Optimized Multi-Attribute Method Workflow Addressing Missed Cleavages and Chromatographic Tailing/Carry-Over of Hydrophobic Peptides

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ABSTRACT: Peptide mapping by liquid chromatography mass spectrometry (LC-MS) and the related multi-attribute method (MAM) are well-established analytical tools for verification of the primary structure and mapping/quantitation of co- and post-translational modifications (PTMs) or product quality attributes in biopharmaceutical development. Proteolytic digestion is a key step in peptide mapping workflows, which traditionally is labor-intensive, involving multiple manual steps. Recently, simple high-temperature workflows with automatic digestion were introduced, which facilitate robustness and reproducibility across laboratories. Here, a modified workflow with an automatic digestion step is presented, which includes a two-step digestion at high and low temperatures, as opposed to the original one-step digestion at a high temperature. The new automatic digestion workflow significantly reduces the number of missed cleavages, obtaining a more complete digestion profile. In addition, we describe how chromatographic peak tailing and carry-over is dramatically reduced for hydrophobic peptides by switching from the traditional C18 reversed-phase (RP) column chemistry used for peptide mapping to a less retentive C4 column chemistry. No negative impact is observed on MS/MS-derived sequence coverage when switching to a C4 column chemistry. Overall, the new peptide mapping workflow significantly reduces the number of missed cleavages, yielding more robust and simple data interpretation, while providing dramatically reduced tailing and carry-over of hydrophobic peptides.

Peptide mapping by LC-MS and multi-attribute method (MAM) are well-established as some of the most powerful tools for characterization of biopharmaceutical product quality.1−3 It is now routinely used for verification of the primary structure as well as site-specific, quantitative evaluation of critical PTMs or quality attributes of biopharmaceutical products.4−7 The information provided by MS-based peptide mapping supports biopharmaceutical development across all development stages, ranging from lead selection, developability assessment, comparability studies, process support, and general product characterization.8−11 Among 80 Biologics License Applications (BLAs) submitted between 2000 and 2015, 79 were found to use MS to support product characterization.12 MAM is currently being applied primarily as a characterization tool to support monitoring of critical quality attributes (CQAs) during biopharmaceutical development. However, in recent years, MAM has advanced into current Good Manufacturing Practice (cGMP) environments, where it is used for release and stability testing of biopharmaceuticals.2,7,13 Conventional impurity assays are typically based on UV or fluorescence detection of product variants resolved at the intact or subunit level by appropriate chromatographic or electrophoretic techniques. The information provided by these assays is thus holistic, i.e., information is typically at the level of the intact molecule. This can be a challenge, given the large complexity of biopharmaceuticals, such as monoclonal antibodies, which can undergo a broad range of degradation pathways, including deamidation, isomerization, oxidation, disulfide scrambling, aggregation, and fragmentation.8 Consequently, multiple orthogonal impurity assays are generally required to capture different product variants when performing cGMP release and stability testing.14 In contrast, MAM provides site-specific (amino acid level) quantitative information on individual CQAs and, as indicated by the name, MAM can monitor a broad range of product variants or CQAs (e.g., deamidation, oxidation, isomerization, and glycoforms) in one assay, which otherwise would require multiple conventional impurity assays.2 Indeed, MAM has now been implemented for
cGMP testing of some biopharmaceutical products, replacing several conventional impurity assays. With the growing importance and adaptation of MAM, an industry-wide consortium has been formed to facilitate knowledge sharing on MS-related assays among the biopharma companies, technology providers, and regulatory agencies (www.mamconsortium.org).

With MAM being broadly adopted across the biopharmaceutical industry, there is a current need and push to implement more robust and simple MAM digestion workflows. The digestion step of conventional MAM workflows remains labor-intensive and involves preparation of multiple non-commercial solutions (some need to be prepared fresh) and several independent steps, such as denaturation with reduction, alkylation, quenching of alkylation, and buffer exchange, before eventually the digestion. The complexity of conventional MAM digestion workflows is not ideal with respect to robustness and transferability between laboratories, including transfer from development to cGMP environments. To address these challenges, semiautomated digestion workflows have been published recently. A key feature of these MAM digestion workflows is they are hands-free and rely on robotics for the digestion step. For instance, Millán-Martín et al. demonstrated good interlaboratory robustness and low levels of protocol-induced PTMs using trypsin immobilized on magnetic beads (SMART digest trypsin) and a small footprint affordable robot. In this workflow, samples are added to row A of a 96 deep-well plate containing a digestion buffer and reducing agent. The robot performs the digestion by transferring the magnetic trypsin beads to the sample well followed by mixing at elevated temperatures for 30 min. At the end of the digestion, the trypsin beads are removed by the robot, which also cools the samples post digestion. The deep-well plate is finally transferred directly to the autosampler of the LC-MS system and analyzed. In this one-step SMART digest workflow, trypsin beads, time, and temperature are precisely controlled by the robot, greatly facilitating robustness and method transfer between laboratories. Furthermore, all chemicals and solutions employed in this MAM workflow are standard, off-the-shelf products, with no solutions needing preparation prior to digestion. In the current manuscript, a modified version of the Millán-Martín protocol is introduced, which significantly reduces the level of missed cleavages. Using the same robot, an automatic two-step SMART digestion protocol is presented in which a short high-temperature digestion step is followed by a lower-temperature digestion step. While the first high-temperature digestion step in the new workflow ensures unfolding and initial digestion of mAbs, the second low-temperature digestion step ensures complete digestion and a significant reduction in the number of missed cleavages, as compared to the original one-step SMART digest workflow. The new protocol is applied to four commercial reference antibodies (NIST mAb, USP mAb1, USP mAb2, and USP mAb3) as well as three internal projects, and the results are compared to the original one-step SMART digest protocol as well as the conventional MAM protocol.

MAM workflows rely on good chromatographic performance to ensure high sequence coverage and robust quantitation of PTMs and quality attributes. A second topic covered by the current paper relates to mitigation of chromatographic tailing and carry-over of hydrophobic peptides from proteolytic digests of biopharmaceutical mAbs. In-house studies have repeatedly shown that some mAbs contain highly hydrophobic regions and/or few trypsin proteolytic cleavage sites in the complementarity-determining regions (CDRs). Endoproteinase Lys-C or trypsin digestion of these mAbs results in highly hydrophobic peptides with poor chromatographic performance on C18 columns due to excessive retentivity (unpublished data). In the current paper, alternative RP columns with lower retentivity were systematically evaluated, and it is demonstrated that the C4 RP columns can significantly reduce tailing and carry-over of hydrophobic peptides while maintaining complete LC-MS/MS sequence coverage and good selectivity for CQAs, such as deamidation.

**EXPERIMENTAL SECTION**

**Chemicals and Reagents.** The NIST mAb was purchased from The National Institute of Standards and Technology (NIST). USP mAb 001, USP mAb 002, and USP mAb 003 were obtained from USP (https://www.usp.org/) as part of USP mAb round-robin study. The remaining mAbs were produced internally at Symphogen. Thermo Scientific SMART digest kits and associated low pH digestion buffer were obtained from Thermo Fisher Scientific. UHPLC MS-grade water and acetonitrile were sourced from Fisher Scientific. MS-grade (IonHance) difluoracetic acid (DFA) was purchased from Waters. Tris(2-carboxyethyl)phosphine hydrochloride (0.5 M, TCEP) and guanidine-HCl (8 M, GuHCl) were obtained from Pierce. DL-Dithiothreitol and iodoacetamide were purchased from Sigma. Ultrapure 1 M Tris-HCl pH 8.0 was purchased from Invitrogen.

**Analytical Instrumentation and LC-MS Data Management.** LC-MS/MS was performed using a Thermo Scientific Vanquish Horizon UHPLC coupled to a Thermo Scientific Orbitrap Fusion Tribrid MS equipped with an Ion Max source and the HESI-II-probe. All data was acquired using Thermo Scientific XCalibur Version 4.4.16.14 and subsequently imported into Thermo Scientific Chromeleon CDS Enterprise version 7.2.10 ES. LC-MS/MS data stored in Chromeleon CDS were automatically synchronized to Protein Metrics Byosphere Enterprise. All MS data processing was performed in Byosphere Enterprise.

**Digestion Protocols.** Three MAM digestion protocols were performed to compare the current, optimized two-step SMART digest protocol head-to-head with the original one-step SMART digest protocol as well as a conventional MAM digestion protocol.

**Optimized Two-Step SMART Digestion Protocol.** Samples were digested in a Thermo Scientific KingFisher Duo Prime robot using Thermo Scientific SMART digest trypsin using Thermo Scientific BindIt software (version 4.0). Samples were mixed with SMART digest buffer (pH 6.5) and TCEP in row A of a KingFisher 96 deep-well plate. The final sample and TCEP concentration were 1 mg/mL and 5 mM, respectively. The final digestion volume was 200 μL. The first and second digestion steps were performed for 15 min at 75 °C and 30 min at 40 °C, respectively, unless otherwise indicated (Figure S1). After digestion, 20% TFA and 8 M GuHCl were added to the sample to a final concentration of 1% and 2 M, respectively. The 96 microwell plate was covered with a Rapid SliT Seal from BioChromato, mixed briefly on Eppendorf ThermoMixer and stored at −80 °C until the time of analysis.

**Original One-Step SMART Digestion Protocol.** Samples were digested in a Thermo Scientific KingFisher Duo Prime robot using Thermo Scientific SMART digest trypsin in row A of a KingFisher 96 deep-well plate. The final sample and TCEP concentration were 1 mg/mL and 5 mM, respectively. The final digestion volume was 200 μL. The first and second digestion steps were performed for 15 min at 75 °C and 30 min at 40 °C, respectively, unless otherwise indicated (Figure S1). After digestion, 20% TFA and 8 M GuHCl were added to the sample to a final concentration of 1% and 2 M, respectively. The 96 microwell plate was covered with a Rapid SliT Seal from BioChromato, mixed briefly on Eppendorf ThermoMixer and stored at −80 °C until the time of analysis.
using Thermo Scientific BindIt software (version 4.0). Samples were mixed with SMART digestion buffer (pH 6.5) and TCEP in row A of a KingFisher 96 deep-well plate. The final sample and TCEP concentrations were 1 mg/mL and 5 mM, respectively. The final digestion volume was 200 μL. Digestion was performed at 75 °C for 30 min (Figure S2). After

**Figure 1.** Base peak chromatograms (BPCs) of NIST mAb. The same scale is used in all three BPCs. Peaks marked with green and red dots represent peptides identified by MS/MS without or with missed cleavages, respectively. Peaks marked with black dots represent trypsin autolysis peptides identified by MS/MS. Examples of peptides identified in all three BPCs are marked with numbers. Smc: peptide 5 carrying a missed cleavage.

**Figure 2.** Venn diagram illustrating the overlap in peptides identified by MS/MS from the three different digestion workflows for eight mAbs (n = 7, one sample contained two mAbs). Thirty-five percent (210) of identified peptides are shared between the three digestion workflows.
digestion, 20% TFA and 8 M GuHCl were added to the samples to a final concentration of 1% and 2 M, respectively. The 96 microwell plate was covered with a Rapid Slit Seal from BioChromato, mixed briefly on Eppendorf ThermoMixer and stored at −80 °C until the time of analysis.

**Conventional MAM Digestion Protocol.** Sample digestion was performed essentially as described by Jakes et al., with the following exceptions. Alkylation was performed using iodoacetamide. Buffer exchange prior to digestion was performed using 0.5 mL of 7 K MWCO Zeba Spin desalting columns from Thermo Scientific. After digestion, the samples were stored at −80 °C until the time of analysis.

**LC-MS/MS Analysis.** Solvent A was 0.1% DFA in water. Solvent B was 95% acetonitrile with 5% water and 0.1% DFA. The LC method includes a gradient from 2 to 45% solvent B from 1 to 52 min and two wash steps, and this method was used throughout (Figure S3). Total run time was 70 min. A 20 μg sample load was used throughout. All samples were analyzed on C4 and C18 versions of Thermo Scientific Hypersil GOLD columns (2.1 × 150 mm², 2.6 μm particles) and Thermo Scientific Accucore columns (2.1 × 150 mm², 1.9 μm particles) to compare C4 and C18 columns of identical dimensions. New columns were acquired for this study. Each new column was conditioned by one blank run followed by a sample run (internal reference mAb) before acquiring data for the study. Identical sample sequences were analyzed sequentially on each of the four columns, i.e., all columns were new, conditioned identically, and exposed to the same samples throughout the entire study.

For Accucore and Hypersil GOLD columns, flowrates of 0.5 and 0.4 mL/min were used, respectively. Source settings were adjusted for the differences in flowrates (Table S1).

LC-MS/MS was performed using data-dependent acquisition (DDA) on an Orbitrap Fusion (Figure S4). Basically, MS was performed in the Orbitrap detector and MS/MS (EThcD and HCD) in the Ion Trap detector, and precursors were excluded for 7 s. LC-MS/MS was performed until the end of LC run to determine which peptides elute of the column during the wash steps.

**Data Processing.** All LC-MS/MS was processed in Byosphere Enterprise using the Byosphere Client Version 4.3. Peptide identification and processing were done using the parameters defined in Figure S5. Since no alkylation step is required in the SMART digest workflow, cysteine residues are searched as reduced forms. In contrast, iodoacetamide was used as an alkylation reagent in the conventional MAM digest workflow, and for those samples, carbamidomethyl was defined as a fixed modification for Cys residues (Table S2).

## RESULTS AND DISCUSSION

This section is divided into two sections covering (1) the optimized two-step SMART digestion workflow and (2) comparative evaluation of C4 and C18 RP columns, respectively.

**Optimized Two-Step SMART Digest Protocol.** SMART digest trypsin workflows originally involve digestion at elevated temperatures under reducing conditions (70–75 °C). Reduction is performed during digestion by adding 5 mM TCEP to the sample prior to digestion. TCEP effectively reduces disulfide linkages during digestion, and these remain reduced during LC-MS analysis due to the low pH of the LC solvents. Typically, for reduced samples > 99% of extracted ion current (XIC) area under the curve (AUC) are from free peptides, with close to 0% being from disulide bonded complexes (unpublished data). The elevated temperatures are required to denature sample proteins during digestion, since no chemical denaturation is done prior to SMART digestion. This is one of the key strengths of the SMART digest workflow; no sample denaturation, reduction, and alkylation are required prior to sample digestion, radically simplifying the digestion workflow. Internal studies have shown that SMART digest trypsin gradually becomes inactive at 75 °C and compared to conventional MAM digestion workflows, SMART digest workflows appeared to have higher levels of missed cleavages (data not shown). While some missed cleavages are commonly observed, it was hypothesized that elevated levels of missed cleavages in SMART digest trypsin most likely stemmed from the high temperature the digestion was performed at, as this may impact enzyme substrate specificity. Furthermore, the gradual inactivation of SMART digest trypsin at high temperatures would also play a part. Based on these considerations, it was decided to evaluate a two-step
SMART digest trypsin workflow in which the first step was performed at the usual high temperature, but for a shorter period, and the second step was performed at lower temperature using fresh SMART digest trypsin resin. The equipment and reagents used in the optimized two-step SMART digest workflow are identical to the original one-step SMART digest workflow. The only difference is that the SMART digest trypsin resin is added to two rows in the 96-well deep-well plate rather than one and that the KingFisher Duo robot performs two digestion steps instead of the usual one-step digestion (Figures S1 and S2).

Some initial experiments were performed using varying time for the first (75 °C) and second digestion (40 °C) steps (data not shown). Based on these experiments, it was decided to perform the first digestion step for 15 min at 75 °C and the second digestion step for 30 min at 40 °C. All data presented here are based on this two-step SMART digest workflow unless otherwise indicated.

A total of eight mAbs were analyzed in the current study. These were the NIST mAb, UPS mAb 001, UPS mAb 002, UPS mAb 003, and three internal Symphogen projects (Project 3 is a mixture of two mAbs). Samples were digested using the optimized two-step SMART digest trypsin workflow, the original one-step SMART digest trypsin workflow, as well as the conventional in-solution MAM digest workflow. Each digested sample was analyzed by LC-MS/MS on four different columns, two C4 columns and two C18 columns of the Accucore (solid core) and Hypersil GOLD (fully porous) brands.

Figure 1 shows base peak chromatograms (BPCs) of NIST mAb digested with the two-step SMART digest workflow, the original one-step SMART digest workflow, and the conventional MAM digest workflow.
tional MAM digest workflow. Overall signal intensities are comparable across the three digest workflows; however, the peak patterns vary substantially, although some peaks, as expected, are present in all digest workflows. In particular, the BPC of the original MAM digest workflow differs clearly from the SMART digest BPCs. This is to be expected as the original MAM digest workflow employs an alkylation step, which results in different chemical properties of all cysteine-containing peptides compared to the two SMART digest workflows (no alkylation step). Overall, fewer peaks are detected in the optimized two-step SMART digest workflow, resulting in a “cleaner” and simpler fingerprint. Fully cleaved peptides dominate the BPC of the two-step SMART digest workflow, and relatively few peptides with missed cleavages are observed. The latter tend to be of lower relative intensity. For

Figure 7. Venn diagram illustrating overlap in peptides identified by MS/MS for C4 and C18 columns (seven samples analyzed on two columns; n = 14). In total, 87% (289) of identified peptides are shared between C4 and C18 columns.

Figure 8. Average MS/MS sequence coverage for eight mAbs analyzed by three MAM digestion workflows (see Experimental Section) (n = 24). Eight mAbs were analyzed by three different digestion workflows, yielding a total of 3 × 8 datapoints for each column.

Figure 9. Base peak chromatograms of blank Accucore C4 (black) and C18 (blue) runs performed after USP mAb3 runs. Identity of the main USP mAb3 carry-over peptides identified by MS/MS is shown.

Table 1. Quantitation of USP mAb3 Carry-Over Peptides (Figure 9) in Blank Run Following Sample Run Using Different C4 and C18 Reversed-Phase Columns

| USP mAb3 peptide | Accucore C4 | Accucore C18 | Hypersil GOLD C4 | Hypersil GOLD C18 |
|------------------|-------------|-------------|-----------------|------------------|
| H20-38           | 0.06%       | 7.65%       | 0.06%           | 0.21%            |
| H44-65           | 0.21%       | 12.06%      | 0.09%           | 0.60%            |
| L60-103          | 0.08%       | 12.50%      | 0.06%           | 0.37%            |

*H = heavy chain; L = light chain.*

the original one-step SMART digest workflow and the conventional MAM digest workflow, a larger number of
peptides with missed cleavages are identified and they tend to be of higher relative signal intensity, as compared to the two-step SMART digest workflow. When looking at the eight analyzed mAbs, only 35% of the identified peptides are shared between all three digestion workflows, in line with the observed differences in chromatograms (Figure 2).

To further investigate the source of this difference in BPCs, the MS/MS results were evaluated, and the MS/MS sequence coverages are summarized in Figure 3. Overall, the most complete sequence coverage is seen for the original SMART digest workflow, which has a slightly higher coverage than the conventional MAM digest workflow, particularly for the heavy chain. The two-step SMART digest workflow has a consistently slightly lower sequence coverage than the other two workflows, which is due to a more complete digestion with fewer missed cleavages (Figure S6). A consequence of fewer missed cleavages is that small proteolytic peptides (typically four amino acids or less) are not identified, as they do not bind sufficiently to the RP columns or are below the lower m/z cutoff (350 m/z in the current paper). Figure S6 highlights some small tryptic NIST mAb peptides that are not identified by MS/MS in the optimized SMART digest workflow. While these fully cleaved peptides are not identified in the original SMART digest workflow either, peptides with missed cleavages cover the sequence region of the missing peptides. In summary, the slightly lower sequence coverage of the two-step SMART digest workflow is a consequence of a more complete digestion with fewer missed cleavages.

Figure 10. Extracted ion currents of hydrophobic peptide (light chain 45–108) from internal project at Symphogen. Digestion was performed with endoproteinase Lys-C. Each red tick at the base of the trace represents a positive identification by data-dependent MS/MS acquisition. Peptides eluting during the increasing and decreasing part of the wash step are indicated as ascending wash peak and descending wash peak, respectively (see Figure S3).

Table 2. Relative Quantitation of USP mAb3 Carry-Over Peptides (Figure 9) in the Main Peak and First Wash Step Using Different C4 and C18 Reversed-Phase Columns

| USP mAb3 peptide peak | Accucore C4 | Accucore C18 | Hypersil GOLD C4 | Hypersil GOLD C18 |
|-----------------------|------------|-------------|-----------------|-----------------|
| H20-38 main           | 99.0%      | 84.9%       | 99.3%           | 93.1%           |
| H20-38 wash           | 1.0%       | 15.1%       | 0.7%            | 6.9%            |
| H44-65 main           | 98.2%      | 73.5%       | 99.3%           | 88.5%           |
| H44-65 wash           | 1.8%       | 26.5%       | 0.7%            | 11.5%           |
| L60-103 main          | 98.6%      | 72.0%       | 99.3%           | 90.1%           |
| L60-103 wash          | 1.4%       | 28.0%       | 0.7%            | 9.9%            |

aH = heavy chain; L = light chain.

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complete digestion with fewer missed cleavages. The slight reduction in sequence coverage could in theory result in missed PTMs, something end users should pay attention to, particularly, if sequence coverage is missing in critical regions (such as CDRs).

Figure 4 illustrates the number of peptides identified by MS/MS and grouped according to the number of missed cleavages. From these results, it is clear that the optimized two-step SMART digest workflow results in a significant reduction in missed cleavages (see numerical data in Table S4). For instance, the percentage of peptides with 0 missed cleavages (blue 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 (for numbers, see Table S5). For peptides with more than two missed cleavages, the two-step SMART digest workflow results in an even more pronounced reduction in peptide count compared to the other digest workflows. In summary, significantly fewer peptides with missed cleavages are identified by MS/MS when using the two-step SMART digest workflow.

In MAM workflows, quantitation is done by comparing XIC AUCs of wildtype and modified peptides (oxidation, deamidation, glycosylation, etc.). To get an overview of the XIC AUC output of the different MAM digest workflows, the XIC AUCs of all peptides identified by MS/MS were summed and grouped into missed cleavages (Figure 5). The summed XIC AUCs for peptides with 0 missed cleavages were on average 89.2, 67.7, and 78.4% for the two-step SMART digest workflow, the one-step SMART digest workflow, and the conventional MAM digest workflow, respectively. These results confirm that the optimized SMART digest workflow results in more complete digestion and that fully cleaved peptides contribute to a larger proportion of total XIC AUC as compared to the other two workflows.

In summary, the two-step SMART digest workflow results in a significant reduction of missed cleavages compared to the original one-step SMART digest workflow as well as the conventional MAM digest workflow. On average, 89% of quantitative peptide signal intensity (XIC AUC) comes from fully cleaved peptides when using the two-step SMART digest workflow, compared to 68 and 78% for the original one-step SMART digest workflow and conventional MAM digest workflow, respectively. Quantitative reporting of PTMs will be simplified using the two-step SMART digest workflow since PTMs will predominantly be present in one peptide (0 missed cleavages) and not distributed over multiple peptides with varying numbers of missed cleavages. The two-step SMART digest workflow uses the same instrumentation and reagents (all available off-the-shelf) as the original SMART digest workflow. The extra digestion step is controlled by additions to the program on the digestion robot. Similar levels of four known stress-sensitive PTMs (in the NIST mAb constant region) were observed in all three digestion workflows, indicating that the optimized two-step workflow, as expected, does not stress mAb more than the other workflows (see Figure S8).

Optimized MAM Chromatographic Conditions. When developing C18-based peptide mapping methods using Lys-C endopeptidase or trypsin for identity testing at Symphogen, chromatographic challenges are sometimes observed with highly hydrophobic peptides derived from CDR spanning regions, which are rich in aromatic residues and/or few cleavage sites. These peptides are characterized by excessive tailing and carry-over between runs. For one project in particular, a large region spanning both CDRI and CDRII of the heavy chain was consistently missing in the chromatogram (data not shown). MS/MS analysis revealed this missing peptide eluted during the wash step of the LC gradient. Changing the LC gradient did not address the issue, as the involved peptide displayed excessive retentivity on a C18 column. Rather than testing alternative proteases, it was decided to evaluate RP columns with lower retentivity (C4 and C8) than the C18 chemistry, which is the de facto standard for peptide mapping in the industry. Initially, RP C8 and C4 columns were evaluated to address carry-over and tailing of hydrophobic peptides. Unexpectedly, all promising results were obtained primarily for the RP C4 columns, so it was decided to carry out a systematic head-to-head comparison of C4 and C18 RP columns for peptide mapping. This section summarizes the key findings from this study on RP C4 columns.

Figure 6 shows a head-to-head comparison of USP mAb3 base peak chromatograms (BPCs) using Hypersil GOLD C4 and C18 columns of identical dimensions using the same LC gradient. USP mAb3 was digested using the two-step SMART digest workflow. Overall, the chromatograms are similar for C4 and C18 columns, with respect to the number of chromatographic peaks and general peak pattern. This is in line with a large 87% overlap in the peptides identified by MS/MS on C4 and C18 columns (Figure 7). A loss of smaller hydrophilic peptides was anticipated from the use of C4 columns, but this assumption was not supported by the obtained chromatograms. To further investigate C4 column performance, the MS/MS sequence coverage was evaluated for the eight mAbs used in the comparison of the three different digestion workflows (Figure 8). No difference in MS/MS average sequence coverage was observed between C4 and C18 columns. This demonstrates that no negative impact is seen on MS/MS sequence coverage when switching to the C4 columns. The smaller and more hydrophilic peptides are retained on C4 columns and detected by LC-MS/MS to a similar extent as when using C18 columns.

We then compared the carry-over for C18 and C4 columns. Figure 9 shows two blank runs following USP mAb3 sample runs on the Accucore C4 and C18 columns, respectively. MS/MS-derived identity of the three major peaks in the C18 blank run are indicated. From Figure 9, it is visually apparent that significantly more carry-over is observed in the blank run using the C18 column. To determine numerical values for the carry-over, the XIC AUCs of the three indicated peptides were compared for the USP mAb3 sample runs and the subsequent blank runs. The results are summarized in Table 1. Generally, low levels of carry-over are observed for the C4 columns (≤0.21%), in contrast to the C18 columns, where up 12.5% carry-over is observed. The amount of carry-over varies between column brands, but more carry-over is consistently observed on the C18 columns.

To evaluate LC-MS behavior of the three indicated carry-over peptides, their MS profiles were evaluated across the full length of the LC-MS runs, including the wash steps. High-resolution accurate mass (HRAM) data-dependent acquisition (DDA) was also performed during the wash steps to confirm detection of the three carry-over peptides in the wash step. XICs of the three carry-over peptides are illustrated in Figure S7. The XICs all illustrate an interesting point, which is that the analyzed peptides “bleed” off the C18 column both during the ascending and descending parts of the wash step; these are
seen as discrete peaks with red tics (positive HRAM MS/MS identification) around $S_4$ and $S_8$ min (Figures 10 and S7). The presence of peptides in the ascending and descending parts of the wash could be due to resin swelling and shrinking during the rapid changes in solvent strength. These “bleeding” peptide peaks are significantly reduced or absent on the C4 columns (Figures 10 and S7). The relative XIC AUC of the main peak and the first wash step were determined for the three peptides (Table 2). The relative XIC AUC of the first wash step is significantly lower on the C4 columns (0.7–1.4%) than on the C18 (6.9–28%). In summary, when using C4 columns, significantly less carry-over is seen in blank runs following a sample run and significantly less bleeding is seen in the wash step of a sample run. When inspecting the XIC traces in detail, more tailing is observed for the main peptide peaks using C18 column. This reflects a general tendency of reduced peak tailing and more symmetrical peaks observed when using C4 columns.

All data generated in the current study was based on trypsin digestion. When using proteases that result in larger, more hydrophobic peptides (e.g., endoproteinase Lys-C), a more pronounced advantage can be expected when using the C4 columns described in the current study. This is illustrated in Figure 10, which shows one of the original observations that triggered the current study. An internal project was digested with endoproteinase Lys-C and analyzed by LC-MS using a Hypersil GOLD C18 column. A critical peptide (light chain C4 columns, a radical reduction in tailing and wash step of a sample run. When inspecting the XIC traces in detail, more tailing is observed for the main peptide peaks using C18 column. This reflects a general tendency of reduced peak tailing and more symmetrical peaks observed when using C4 columns.

A significant reduction in missed cleavages was obtained using an optimized two-step SMART digest workflow. On average, 89, 68, and 78% of the total peptide signal intensity (XIC AUC) was derived from fully cleaved peptides in the optimized two-step SMART workflow, the original one-step SMART workflow, and conventional MAM workflow, respectively. For the number of identified peptides, the relative distribution of peptides with no missed cleavages was 60, 32, and 34% for the optimized two-step SMART workflow, the original SMART workflow, and conventional MAM workflow, respectively. The significant reduction in missed cleavages resulting from the two-step SMART digest workflow results in simpler data interpretation and reporting, as more PTMs or quality attributes only will be present on the peptides (no missed cleavages), rather than distributed over multiple peptides derived from different missed cleavages. The digestion procedure is simple and automated, with no requirement for manually prepared reagents, which results in very high reproducibility. This is an important factor when transferring methods to cGMP environments. This should also enhance the ease of deployment of new peak detection if implemented during MAM analysis.

Switching from C18 to C4 reversed-phase columns resulted in significant reduction of hydrophobic peptide bleeding in chromatographic wash steps, as well as significant reduction of hydrophobic peptide carry-over to subsequent runs. Generally, less tailing and more symmetrical peaks were obtained when switching to C4 column chemistries. Average MS/MS sequence coverages for eight mAbs did not change when switching from C18 to C4 column chemistries. Concerns were raised internally about potential loss of smaller hydrophilic peptides when using columns of lower retentivity, but as demonstrated in the current study, this concern was unfounded. Consequently, it has been decided to transition to a C4 reversed-phase column chemistry for all peptide mapping and MAM workflows using LC-MS internally at Symphogen.

CONCLUSIONS
A significant reduction in missed cleavages was obtained using an optimized two-step SMART digest workflow. On average, 89, 68, and 78% of the total peptide signal intensity (XIC AUC) was derived from fully cleaved peptides in the optimized two-step SMART workflow, the original one-step SMART workflow, and conventional MAM workflow, respectively. For the number of identified peptides, the relative distribution of peptides with no missed cleavages was 60, 32, and 34% for the optimized two-step SMART workflow, the original SMART workflow, and conventional MAM workflow, respectively. The significant reduction in missed cleavages resulting from the two-step SMART digest workflow results in simpler data interpretation and reporting, as more PTMs or quality attributes only will be present on the peptides (no missed cleavages), rather than distributed over multiple peptides derived from different missed cleavages. The digestion procedure is simple and automated, with no requirement for manually prepared reagents, which results in very high reproducibility. This is an important factor when transferring methods to cGMP environments. This should also enhance the ease of deployment of new peak detection if implemented during MAM analysis.

Switching from C18 to C4 reversed-phase columns resulted in significant reduction of hydrophobic peptide bleeding in chromatographic wash steps, as well as significant reduction of hydrophobic peptide carry-over to subsequent runs. Generally, less tailing and more symmetrical peaks were obtained when switching to C4 column chemistries. Average MS/MS sequence coverages for eight mAbs did not change when switching from C18 to C4 column chemistries. Concerns were raised internally about potential loss of smaller hydrophilic peptides when using columns of lower retentivity, but as demonstrated in the current study, this concern was unfounded. Consequently, it has been decided to transition to a C4 reversed-phase column chemistry for all peptide mapping and MAM workflows using LC-MS internally at Symphogen.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c03820.

KingFisher Duo Prime program for the optimized two-step SMART digest workflow (Figure S1); KingFisher Duo Prime program for the original one-step SMART digest workflow (Figure S2); LC gradient used throughout the current study for LC-MS analysis (Figure S3); Orbitrap Fusion MS method settings used in the current study (Figure S4); Byosphere settings for processing MS data in the current study (Figure S5); sequence coverage map of NIST mAb light chain using original one-step SMART digest workflow and the optimized two-step SMART digest workflow (Figure S6); extracted ion currents of major USP mAb3 peptides on Accucore C4 and C18 reversed-phase columns (Figure S7); levels of four known post-translational modifications from the constant region of the NIST mAb (Figure S8); LC-MS source settings for Accucore and Hypersil GOLD columns (Table S1); Byosphere modification settings for the different digestion workflows (Table S2); MS/MS sequence coverage determined by Byosphere Enterprise (Table S3); peptide count for each of the employed digestion workflows grouped into the number of missed cleavages (Table S14); and relative peptide count for different MAM digest workflows (Table S5) (PDF)

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Notes
The authors declare no competing financial interest.

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