Enhanced HCP quantitation LC-MS/MS workflow solution using a MS sample prep method

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Abstract

Purpose: The research aims to enhance HCP quantitation in biotherapeutics using an improved MS workflow with protein A depletion and Thermo Scientific™ HeavyPeptide™ AQUA standards.

Methods: Samples of rituximab and trastuzumab were prepared using the protein A-depleted Thermo Scientific™ EasyPep™ method and native digestion, followed by LC-MS/MS analysis to quantify 28 high-risk HCPs.

Results: The protein A-depleted EasyPep method identified significantly more HCPs than native digestion, demonstrating superior performance in HCP detection and quantitation.

Introduction

Host cell proteins (HCPs) are a complex array of impurities found in biotherapeutics that can compromise the safety of biologic drug products. Monitoring and controlling these contaminant proteins during biologics production is crucial. While ELISA is the established method for detecting HCPs, its effectiveness depends on antibody affinity, potentially missing non-immunogenic HCPs and only providing collective identification. In contrast, LC-MS/MS can identify and quantify individual HCPs, offering a significant advantage as an orthogonal method[1].

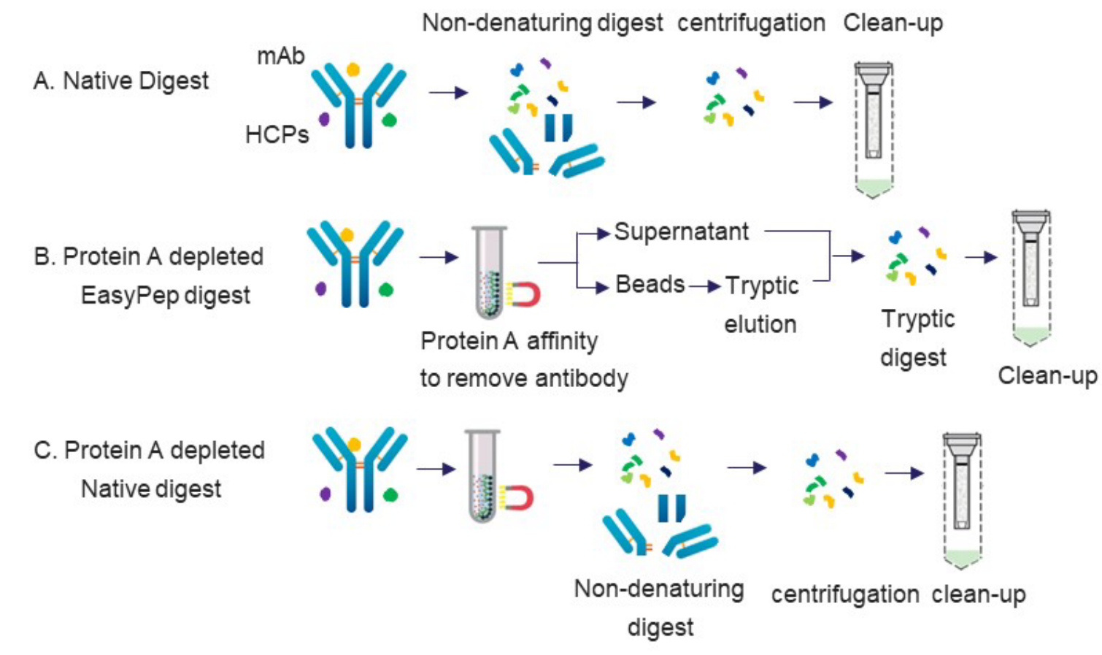
We have developed an enhanced EasyPep sample preparation method that incorporates protein A-based depletion and heavy-labeled peptide standards for HCP detection and quantitation. A key challenge with this method is the potential loss of low abundant HCPs during depletion due to non-specific interactions with the biologic drug or the depletion resin. To mitigate this issue, we developed a "trypsin elution" protocol, which uses trypsin to partially digest and recover HCPs from the depleted sample. Additionally, we have created a targeted MS assay to monitor 28 critical HCPs[1] from CHO cell lines, optimizing the MS platform's acquisition efficiency. This novel approach enables effective monitoring of HCPs throughout the downstream processes

Materials and methods

Sample Preparation

We used 1 mg of downstream process intermediate or UF/DF, equivalent to the drug substance. We employed Thermo Scientific™ Pierce™ high-capacity protein A MagBeads for antibody depletion before the EasyPep digest and compared it with native digestion with and without protein A depletion.

Figure 1. Sample preparation workflows



LC-MS/MS and data analysis

We developed a CHO critical HCP AQUA peptide panel to quantify 28 high-risk HCPs. Two hundred fmol of internal standard peptide mixture was added to 0.5µg of digested sample. Data acquisition was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System coupled with a Thermo Scientific™ Q Exactive Plus™ Mass Spectrometer, and raw files were analyzed with Thermo Scientific™ Proteome Discoverer™ 3.0 Software or Skyline software.

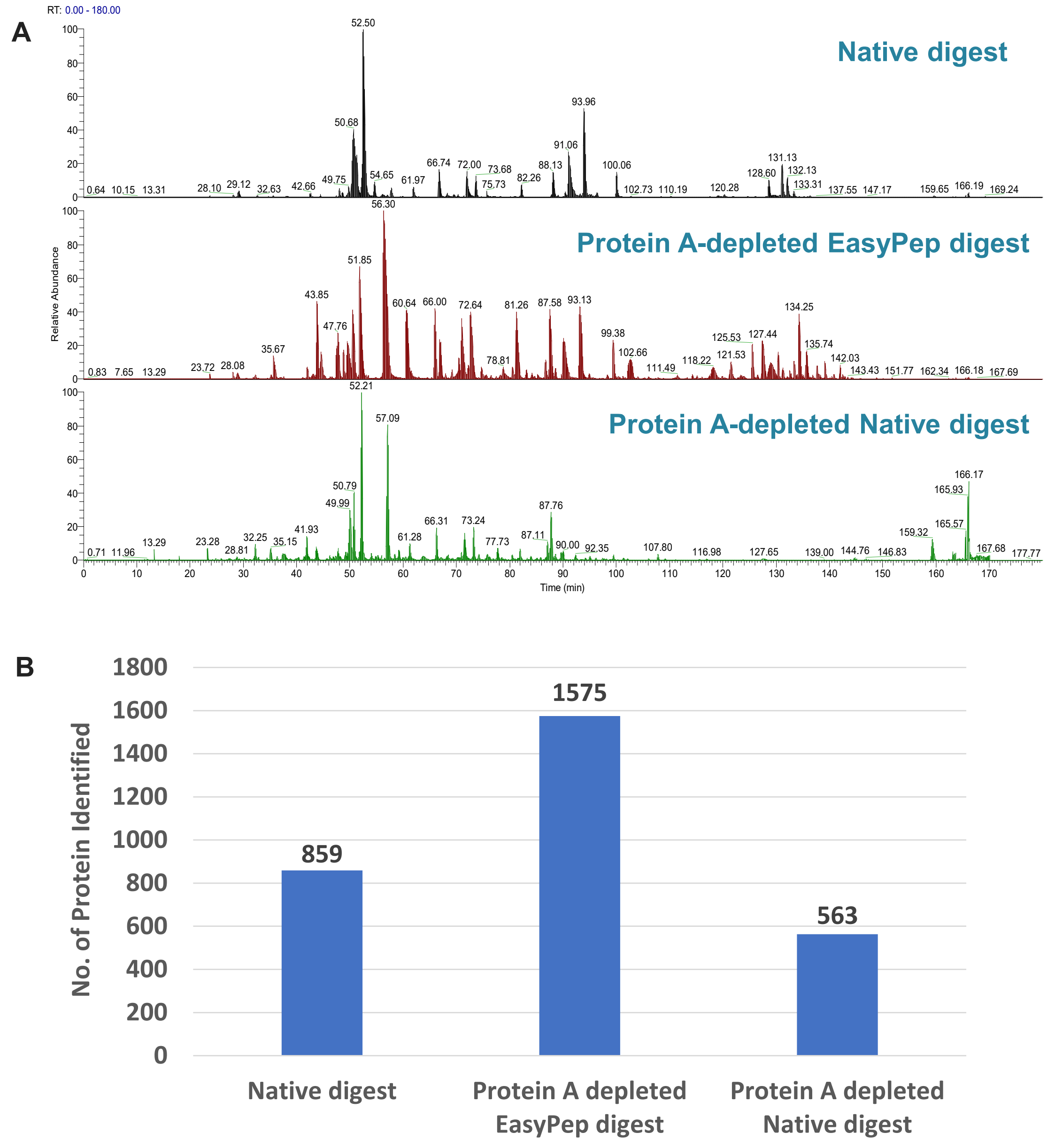
Results

Comparison of various sample preparation techniques for HCP analysis

We began with 1 mg of trastuzumab that had been purified using an affinity column. For antibody depletion, we utilized Pierce high-capacity protein A MagBeads prior to performing the EasyPep digest (Figure 1B). This method was compared against native digestion both with and without protein A depletion (Figures 1A and 1C).

Figure 2A illustrates the Base Peak Chromatogram (BPC) profiles for three trastuzumab samples prepared using different methods. The chromatograms in the upper, middle, and lower panels represent the native digest, protein A-depleted EasyPep digest, and protein A-depleted native digest samples, respectively. For each analysis, 4 µL (0.5 µg) of the digested sample was injected onto the column. Data analysis indicated that the protein A-depleted EasyPep digest sample (Figure 2B) identified the highest number of HCPs, with 1575 HCPs detected. This was significantly higher compared to the native digest sample, which identified 859 HCPs, and the protein A-depleted native digest sample, which identified 563 HCPs. Our findings suggest that both protein A depletion and semi-tryptic elution from the beads are effective in minimizing dynamic range differences and enhancing the recovery of HCPs from the beads, thus improving HCP identification. Moreover, the protein A-depleted EasyPep digest sample yielded the highest number of unique HCPs among the three sample preparation methods.

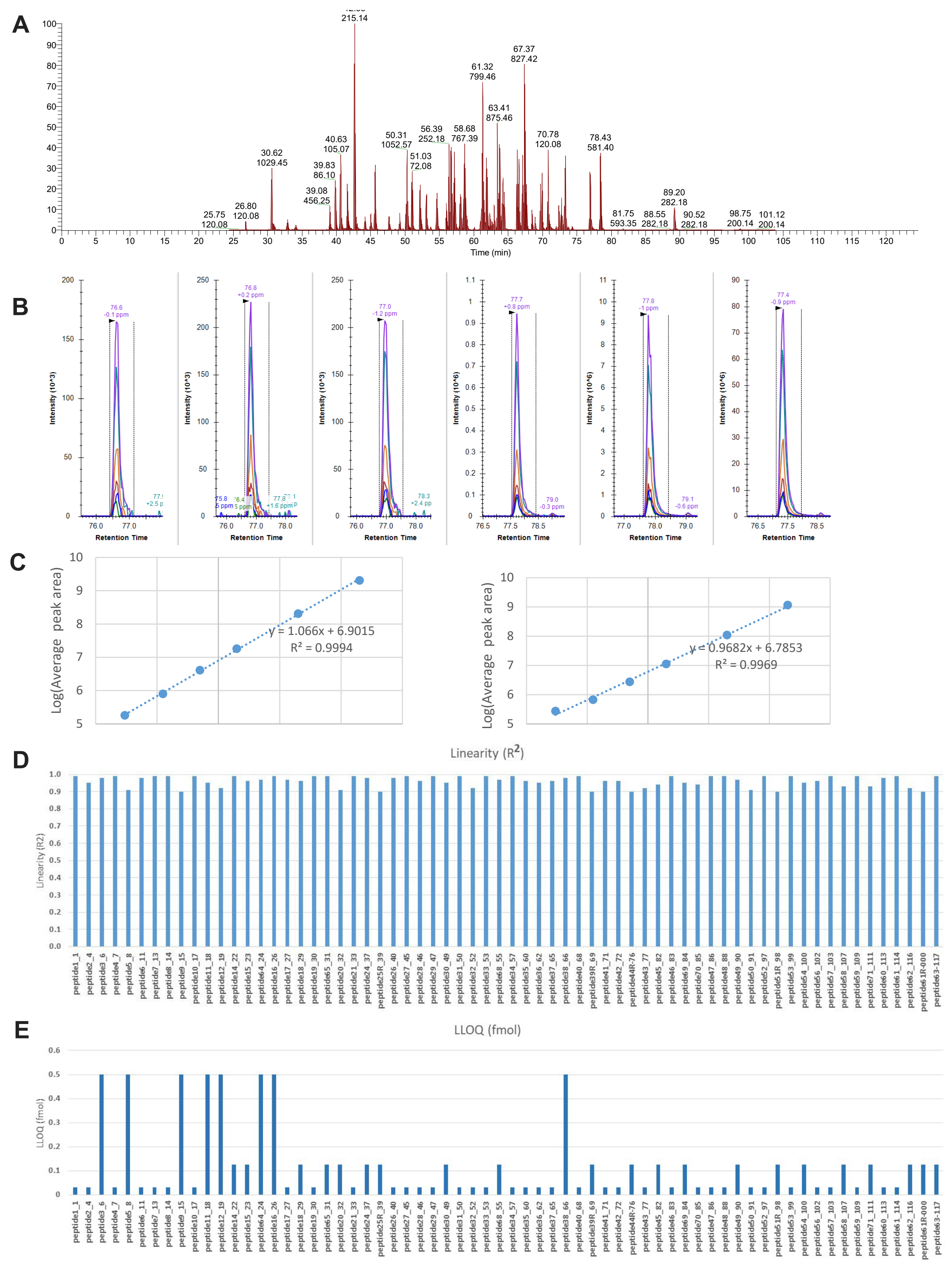
Figure 2. HCP results of trastuzumab prepared using three different sample prep methods. A. BPC profiles of three samples, B. The number of HCPs identified from three different methods.



Linearity and LLOQ

To assess the suitability of HeavyPeptide™ AQUA standards for targeted HCP quantitation, we first performed PRM analysis for peptide quantitation by evaluating the linearity, range, and LLOQ. Figure 3A displays a sample chromatogram in PRM mode for a mixture of 65 heavy peptides. We analyzed the range and linearity for each heavy peptide by preparing six-point dilutions at concentrations ranging from 0.03 fmol to 200 fmol. Linearity was defined as the concentration range for each peptide that produced a linear fit with an R^2 value of at least 0.95. The LLOQ was identified as the lowest concentration that met the linearity acceptance criteria. Figures 3B and 3C show the representative PRM peak area of a peptide in Skyline and the linearity plot for selected peptides. The linearity R^2 value and LLOQ for the entire heavy peptide panel are presented in Figures 3D and 3E, demonstrating excellent linearity and a 0.03 fmol LLOQ for the majority of heavy peptides.

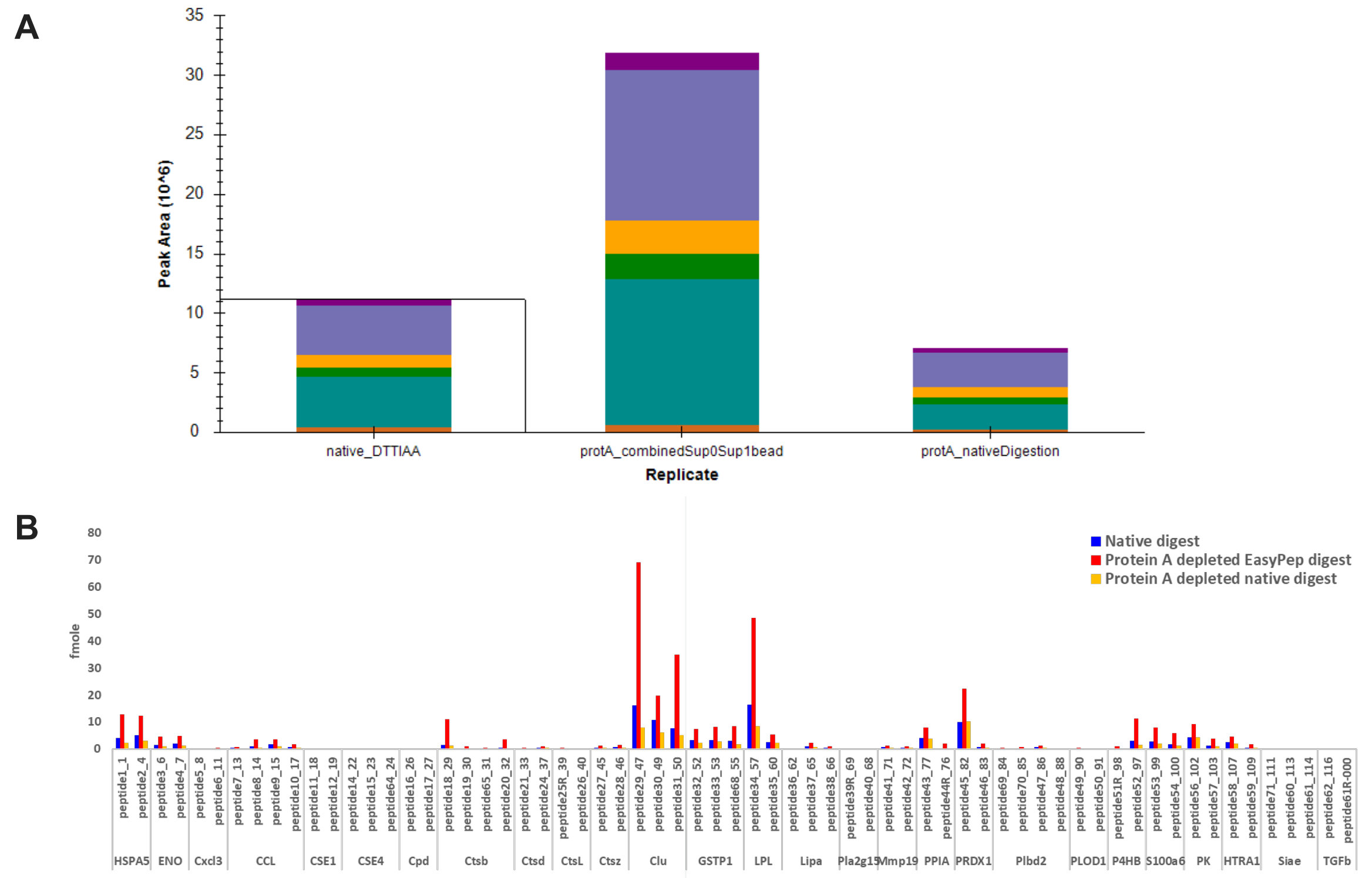
Figure 3: Evaluation of PRM Method for Quantification of Heavy Peptides. (A) Sample chromatogram in PRM mode for a mixture of 65 heavy peptides, (B) Representative PRM peak area of a peptide in Skyline.
(C) Linearity plot for selected peptides, showing the concentration range and linear fit with an R^2 value, (D) Linearity R^2 values for the entire heavy peptide panel. (E) LLOQ for the entire heavy peptide panel.



HCP quantification of CHO HCPs in affinity-purified trastuzumab

We employed the PRM method to quantify 28 key HCPs in four distinct affinity-purified trastuzumab samples, each prepared using a different technique (see Figure 1). To aid this analysis, we added 1 µL of heavy peptides (200 fmol) to each 0.5 µg of digested trastuzumab sample, prepared using various methods (Figure 1). For the quantification of endogenous light peptides, we utilized a standard curve created by serially diluting the heavy peptides (see Figure 3C). Our results indicated that the protein A-depleted EasyPep digest sample had the highest quantification value among the three different preparation methods (Figure 4A). This suggests that the protein A-depleted EasyPep method provides a more precise quantification of target HCPs compared to other preparation techniques. Figure 4B illustrates the quantification of endogenous HCPs for all 65 peptides in the post-affinity purified trastuzumab.

Figure 4: Quantification of HCPs in affinity-purified trastuzumab samples using PRM Method. (A) Quantification values of an HCP peptide (YITLIYTK) in affinity-purified trastuzumab samples prepared using 3 different methods.
(B) Quantification of endogenous HCPs for all 65 peptides in post-affinity purified trastuzumab samples, highlighting the differences in HCP levels across the various sample preparation techniques.



HCP quantification of CHO HCPs in rituximab (DS)

We began with 1.3 mg of post-ultrafiltration and diafiltration (UF/DF) rituximab, equivalent to the final drug substance (DS). Digest samples were prepared to compare the protein A-depleted EasyPep method with a native digest method. Our analysis showed that the protein A-depleted EasyPep method identified 13 unique HCPs, whereas the native digest method identified only 3, indicating the effectiveness of protein A depletion in enhancing HCP identification (Table 1).

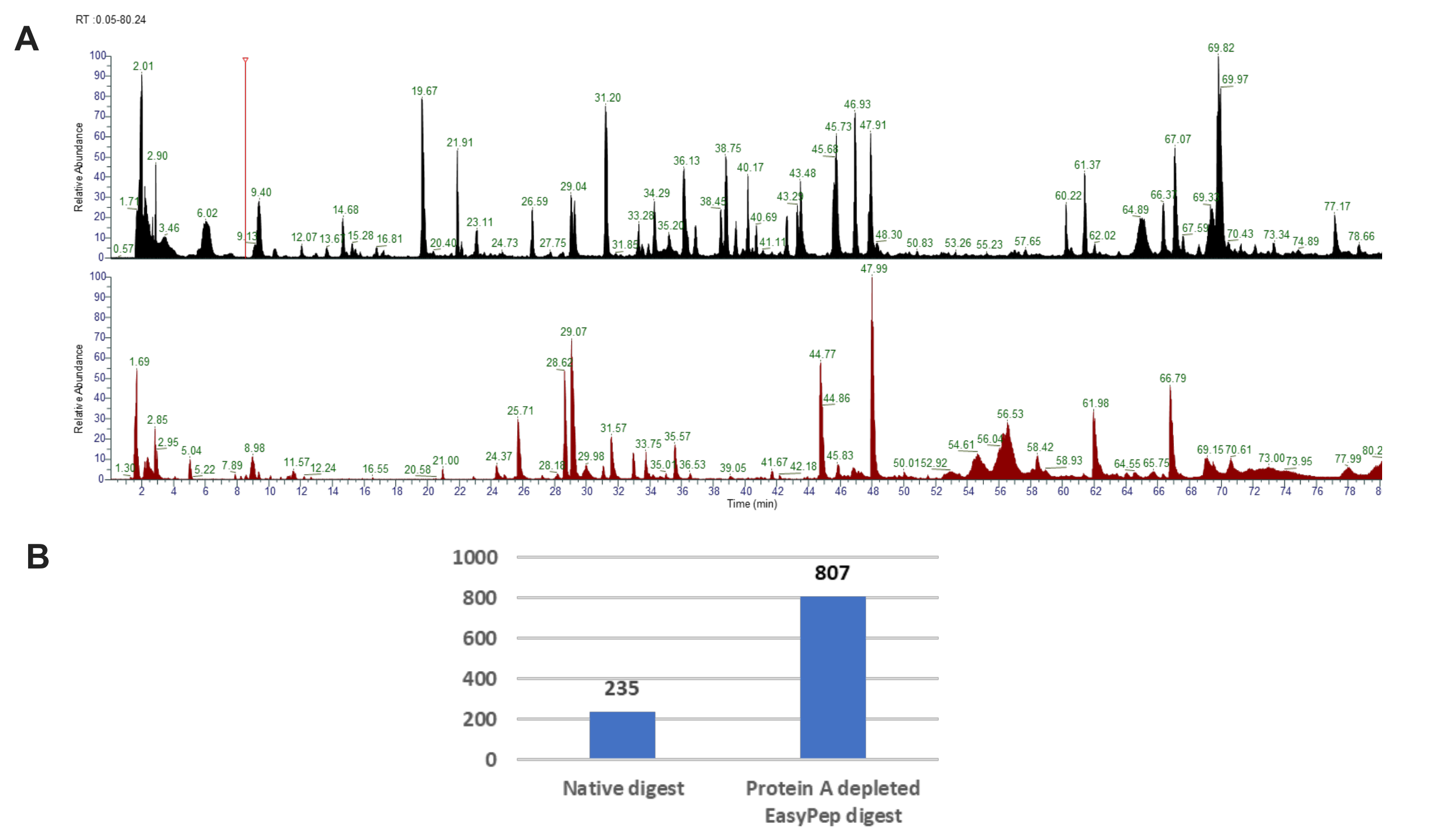
Table 1. Comparison of HCP identification in rituximab DS samples using protein A-depleted EasyPep method vs. native digest method

Native digest unique HCPs		Overlap HCPs		Protein A depleted EasyPep digest unique HCPs	
Accession	Description	Accession	Description	Accession	Description
G3I3X4	Fructose-bisphosphate aldolase		Rituximab-mAb-HC	G3HUA1	Anionic trypsin-2-like protein
G3HHM2	Histone H3.		Rituximab-mAb-LC	G3I9K7	Arylacetamide deacetylase
G3IBN9	Keratin, type II cytoskeletal 71	P13645	Keratin, type I cytoskeletal 10	G3IBN5	ATP-dependent RNA helicase DDX18
		G3IBP3	Keratin, type II cytoskeletal 2 epidermal	G3H0V0	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1
				G3HWB6	NACHT, LRR and PYD domains-containing protein 9A
				G3HC31	Protein S100
				G3I298	Serpin A9
				G3HIA8	Semaphorin-4D
				G3HTC3	Beta-crystallin B1
				G3IG05	Annexin
				G3HFA8	AP-3 complex subunit delta-1
				D7RJW0	Solute carrier family 35 member C2
				G3HU72	Vacuolar protein sorting-associated protein 13B

HCP analysis of NISTmAb using protein A-depleted EasyPep vs native digestion

Figure 5A presents the Base Peak Chromatogram (BPC) profiles for NISTmAb injections, comparing the protein A-depleted EasyPep method (upper panel) with the native digestion method (lower panel). In this study, the protein A-depleted EasyPep method demonstrated superior performance, identifying a total of 807 HCPs in the NISTmAb samples. In contrast, the native digestion method identified only 235 HCPs (Figure 5B). This significant difference highlights the enhanced capability of the protein A-depleted EasyPep method in detecting a broader range of HCPs. Overall, the protein A depletion process proves to be more effective in improving HCP identification compared to the native digestion approach.

Figure 5. Enhanced HCP Identification in NISTmAb Using Protein A-Depleted EasyPep Method Compared to Native Digestion. (A) Base Peak Chromatogram (BPC) profiles for NISTmAb injections, comparing the protein A-depleted EasyPep method (upper panel) with the native digestion method (lower panel). (B) Comparison of HCP identification in NISTmAb samples



Conclusions

- The protein A-depleted EasyPep method identified more unique HCPs in both trastuzumab and rituximab samples compared to native digestion methods.
- The protein A-depleted EasyPep method significantly outperforms native digestion in HCP identification, detecting 807 HCPs in NISTmAb compared to 235 HCPs with the native method.
- The linearity and LLOQ assessments of heavy peptides using the PRM method demonstrated excellent linearity and a 0.03 fmol LLOQ for the majority of peptides

References

1. Jones et al (2021): “High-risk” host cell proteins (HCPs): A multi-company collaborative view. Biotechnol Bioeng. 2021 Aug;118(8):2870-2885.

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