

INTRODUCTION & MOTIVATION

Multi-Attribute Method (MAM) workflows enable simultaneous assessment of biotherapeutic identity, purity, and post-translational modifications (PTMs). They have become for peptide mapping and monitoring of critical quality attributes (CQAs). In such workflows, sample preparation is often the largest source of variability where a wide variety of contaminants must be reproducibly removed and enzymatic processing enabled without introducing artifactual changes in samples.

ProtiFi's Multi-Attribute Method Kit standardizes MAM sample preparation for samples from development through final quality control (QC). Its general-purpose approach eliminates the need for product-specific method development and is designed for use across multiple products and laboratories. It efficiently removes a broad range of contaminants including detergents, salts, buffers, stabilizers, excipients, and related interferents, and minimizes or prevents artifactual deamidation and oxidation. Compared with standard published workflows, the kit delivers 14.4-fold higher monoclonal antibody (mAb) protein ion current, more than 35-fold more direct expected host-cell protein (HCP) peptide identifications, and a lower burden of non-antibody signal to give high-confidence analysis even with challenging matrices.

Here, we compare the published National Institute for Bioprocessing Research and Training (NIBRT) MAM1 sample preparation workflow with the ProtiFi Multi-Attribute Method Kit workflow using NISTmAb 8671 challenged with 41 bioprocess-relevant contaminants. These common foulants can compromise digestion efficiency, chromatographic performance, and mass spectrometric detection, all of which can reduce the reliability and quality of MAM data. We systematically evaluated both workflows for robustness, contaminant removal, peptide coverage, PTM preservation, reproducibility, and overall suitability for peptide mapping-based MAM under contaminated sample conditions.



Fig. 1
The 96-well Multi-Attribute Method (MAM) Mini Plate Kit standardizes the rapid analysis of biological medicines at every stage of manufacturing, eliminating the need for protocol development. The kit fully removes a broad range of contaminants including detergents, salts, buffers, stabilizers, excipients and others for samples of 10 µg – 250 µg of biological drug sample. The kit minimizes or eliminates deamidation and chemical artifact formation.

METHODS

Matched NISTmAb 8671 samples were prepared using either the published NIBRT MAM workflow or the ProtiFi Multi-Attribute Method Kit protocol, with or without 41 foulants commonly encountered in biologics manufacturing, including salts, buffers, surfactants, stabilizers, and other process-related contaminants. The workflows differ in denaturation, cleanup, and digestion strategy. The NIBRT workflow uses chaotropic denaturation, size-exclusion desalting, and in-solution digestion, whereas the ProtiFi workflow uses detergent-based denaturation, optional reduction and alkylation, protein trapping and washing, and in-plate digestion in TruPROTTM Solution to reduce chemical artifacts such as deamidation and oxidation. Workflow performance was evaluated by LC-MS for foulant removal, sample cleanliness, sequence coverage, reproducibility, robustness, and product quality attribute characterization.

NISTmAb tryptic digests were analyzed by nano-flow LC-MS/MS using a Dionex UltiMate 3000 RSLCnano coupled to a Q Exactive HF Orbitrap mass spectrometer via a Nanospray Flex source. Peptides were separated on a PepMax XHP1 C18P column using 0.1% formic acid in water and acetonitrile with 0.1% formic acid. Unless otherwise stated, 150 ng of peptide was injected and separated over a 75-minute gradient from 1% to 80% mobile phase B at 350 nL/min, with MS acquisition from 5 to 75 minutes.

Data-dependent MS/MS was performed in positive ion mode. Full MS scans were acquired at 60,000 resolution over m/z 200–2,000 with an AGC target of 3×10^6 . Precursors were isolated with a 1.8 m/z window and fragmented by HCD at 27% normalized collision energy. MS² scans were acquired at 30,000 resolution with an AGC target of 2×10^5 , a maximum injection time of 120 ms, and a fixed first mass of 150 m/z.

Peptide identification and PQA characterization were performed in BioPharma Finder 5.4 using a modified two-phase MAM workflow. Discovery searches used the NISTmAb sequence with trypsin specificity and a 1.0×10^5 count MS signal threshold. Fixed modifications were carboxymethylation of cysteine for NIBRT samples and methyl thiolation for MAM Kit samples. Variable modifications included deamidation, oxidation, succinimide formation, N-terminal pyroglutamate formation, C-terminal lysine clipping, and lysine glycation. Results were filtered to include peptides with up to two missed cleavages while excluding adducts and gas phase-generated ions; components with nonspecific modifications were retained. A target peptide workbook was generated from the filtered component list. Host cell proteins were detected using Mascot.

To improve sequence coverage, a proteolytic NISTmAb peptide ladder was generated using the ProtiFi MAM Kit with trypsin and Tryp-N at protease-to-protein ratios of 1:200, 1:100, 1:50, 1:25, and 1:10. Samples were incubated for 1 hour at 37°C for trypsin or 55°C for Tryp-N, then pooled and analyzed by LC-MS/MS.

RESULTS

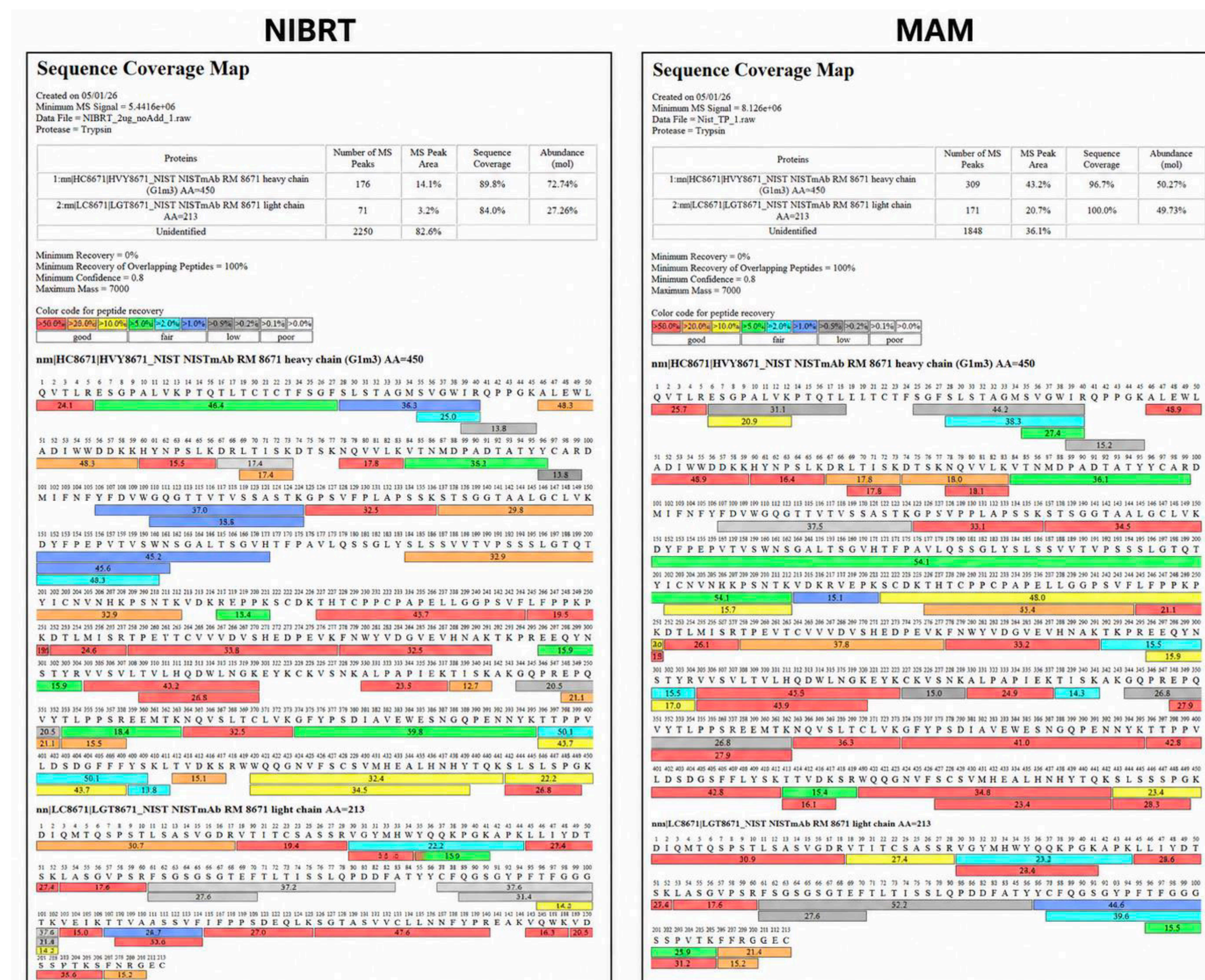


Fig. 2
Sequence coverage analysis of NISTmAb tryptic peptides. Sequence coverage was determined using Thermo Scientific BioPharma Finder 5.4. Data were collected using acquisition parameters adapted from Millán-Martín et al., with DDA data acquisition. Nist mAb tryptic digests were prepared either using the NIBRT protocol, which has 89.8% sequence coverage of the heavy chain and 84% for the light chain, or the MAM kit, yielding 96.7% coverage for the heavy chain a 100% for the light.

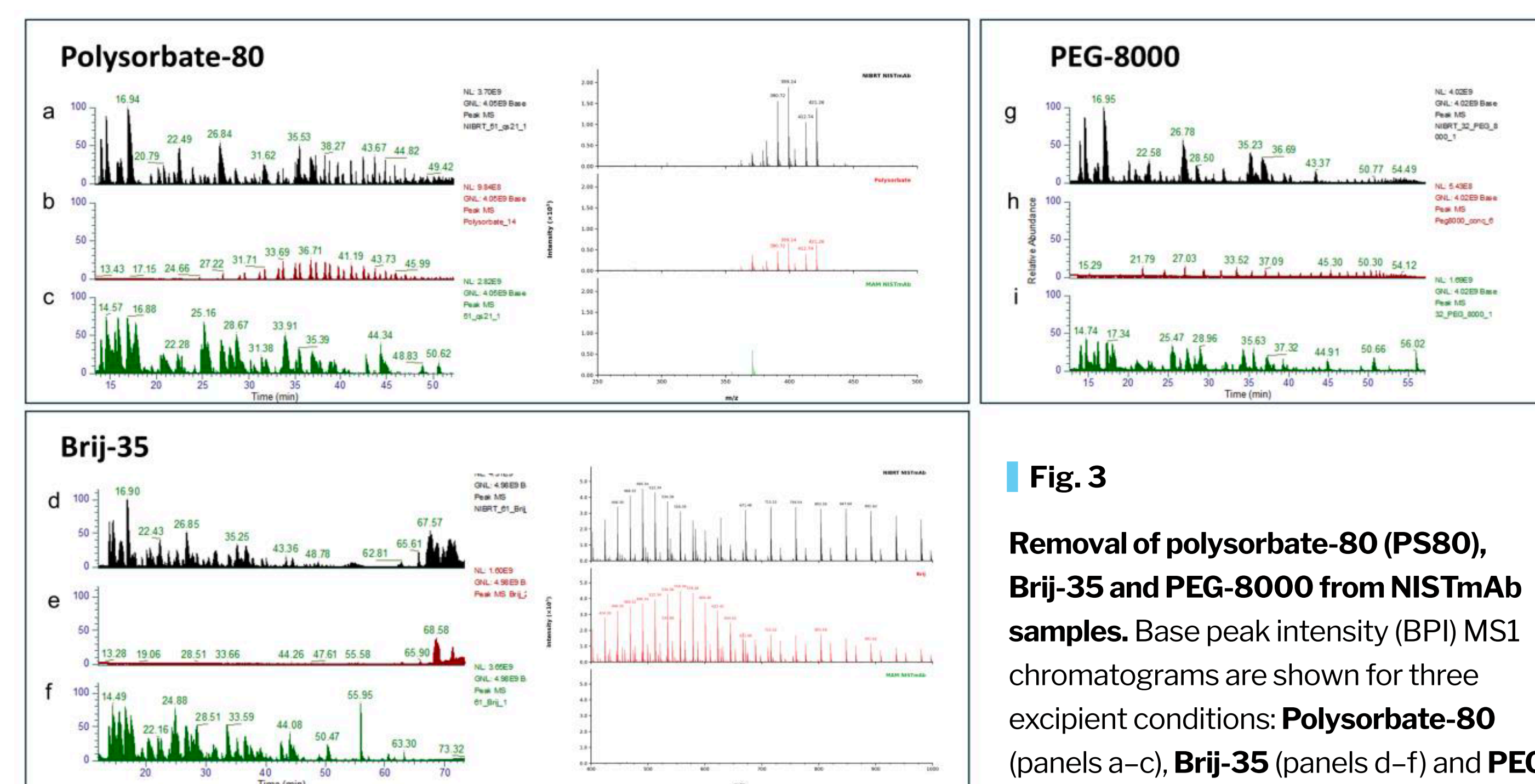


Fig. 3
Removal of polysorbate-80 (PS80), Brij-35 and PEG-8000 from NISTmAb samples. Base peak intensity (BPI) MS1 chromatograms are shown for three excipient conditions: **Polysorbate-80** (panels a–c), **Brij-35** (panels d–f) and **PEG-8000** (panels g–i) and corresponding averaged full-scan MS1 spectra are shown for Polysorbate-80 and Brij-35. For each condition, chromatograms are displayed for **(top, black)** NIBRT prepared NISTmAb digest, **(middle, red)** excipient alone, and **(bottom, green)** ProtiFi MAM kit prepared NISTmAb digest. The MS1 spectra displayed between 13.5–53 min (Polysorbate-80 and PEG-8000) or 13.5–73 min (Brij-35) to capture the full elution range of each excipient. Averaged MS1 spectra were generated by averaging all full-scan spectra acquired within defined retention time windows corresponding to peak excipient elution: 40.5–43.0 min (Polysorbate-80, m/z 250–500), and 68–69 min (Brij-35, m/z 400–1000). Labeled peaks in the NIBRT NISTmAb and excipient spectra indicate shared m/z values, confirming excipient carry-over or co-elution in the NISTmAb formulation samples. All spectra were visualised and exported using Thermo Scientific FreeStyle.

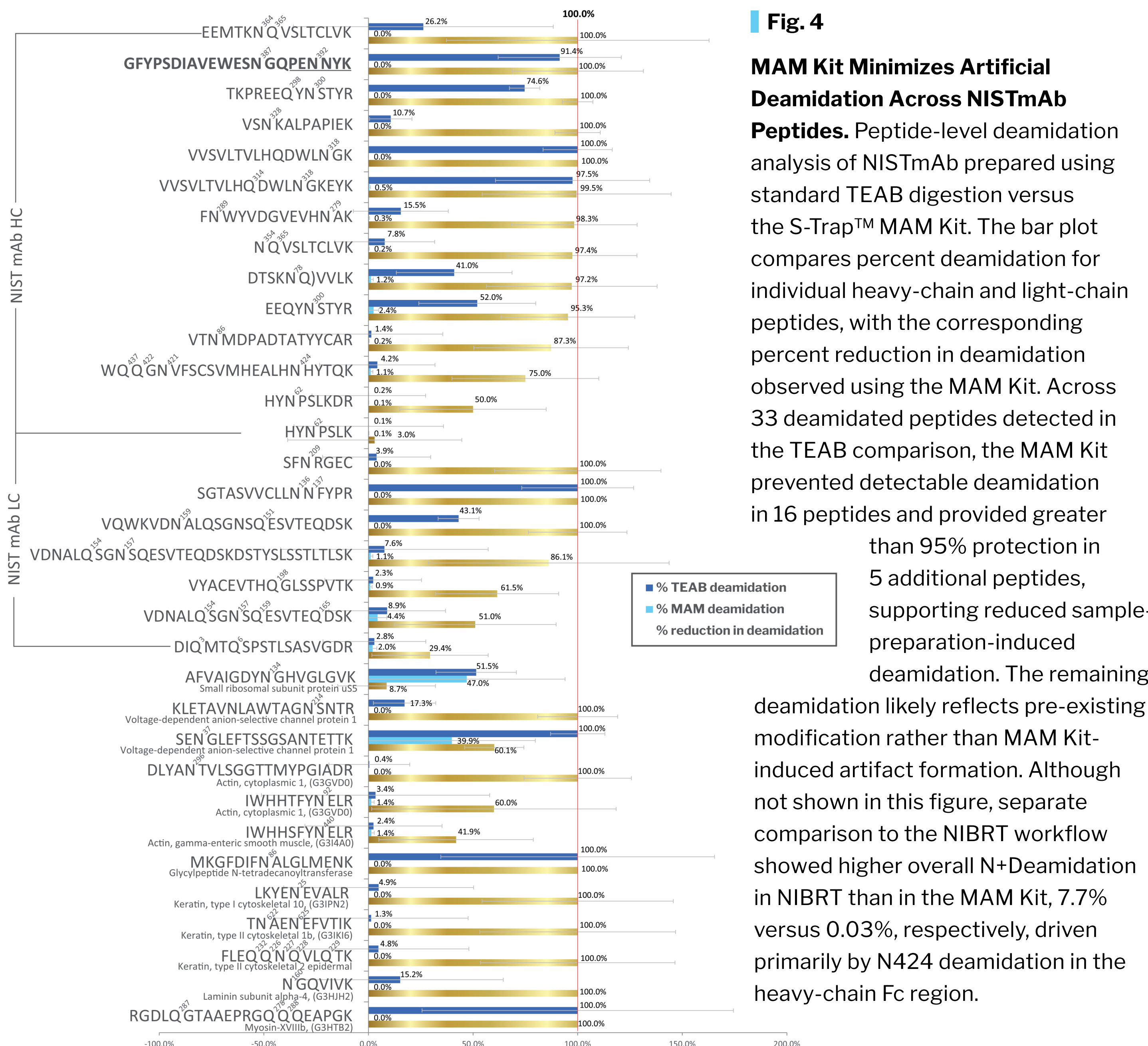


Fig. 4
MAM Kit Minimizes Artificial Deamidation Across NISTmAb Peptides. Peptide-level deamidation analysis of NISTmAb prepared using standard TEAB digestion versus the S-TrapTM MAM Kit. The bar plot compares percent deamidation for individual heavy-chain and light-chain peptides, with the corresponding percent reduction in deamidation observed using the MAM Kit. Across 33 deamidated peptides detected in the TEAB comparison, the MAM Kit prevented detectable deamidation in 16 peptides and provided greater than 95% protection in 5 additional peptides, supporting reduced sample-preparation-induced deamidation. The remaining deamidation likely reflects pre-existing modification rather than MAM Kit-induced artifact formation. Although not shown in this figure, separate comparison to the NIBRT workflow showed higher overall N+Deamidation in NIBRT than in the MAM Kit, 7.7% versus 0.03%, respectively, driven primarily by N424 deamidation in the heavy-chain Fc region.

RESULTS (CONT.)

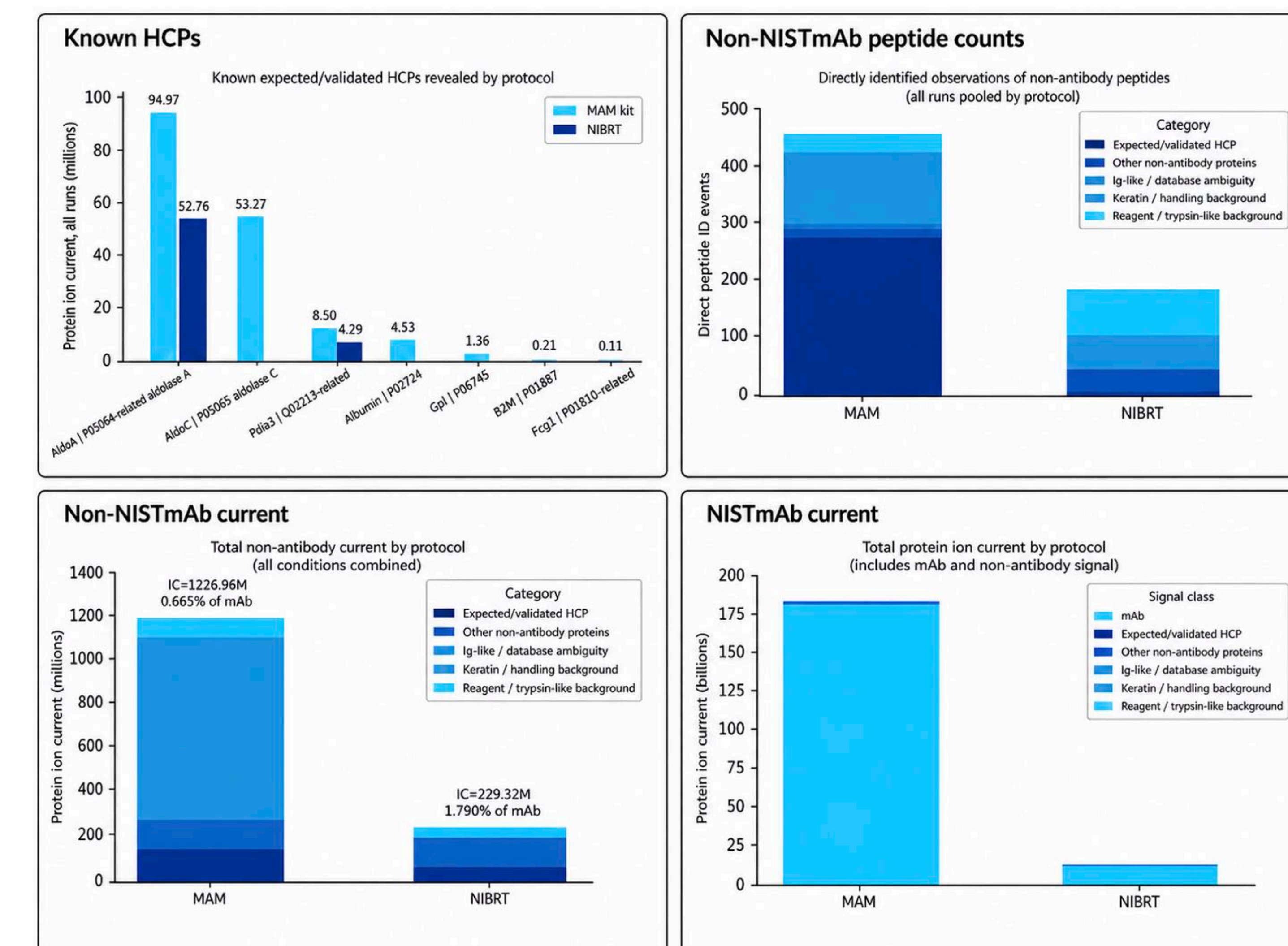


Fig. 5
Comparison of NISTmAb recovery and host cell protein (HCP) background between sample preparation methods. The NISTmAb, expressed in murine suspension cell culture, was analyzed by Mascot database searching against a combined NISTmAb and mouse proteome. The MAM kit yielded a higher total protein ion current and enabled detection of a greater number of expected/validated HCPs and non-antibody peptides compared to the NIBRT protocol. Despite the increased absolute non-antibody signal, the relative contribution of non-NISTmAb signal remained lower for the MAM kit (0.665%) than for NIBRT (1.790%). This indicates improved antibody recovery, proportionally reduced background interference and more sensitive HCP detection using the MAM workflow.

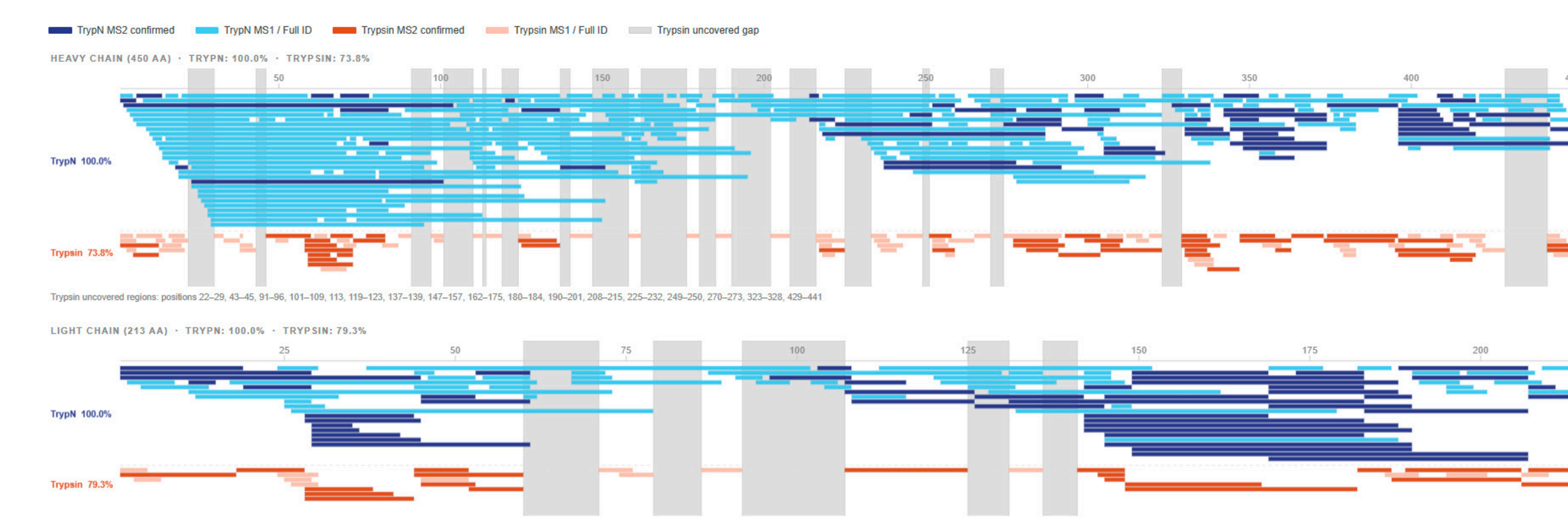


Fig. 6
NISTmAb ladder peptides from trypsin and Tryp-NTM. NISTmAb RM 8671 was digested with either Tryp-NTM or trypsin, analyzed by LC-MS/MS and peptide sequence coverage was mapped with Thermo BioPharma Finder. Tryp-N provided complete sequence coverage of both the heavy and light chains, reaching 100% coverage for each chain, while Trypsin produced lower overall coverage, with 73.8% heavy-chain coverage and 79.3% light-chain coverage. Gray regions indicate sequence positions not covered by tryptic peptides, highlighting gaps that were resolved by Tryp-N digestion. Overall, Tryp-N generated broader and more continuous NISTmAb sequence coverage than trypsin.

CONCLUSION

The Multi-Attribute Method (MAM) Kit improved NISTmAb 8671 peptide mapping relative to the National Institute for Bioprocessing Research and Training (NIBRT) workflow by increasing sequence coverage, enhancing protein recovery, and providing more effective contaminant removal. Under foulant-challenged conditions, the kit removed all tested sample preparation interferents, including even large PEG 8000, Brij-35 and extremely common polysorbate-80 (PS80), which can otherwise easily compromise LCMS performance and data quality. The MAM Kit showed significantly increased sensitivity in detection of low-abundance non-monoclonal signals like host cell proteins (HCPs) as well as enhanced NISTmAb signal. Incorporation of Tryp-NTM ladder digestion enabled complete heavy- and light-chain sequence coverage, complementing trypsin-based peptide mapping. Together, these results support the ProtiFi MAM Kit is a robust, standardized workflow for biotherapeutic characterization, including sequence coverage, HCP assessment, foulant removal, and peptide-level product quality attribute (PQA) monitoring.

REFERENCES

1. Millán-Martín, S., Jakes, C., Carillo, S., Rogers, R., Ren, D., & Bones, J. (2023). Comprehensive multi-attribute method workflow for biotherapeutic characterization and current good manufacturing practices testing. *Nature Protocols*, 18(4), 1056-1089.