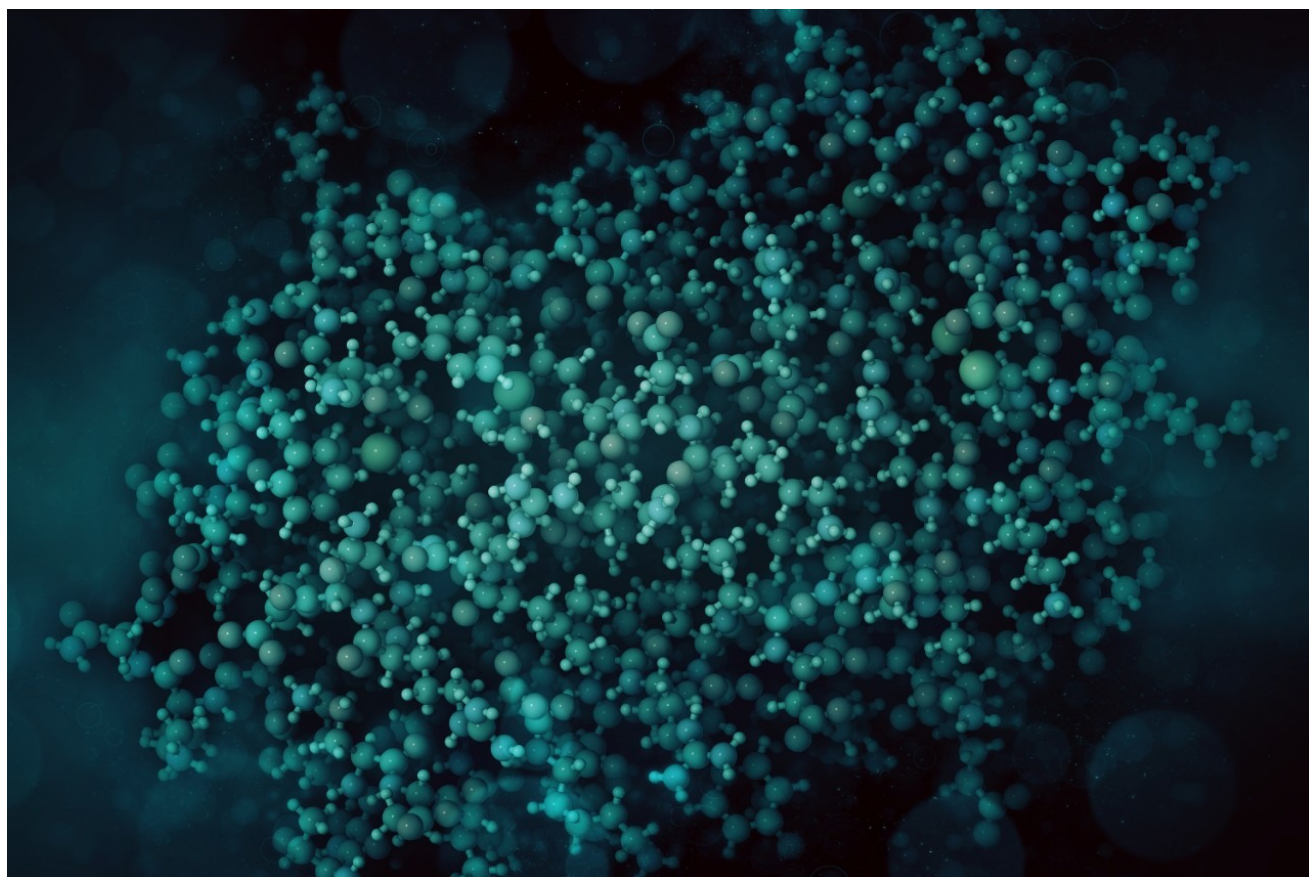


## Improved Recovery and Peak Shape of Sialylated O-glycopeptides of Erythropoietin (EPO) with ACQUITY PREMIER CSH Column

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This is an Application Brief and does not contain a detailed Experimental section.

## Abstract

Peptide mapping via LC-MS is a powerful characterization technique commonly used for protein sequence confirmation, post translational modification (PTM) analysis, and relative quantitation. To facilitate in-depth characterization, one needs to achieve adequate chromatographic peak resolution and minimize analyte loss during sample preparation and data acquisition. This can become increasingly challenging for some peptides, such as sialylated O-glycopeptides, which may exhibit adsorptive losses brought on by non-specific interactions with surfaces encountered during sample preparation and experiments. More specifically, highly sialylated glycopeptides can adsorb to metal surfaces in the column and LC system, leading to peak tailing and analyte loss. This application brief demonstrates the benefits of ACQUITY PREMIER Column featuring MaxPeak High Performance Surfaces and its ability to improve the separation and recovery of sialylated O-glycopeptides in the characterization of biologics.

## Benefits

- Reduced non-specific sample adsorption compared to stainless-steel columns
- Improved sample recovery, LC peak symmetry, and retention time reproducibility

## Introduction

In the biopharmaceutical industry, peptide mapping is often a critical analytical method for the characterization and quality control of drug products. It is therefore essential to have robust and reproducible methods for accurate characterization, monitoring, and comparability of these protein analytes. For most peptides, currently available technologies meet these needs, but in some cases, challenges still exist with acidic peptides<sup>1</sup>, such as phosphorylated peptides<sup>2</sup> and sialylated glycopeptides, which can exhibit poor peak shape and lower recovery under certain conditions. These peptides commonly carry a net negative charge in typical reversed phase separations using formic acid as the mobile phase additive, which makes them prone to non-specific adsorption to the metal surfaces of the column and LC system brought on by a Lewis acid/base-like interaction. This leads to chromatographic peak tailing and analyte loss, which in turn produces inaccurate and less reproducible results. In response to these observations, Waters developed a series of PREMIER columns featuring MaxPeak High Performance Surfaces (HPS) Technology, which provides a barrier layer that helps mitigate unwanted

interactions with metal surfaces.<sup>3</sup>

In this study, we investigate the chromatographic performance of an ACQUITY PREMIER Column with MaxPeak HPS Technology versus a traditional stainless-steel column in terms of recovery and peak shape of sialylated glycopeptides. Erythropoietin (EPO) will be used for this case study given its dynamic glycosylation profile and clinical relevance as a therapeutic biomolecule.

## Results and Discussion

As a small glycoprotein, EPO contains 3 N-glycosylation sites and 1 O-glycosylation site (Figure 1) increasing its susceptibility to exhibit chromatographic artifacts induced by metal surface interaction. The tryptic O-glycopeptide (T13) is found in four forms: T13 aglycosylated, T13 + HexNAc(1)Hex(1), T13 + HexNAc(1)Hex(1)NeuAc(1) (singly sialylated), and T13 + HexNAc(1)Hex(1)NeuAc(2) (doubly sialylated). In this study, a sample of EPO (a biosimilar candidate) was denatured, reduced, alkylated, and digested with trypsin prior to analysis using the BioAccord System (ACQUITY UPLC I-Class coupled with ACQUITY RDa Detector). To facilitate comparison of chromatographic performance, a separation of the peptide digest (0.5 µg load) was carried out at 60 °C using a conventional method on both an ACQUITY PREMIER Column with MaxPeak HPS Technology (ACQUITY PREMIER CSH C<sub>18</sub>, 130 Å, 1.7 µm, 2.1 x 100 mm, P/N [186009488 <https://www.waters.com/nextgen/us/en/shop/columns/186009488-acquity-premier-peptide-csh-c18-column-130a-17--m-21-x-100-mm-1-.html>](https://www.waters.com/nextgen/us/en/shop/columns/186009488-acquity-premier-peptide-csh-c18-column-130a-17--m-21-x-100-mm-1-.html)) and an equivalent stainless-steel column (ACQUITY UPLC CSH C<sub>18</sub>, 130 Å, 1.7 µm, 2.1 x 100 mm, P/N [176002141 <https://www.waters.com/nextgen/us/en/shop/columns/176002141-acquity-uplc-csh-c18-column-130a-17--m-21-mm-x-100-mm-3-pk.html>](https://www.waters.com/nextgen/us/en/shop/columns/176002141-acquity-uplc-csh-c18-column-130a-17--m-21-mm-x-100-mm-3-pk.html)). Briefly, the charged surface hybrid (CSH) chemistry was used given its demonstrated performance for enhanced peak shape and increased peak capacity of peptides in RPLC-based separations using formic acid (FA). Standard mobile phases were prepared as water, 0.1% FA (MP A) and acetonitrile, 0.1% FA (MP B). A gradient of 1-35% MP B over 50 minutes was employed for the separation. MS data was collected in positive ESI mode, at *m/z* 50–2000. The ACQUITY RDa MS source was set to 1.2kV capillary voltage, 30V cone voltage, 350 °C desolvation temperature, and 60–120V energy ramp for collisional induced fragmentation under Data Independent Mode (DIA).

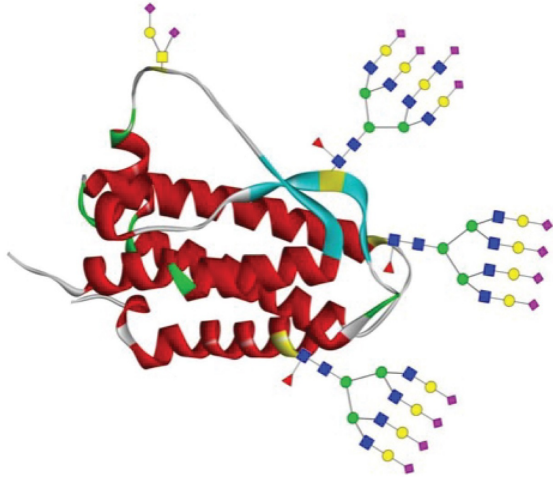


Figure 1. Structure of Erythropoietin (EPO) with examples of N- and O-glycosylation present.

Extracted ion chromatograms (XIC) of the most intense charge state for peptides of interest were used to assess the peak shape and recovery of the O-glycosylated peptides. Figure 2 displays overlays of each XIC for expected masses in Table 1. Peak 1 (green) corresponds to T13 core O-glycan species (+HexNAc(1)+Hex(1)), Peak 2 (black) corresponds to aglycosylated T13 peptide, Peak 3 (red) corresponds to singly sialylated T13 species, and Peak 4 (blue) corresponds to doubly sialylated T13 species. Resulting XIC chromatograms indicate a significant improvement in sensitivity (detector response) across all XIC peaks when using the ACQUITY PREMIER Column with MaxPeak HPS Technology. Peak areas for all species increased by at least 2.5-fold indicating improved recovery of O-glycosylated peptides in general when using ACQUITY PREMIER with MaxPeak Technology. Of notable interest was an observed 4-fold increase in the doubly sialylated T13 O-glycopeptide area, demonstrating the ability of this column technology to significantly increase recovery of sialylated glycans which may be more susceptible to metal induced adsorption artifacts. The enhanced MS signal of the O-glycopeptides is predicted to help improve the quality of MS/MS data for peptide identification and structural elucidation in LC-MS analysis, especially when the abundance of the peptides is low.

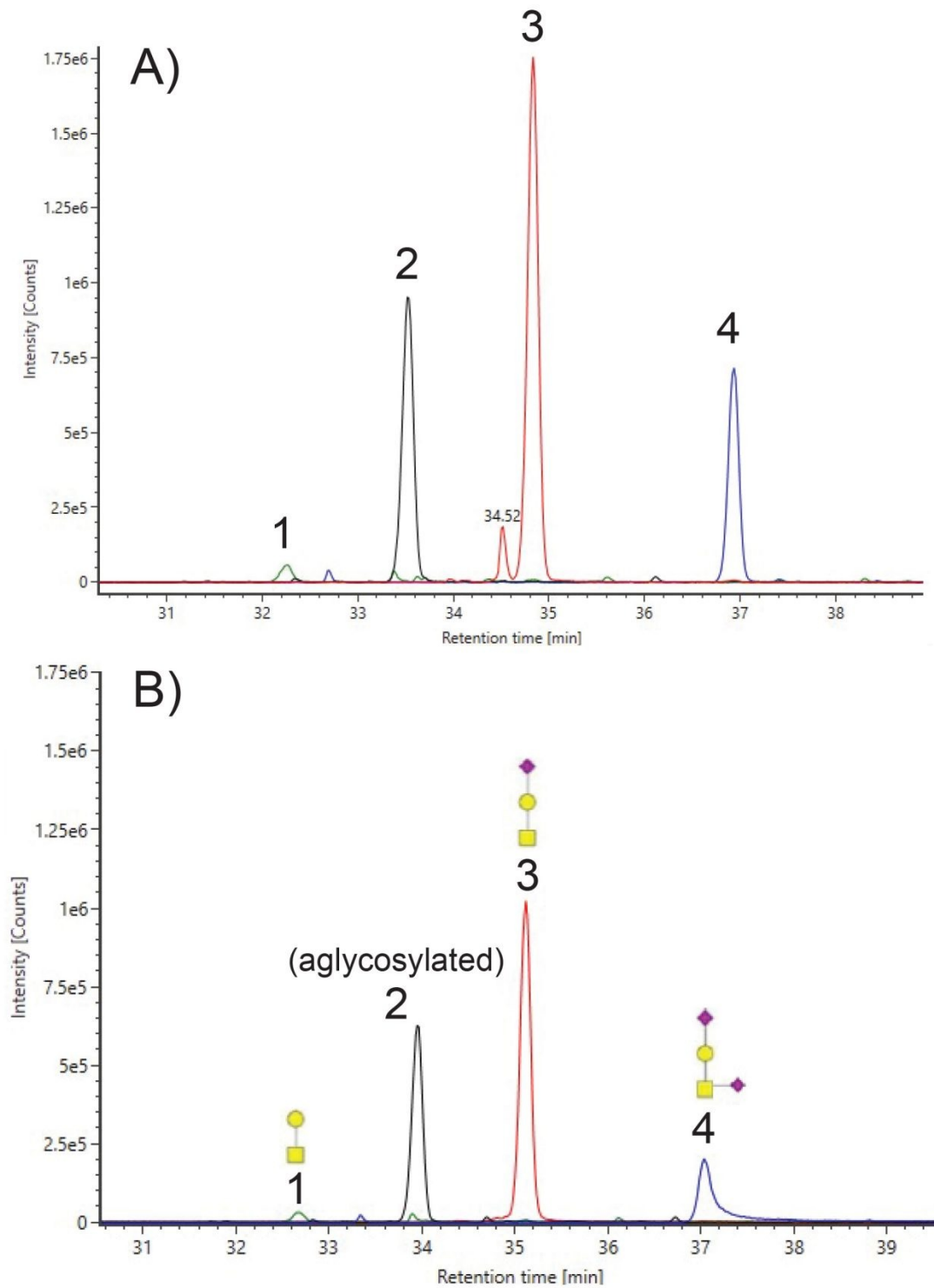


Figure 2. Extracted ion chromatogram overlays of T13 O-glycopeptide (EAISPPDAASAAPLR) for A) ACQUITY PREMIER CSH and B) stainless-steel ACQUITY CSH.




Peptide T13: EAISPPDAASAAPLR	Expected mass (M+H <sup>+</sup> )	Charge	Expected m/z
Aglycosylated	1465.7645	2	733.3859
		3	489.2597
+ HexNAc(1)Hex(1) 	1830.8967	2	915.9520
		3	610.9704
+ HexNAc(1)Hex(1)NeuAc(1) 	2121.9921	2	1061.4997
		3	708.0022
+ HexNAc(1)Hex(1)NeuAc(2) 	2413.0876	2	1207.0474
		3	805.0340

Table 1. List of T13 O-glycopeptides with expected masses and m/z values which were used to generate extracted ion chromatograms (XICs) for analysis.

Furthermore, the peak shape of the doubly sialylated species was observed to improve significantly when using ACQUITY PREMIER with MaxPeak HPS Technology. As shown in Figure 3, peak asymmetry (calculated at 10% peak height) was reduced by 6-fold ( $A_s \approx 6$  vs.  $A_s \approx 1$ ) with peak shape of the sialylated glycopeptide approaching Gaussian distribution. This newly observed peak symmetry enables clear and reproducible peak integration, and thus more accurate relative quantitation. The values obtained from these peptide mapping experiments were compared with the relative quantitation for a previously analyzed\* sample of intact de-N-glycosylated EPO of the same batch. At an intact level, the protein is not expected to exhibit adsorption artifacts as pronounced as the peptides, as the physicochemical properties of the macrostructure dominate the elution profile. Given this, the O-glycosylation profile at an intact level is expected to be a more accurate representation of the O-glycosylation content. As shown in Table 2, the relative quantitation values for the ACQUITY PREMIER Column with MaxPeak HPS Technology agree more closely to the values observed from intact protein analysis, most notably for the doubly sialylated species demonstrating ACQUITY PREMIER able to accurately reflect sample composition when compared to orthogonal complementary methods. Collectively, these results demonstrate the value of ACQUITY PREMIER columns with MaxPeak HPS Technology to enable more robust and accurate methods for the relative quantitation of simple O-glycopeptides.

\*Previously analyzed intact EPO sample was deglycosylated with PNGaseF prior to RPLC-MS analysis.

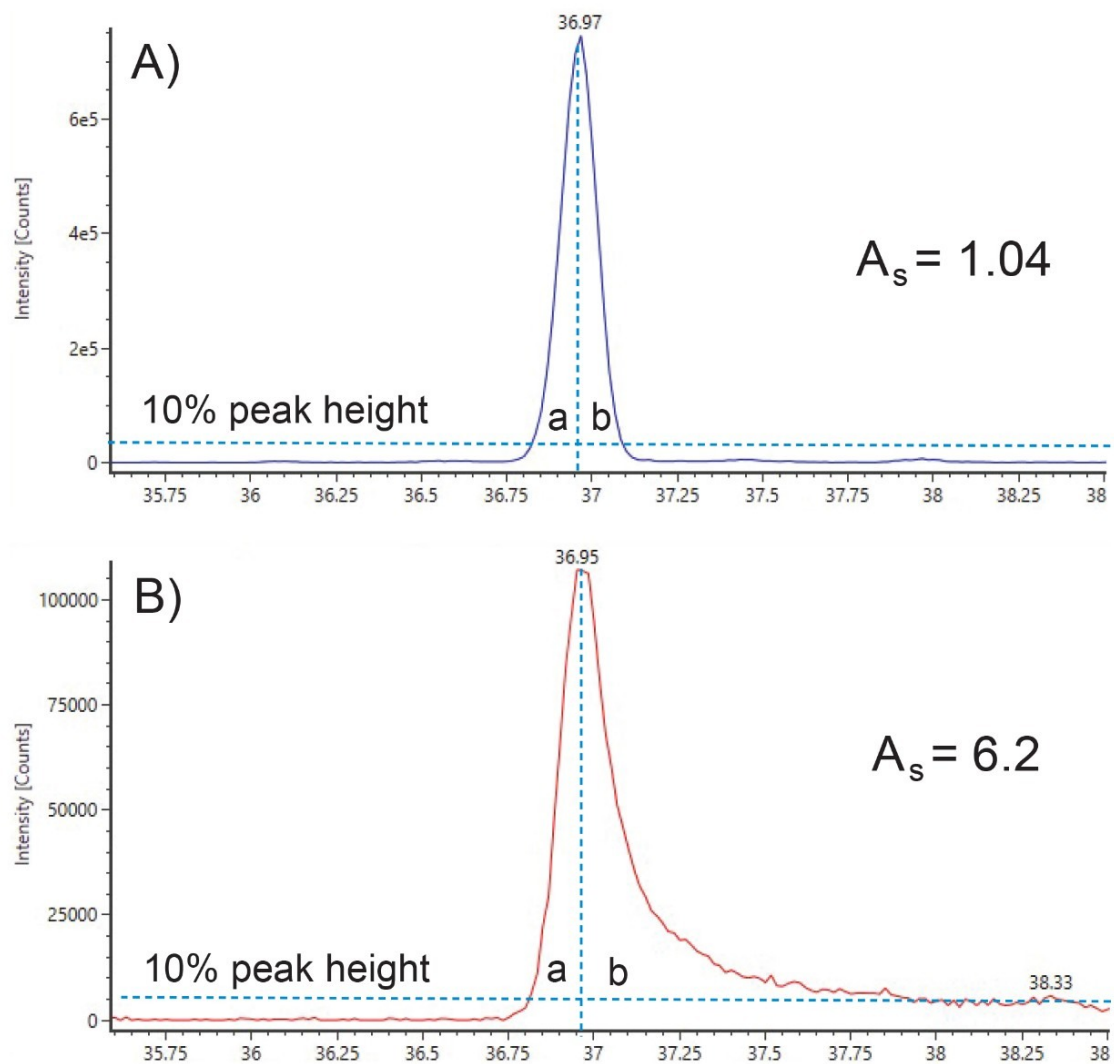


Figure 3. Extracted ion chromatograms for doubly sialylated T13 O-glycopeptide on and A) ACQUITY PREMIER CSH and B) stainless-steel ACQUITY CSH, highlighting peak asymmetry (Calculation:  $A_s = b / a$  at 10% peak height).




Peptide T13: EAISPPDAASAAPLR	Relative %		
	ACQUITY Premier CSH C <sub>18</sub>	Stainless steel CSH C <sub>18</sub>	Intact De-N-glycosylated EPO
Aglycosylated	27.0%	31.0%	29.6%
+ HexNAc(1)Hex(1) 	2.9%	3.0%	3.1%
+ HexNAc(1)Hex(1)NeuAc(1) 	52.2%	54.6%	50.1%
+ HexNAc(1)Hex(1)NeuAc(2) 	17.5%	11.4%	17.9%

Table 2. Relative quantitation for T13 O-glycopeptides and intact De-N-glycosylated EPO sample.

## Conclusion

Non-specific interactions and adsorption of peptide analytes on traditional stainless-steel LC-MS column surfaces contribute to poor analyte recovery and asymmetrical peaks. This presents significant challenges for characterization of metal-sensitive analytes such as O-glycopeptides with sialylated glycans. Waters ACQUITY PREMIER Technology featuring MaxPeak High Performance Surfaces addresses the challenge of metal-induced adsorption artifacts. In the case of EPO T13 O-glycopeptide the ACQUITY PREMIER CSH Column, when compared to traditional stainless-steel hardware, provides an overall increase in sensitivity and significant improvement to the peak shape and relative quantitation of the doubly sialylated T13 O-glycopeptide. This is a significant milestone in creating robust and reproducible methods for the analysis of O-glycopeptides.

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