMART digest method, the original one-step SMART digest method, and the conventional MAM digest method. Missed cleavages are indicated in red (5 mc highlighted to show variation in intensity of a constant missed cleavage across all methods).

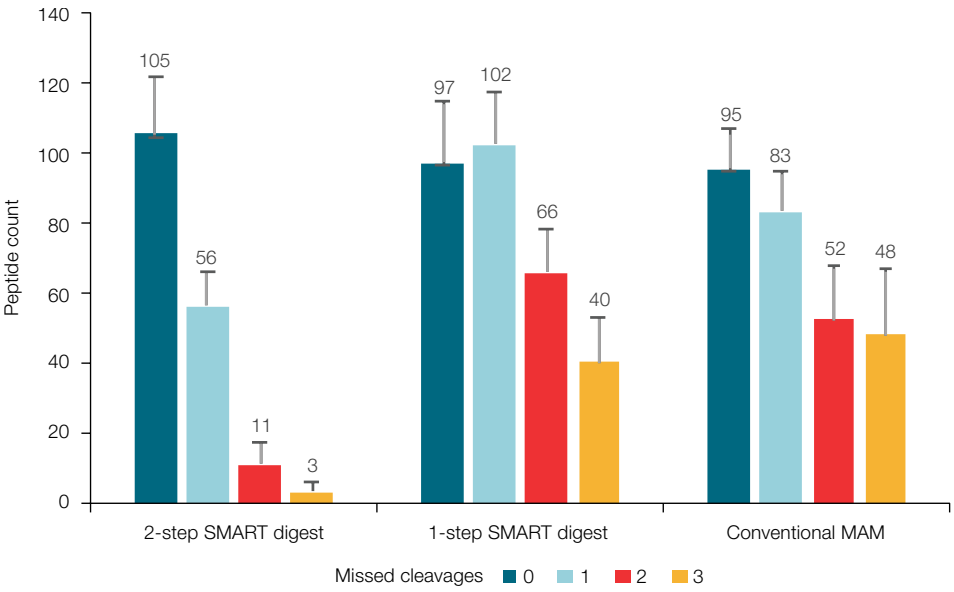

Figure 2. Illustration of the number of identified peptides by MS/MS and grouping according to the number of missed cleavages in the range from 0 to 3.

Optimized chromatographic conditions

Comparison of peptide retention

During digestion for peptide mapping methods, hydrophobic peptides are typically obtained when using Lys-C or trypsin enzyme. These present chromatographic challenges on C18 column chemistries such as long retention times, poor peak tailing, and significant carry-over.

C18 reverse phase columns have historically been the standard choice for peptide mapping. Here we asses less hydrophobic C4 chemistry for peak shape, carryover, and retention of early eluting peptides.

Upon initial comparison the base peak chromatograms appeared similar in regards to the number of identified peptides and the overall peak pattern.

However, it was anticipated that smaller, more polar peptides would be lost with the C4 columns lower retentivity. However, no difference was observed in the sequence coverage obtained from data acquired using the C18 and C4 columns, confirming that both columns retain hydrophilic peptides to a similar extent.[2]

Typically, the LC flow is set to 'waste' for the first 3 min of the run to avoid contamination of the MS source by salts and buffers from the digest. However, it was anticipated that the number of smaller, polar peptides would increase with improved digestion efficiency. Consequently, it was investigated whether small polar peptides were eluting before 3 min with samples obtained using the new and optimized digestion protocol. As Figure 3 demonstrates, several early eluting peptides were identified between 1.3 and 3 min. In due consequence, the time point for switching the flow from waste to the MS was reduced from 3 to 1.2 min.

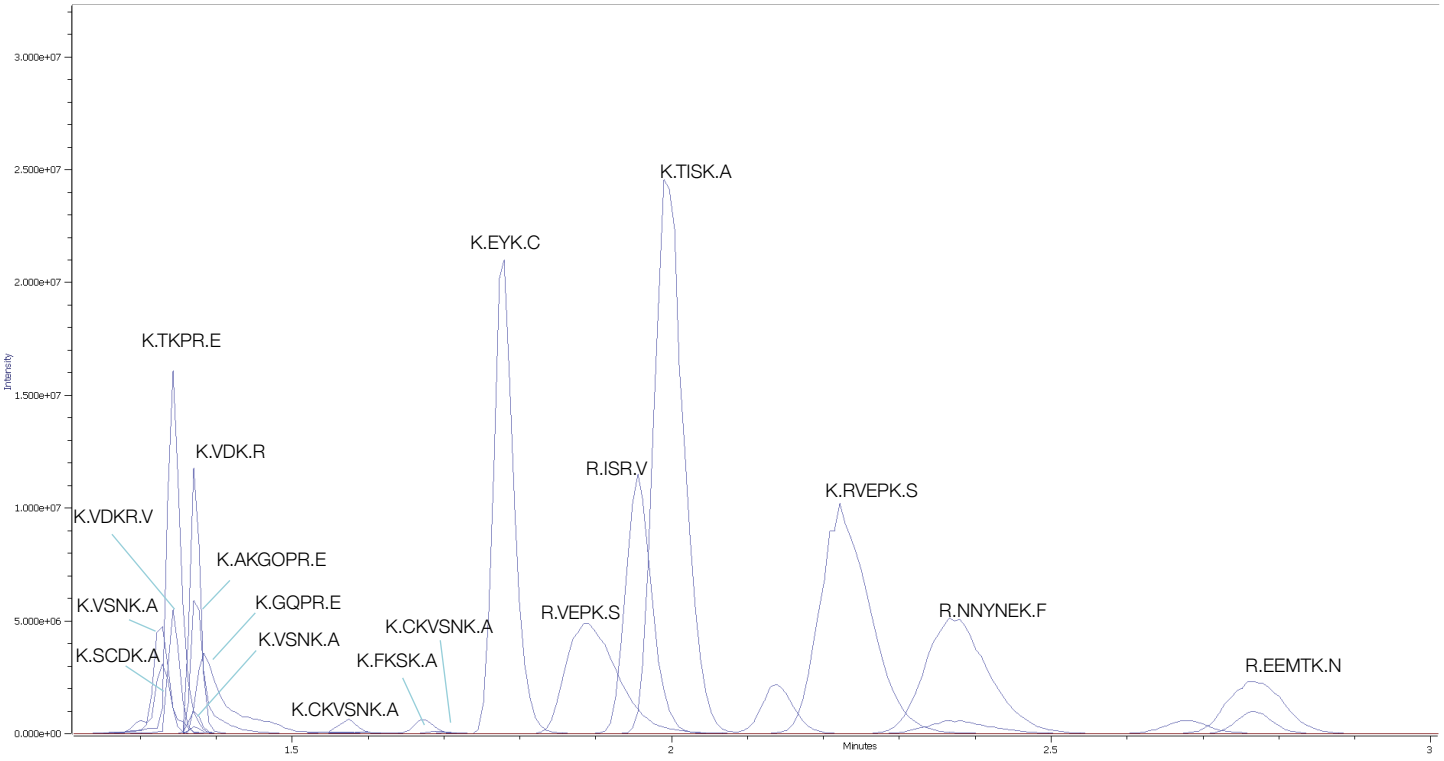

Figure 3. Identified early eluting peptides from 1 to 3 min on C4 column, confirming the MS switching time is required to be optimized to include peptides eluted earlier than the original 3 min switching valve time.

Effect on peak shape and carry-over

The comparison of chromatograms obtained from water blank injections immediately after each sample shows significantly reduced carry-over when using the C4 column (black trace) versus the C18 column (blue trace) as highlighted in the chromatogram overlay shown in Figure 4. MS/MS-based identification of the three major peaks in the blank run using the C18 column are assigned based on identification via MS/MS.

For a later eluting peptide excessive peak tailing was observed on the C18 column, along with a carryover peak in the 'washing' portion of the chromatography, indicating strong retentivity, and poor elution of the peptide. By comparison the C4 column showed significantly reduced peak tailing and carryover (Figure 5).

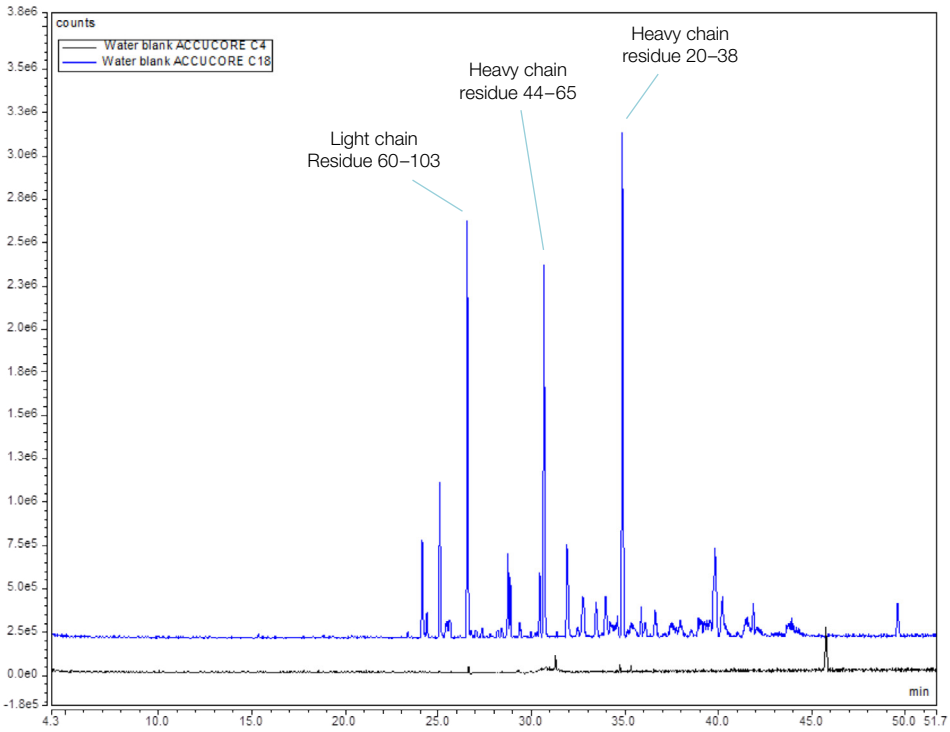

Figure 4. Base peak chromatograms obtained from runs with blank injections using the C4 (black) and C18 (blue) columns, each performed right after a series of injections of Project 3 mAb 2 digest sample. Identities of the three main peaks in the blue chromatogram were assigned via MS/MS.

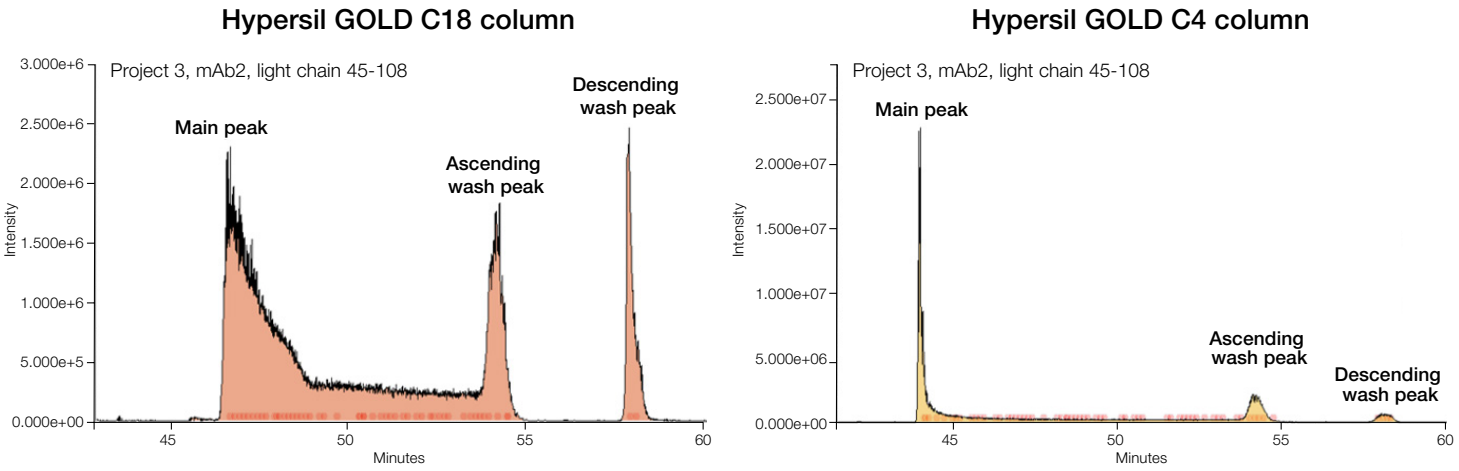

Figure 5. Project 3 mAb2 Extracted ion chromatograms (XICs) of the hydrophobic peptide (light chain 45–108).

Adjustment to the sample preparation based on LC observation

Early observation showed a time dependent loss of hydrophobic peptides in the 96 deep-well plate (Figure 6). Evaluation of different plastics did not solve the issue, but addition >2 M GuHCl effectively recovered the lost peptide as shown in Figure 6 bottom trace. Based on this observation it was decided to add 2 M GuHCl to the sample lane just after digestion, thus effectively preventing time dependent loss of hydrophobic peptides to the plastic surface.

To further evaluate the impact of GuHCl on hydrophobic peptides, it was assessed if peptides were sticking to any other surfaces that the sample is exposed to during the digestion procedure. These surfaces include the KingFisher plastic comb (used for sample mixing and transfer of magnetic beads) as well as the SMART Digest magnetic resin with immobilized trypsin. For investigation, a wash step in 8 M GuHCl was included in the KingFisher digestion program (see Digestion Protocols). After each of the two digestion steps, the SMART resin was collected from the sample lane and washed in 100 µL 8 M GuHCl in Lane F. During this procedure both the SMART Digest resin and the KingFisher plastic comb were exposed to 8 M GuHCl, ensuring release of peptides potentially sticking to the comb or SMART Resin.

As Figure 7 demonstrates based on a comparison of chromatograms, the additional wash steps with 8 M GuHCl results in increased signal intensities of some hydrophobic peptides. Consequently, the wash step in 8 M GuHCl was implemented within the kingfisher method, to ensure exposure of all relevant surfaces to GuHCl.

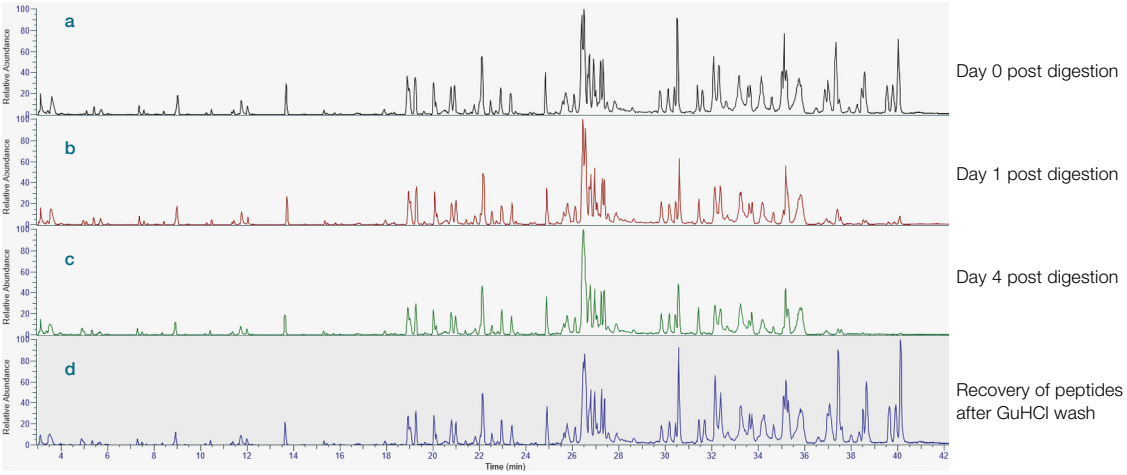

Figure 6. Time course experiment showing the loss of hydrophobic peptide over time (traces a,b,c) and recovery of hydrophobic peptides by addition of 4 M GuHCl (trace d).

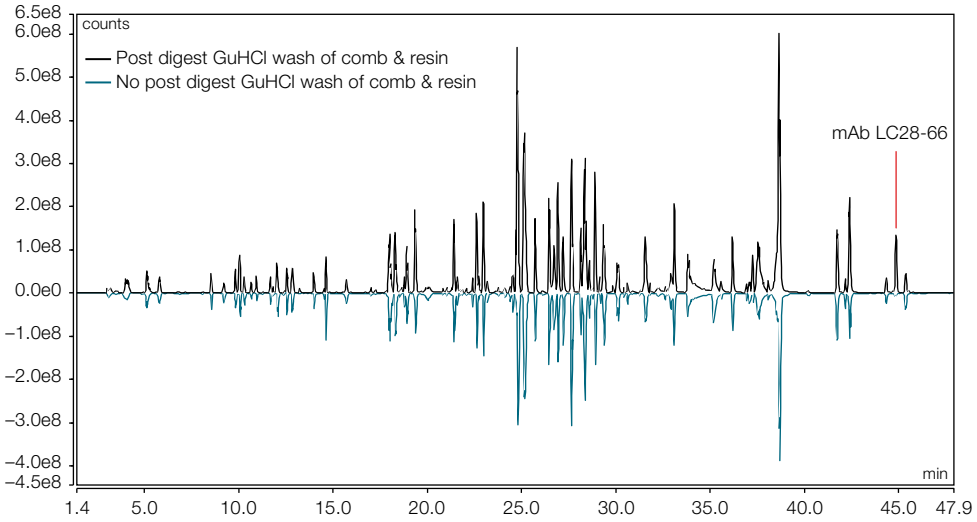

Figure 7. Comparison of base peak chromatograms obtained from procedures with (black trace) and without (teal trace) additional 8 M GuHCl wash steps. The strongest effect upon including the GuHCl wash step is observed for the late-eluting peptide mAb LC28-66 showing a significantly increased signal intensity. GuHCl wash step (top, black trace) versus much lower intensity of LC25-66 without the GuHCl wash (lower, teal trace).

Conclusion

Here, we developed an automated sample digestion protocol upon stepwise optimization of existing protocols. This protocol achieved a much-improved digestion performance resulting in a significant reduction of the level of missed cleavages. For chromatographic separation, we compared a C18 column chemistry—most commonly applied for peptide separations—with a C4 column chemistry which revealed to be the superior option with regard to improved peak shape and reduced carry-over.

Digestion protocol assessment

- Significant reduction of missed cleavages was observed with the optimized two-step SMART digest protocol described (60% with no missed cleavages compared to 32–34% for alternative protocols). This simplifies PTM monitoring as the modifications are present across fewer peaks due to more complete cleavage.

Column chemistry assessment

- C4 column significantly reduced peak tailing of hydrophobic peptides.
- Carry-over is reduced on C4 columns both within the chromatographic run, and injection to injection carry-over.

Reducing loss of hydrophobic peptides

- Addition of GuHCl wash step and GuHCl in samples improves recovery and in-solution stability of hydrophobic peptides.

References and acknowledgements

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