

## Improved Separation Performance of Monoclonal Antibody and Antibody Drug Conjugates Using Waters XBridge Premier Protein SEC 250 Å, 2.5 µm Column

---

Kenneth D. Berthelette, Jennifer M. Nguyen, Lavelay Kizekai, William J. Warren, Steve Shiner

Waters Corporation

---

### Abstract

Monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs) have been used as biopharmaceutical products for over twenty years. Of the top fifty grossing pharmaceuticals of 2020, mAbs account for eleven, including the top two highest grossing products, Humira and Keytruda respectively.<sup>1</sup> Size exclusion chromatography (SEC) is one of main techniques used to separate protein size variants as part of their characterization. However, undesired secondary interactions between the proteins and the liquid chromatography (LC) column hardware and/or packed material can compromise the quality of the obtained data. Waters XBridge Premier Protein SEC 250 Å, 2.5 µm Column is uniquely designed to mitigate these secondary interactions. Three different protein standards were separated on a Waters XBridge Premier Protein SEC 250 Å, 2.5 µm Column and a silica-based SEC (dSEC-2) column to highlight the improvements provided by Waters MaxPeak Premier SEC Technology in these defined application areas.

### Benefits

---

- Improved USP resolution and detection of the low molecular weight species (LMWS) found in two mAb standards
- Improved relative (percent) peak area for HMWS vs monomer compared to silica-based columns in three different standards
- Higher five sigma efficiency for the main mAb monomer peak in three different standards
- Simplified SEC eluent method development using phosphate buffered saline (PBS)

---

## Introduction

Therapeutic biomolecules are experiencing rapid growth due to advances in their synthesis, characterization, manufacturing, and efficacy. Of the fifty drugs approved by the Food and Drug Administration (FDA) in 2021, sixteen were biologics.<sup>2</sup> In order to properly characterize the compound of interest and ensure aggregates and fragments are tracked, analysts can employ size exclusion chromatography (SEC). First introduced by Wheaton and Bauman in the 1950s, SEC is a technique that separates compounds based on their hydrodynamic radius.<sup>3</sup> Since then, improvements to stationary phase design and manufacturing including consistent pore sizes on a particle, new pore size offerings, and reproducible particle manufacturing processes and testing have made SEC more reliable and accurate while facilitating method development and validation for novel compounds.

Existing SEC methods are particularly susceptible to interactions between the analyte and the separation platform that includes both the column hardware and SEC packed particles. These interactions can be ionic and/or hydrophobic depending on the assay conditions, the analyte of interest and the system configuration. The complexity of biological analytes can lead to a variety of secondary interactions, producing poor peak shape, wider peaks, and potentially lower recovery of aggregates or fragments. Acidic moieties can interact with the metal surfaces of a liquid chromatography (LC) system leading to decreased peak areas.<sup>4-6</sup> MaxPeak Premier High Performance Surface (HPS) hardware was introduced in 2020 to mitigate the ionic interactions between analyte, metallic column, and system hardware for small molecule analytes. Proteins are highly susceptible to ionic interactions, and thus require additional special considerations. Measures must also be taken to mitigate ionic and hydrophobic interactions with active sites on packing material. To that end, a novel ethylene bridged-hybrid particle with high coverage hydroxy-terminated polyethylene oxide (BEH-PEO) bonding was paired with new hydrophilic MaxPeak HPS hardware provide a solution to the problem of undesired secondary interactions.<sup>7</sup>

The work shown compares the performance of the Waters XBridge Premier Protein SEC 250 Å, 2.5 µm Column to a 200 Å, 2.5 µm silica-based (dSEC-2) column using an Agilent 1260 Infinity Bio system.

---

## Experimental

### Sample Description

Waters mAb size variant standard (p/n: 186009429 <<https://www.waters.com/nextgen/global/shop/standards--reagents/186009429-mab-size-variant-standard.html>> ) reconstituted in 70 µL water (2.28 mg/mL) and vortexed for several seconds before injection. NISTmAb Reference Material 8671 working solution (2 mg/mL) prepared with histidine formulation buffer (12.5 mM Histidine/12.5 mM Histidine-HCl). Ado-trastuzumab emtansine (KADCYLA), an antibody drug conjugate (ADC) with working solution (5 mg/mL) prepared in Milli-Q water.

### LC Condition

LC system:	Agilent 1260 Infinity Bio-Inert system Quaternary LC, HiP ALS (High Performance Autosampler), and DAD with a BioInert Flowcell (10 mm, 13 µL). 5-sigma ( $5\sigma$ ) system dispersion measured at 37 µL
Detection:	UV @ 280 nm, 5 Hz
Column(s):	XBridge Premier Protein SEC 250 Å, 7.8 x 300 mm, 2.5 µm  Comparison Column: dSEC-2 200 Å, 7.8 x 300 mm, 3 µm
Column temperature:	Ambient
Sample temperature:	8 °C

---

Injection volume:	10.0 $\mu$ L (mAb standards), 7.2 $\mu$ L (KADCYLA)
Flow rate:	0.58 mL/min
Isocratic mobile phase:	2x Phosphate Buffered Saline (20 mM Phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4)
Sample manager wash:	18.2 M $\Omega$ water
Sample manager purge:	18.2 M $\Omega$ water

## Data Management

Chromatography software:	Empower 3 Feature Release 4
--------------------------	-----------------------------

---

## Results and Discussion

An Agilent 1260 Infinity Bio system with a diode array detector (DAD) was configured with a Bio-Inert 10 mm, 13  $\mu$ L flow cell. Prior to testing, the system bandspreading was assessed using injections of uracil, and the dispersion was measured to be 37  $\mu$ L using peak width at 4.4% ( $5\sigma$ ). Each column tested was equilibrated to assay conditions for 40 minutes prior to sample injections.

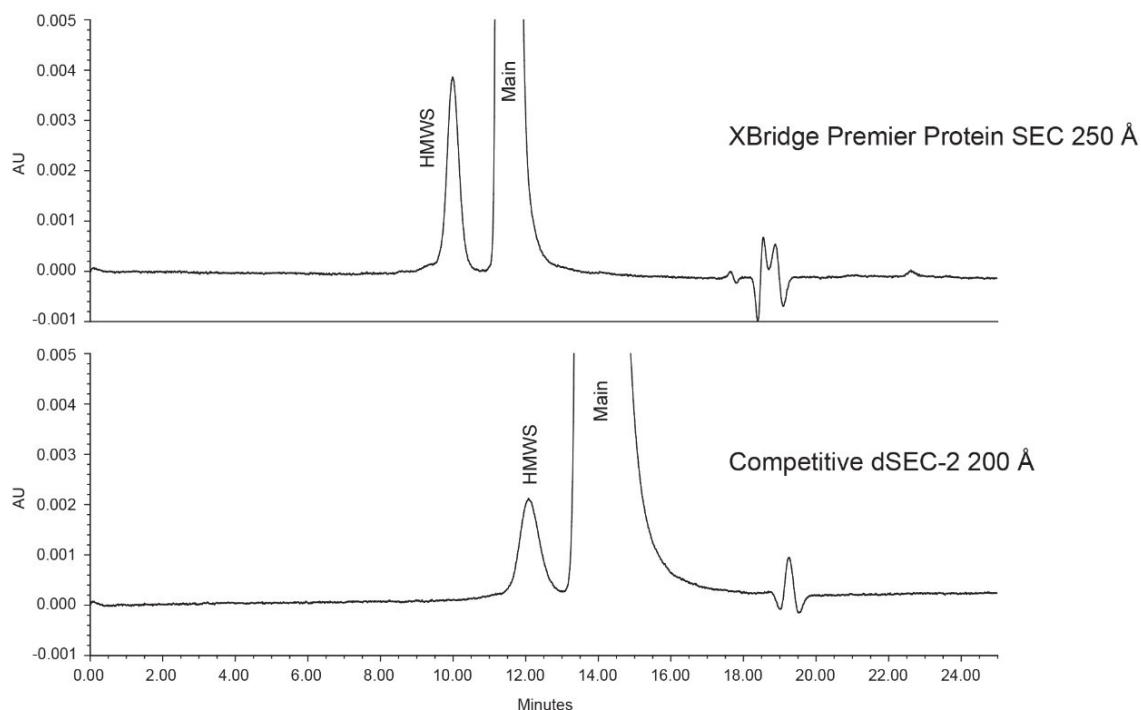


Figure 1. Analysis of ADC on two columns. Isocratic separation using 2x PBS in water, 0.57 mL/min, with UV detection at 280 nm.

The separation of the ADC ado-trastuzumab emtansine (KADCYLA) is shown in Figure 1. For both columns, the high molecular weight species (HMWS) is well separated from the main peak. The competitive dSEC-2 column produces slightly wider peaks compared to the XBridge Premier Protein SEC Column, as well as higher retention times. The increased retention time is likely due to differences in pore size between the two phases. Critical attributes of the separation, including percent peak area of the HMWS are shown in Table 1. The XBridge Premier Protein SEC Column gave slightly higher percent area for the HMWS (1.72%) compared to the competitive dSEC-2 column (1.51%). These values are both within the agreed upon ranges for this species, with the competitive column being just within range. Higher resolution at half height for the main peak are also achieved for the Waters column compared to the competitive column, 2.68 and 1.71 respectively. Reliable measurement of the HMWS is critical throughout the product lifecycle, from development and efficacy testing through quality control at production to ensure safety and efficacy of the product.

Similar improvements using the XBridge Premier Protein SEC Column can be seen in the analysis of the Waters mAb size variant standard in Figure 2. The Waters mAb size variant standard contains not only HMWS but also

LMWS, or fragments. Based on the reference material NISTmAb (8671), the Waters mAb size variant standard has been enriched with IdeS digest fragments to bring the ~100 kDa species to roughly 1% of the total mAb. The 100 kDa fragment is thus referenced here as LMWS1&2, as it contains both a hydrolysis and IdeS digested species that elute very closely together, being completely unresolved.<sup>8</sup>

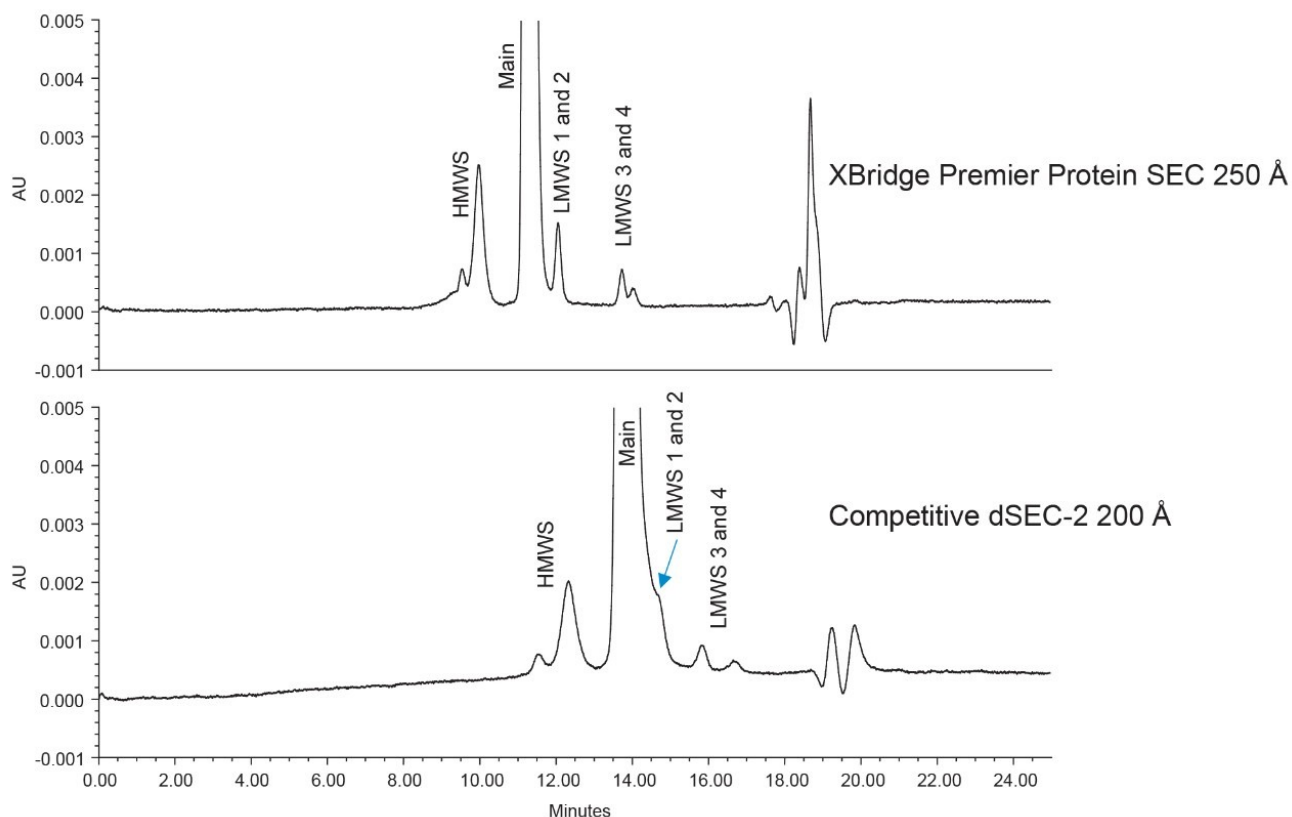


Figure 2. Analysis of Waters mAb size variant standard on the XBridge Premier Protein SEC 250Å and the competitive silica dSEC-2 column Isocratic separation performed using a mobile phase of 2x PBS in water, flow rate of 0.57 mL/min, and UV detection at 280 nm.

The XBridge Premier Protein SEC Column effectively separates the main peak from the LMWS1&2 peak, while the competitive column produces only a shoulder on the tail of the main peak. That the monomer (~150 kDa) is less than two-fold greater in molecular weight than the first fragment (~100 kDa), and that the much lower abundance of LMWS elutes in the tail of the monomer peak, makes this a very challenging standard to separate. Resolving the LMWS1&2 from the main peak, along with higher recoveries for the HMWS, while still within

guidelines for the standard, provides a more thorough characterization of the sample. The XBridge Premier Protein SEC Column yielded higher percent area for the HMWS (2.47%) compared to the competitive dSEC-2 column (2.28%) along with other improvements as outlined in Table 1. Overall, the separation efficiency achieved with the XBridge Premier Protein SEC Column is superior to the results on the competitive column, particularly for HMWS and the LMWS1&2.

The last standard to be analyzed was the NISTmAb reference material (RM) 8671. Chromatograms for this standard on both columns are shown in Figure 3. This standard contains the HMWS as well as minor peaks for LMWS1 and LMWS4 and does not contain the LMWS2 fragment. Peak identification based on chromatograms shown in the Waters mAb size variant standard care and use manual.<sup>7</sup>

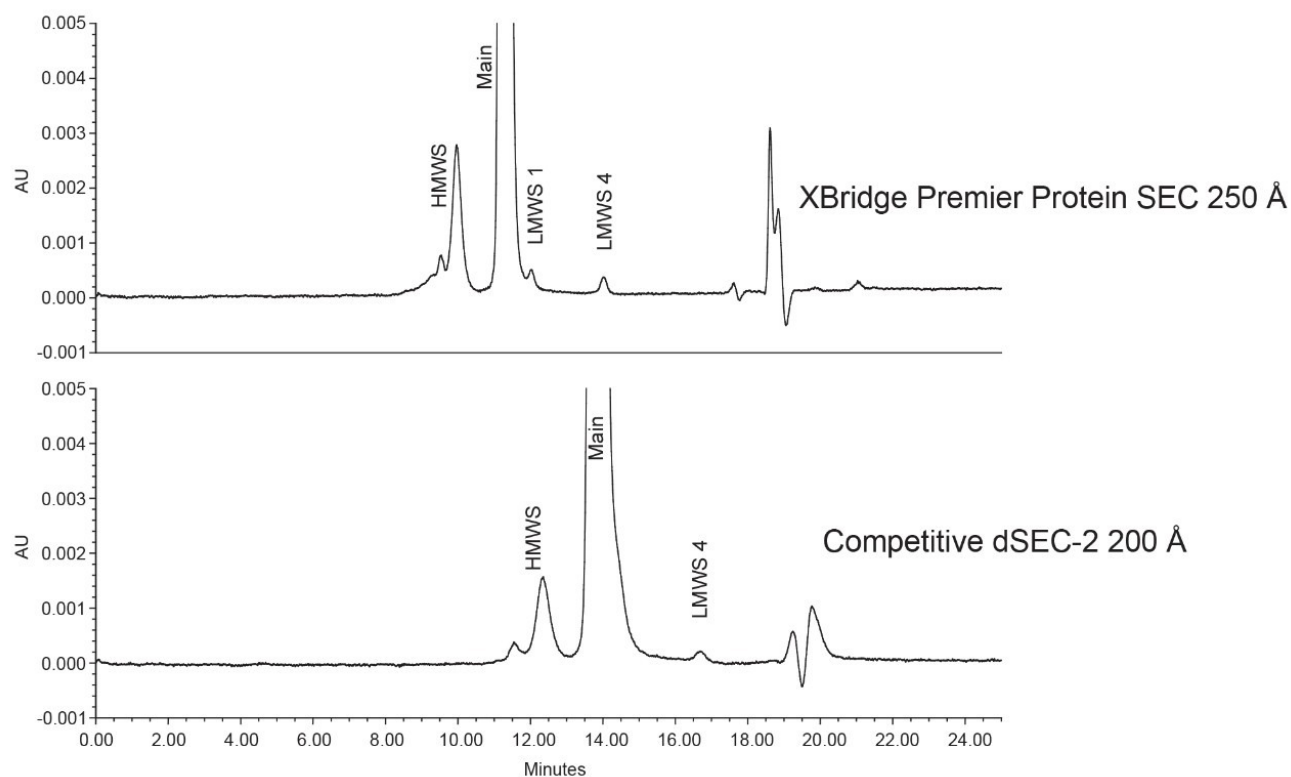


Figure 3. Analysis of NISTmAb reference material 8671 on the XBridge Premier Protein SEC 250 Å and the competitive silica dSEC-2 column Isocratic separation performed using a mobile phase of 2x PBS in water, flow rate of 0.57 mL/min, and UV detection at 280 nm.

The LMWS1 shows partial resolution from the main peak on the XBridge Premier Protein SEC Column at a low

relative abundance (0.26%). As a result, USP resolution at half height for this peak could not be calculated. In contrast, the dSEC-2 column does not show the LMWS1 peak, but rather a slight shoulder of the main peak similar to what was seen with the Waters mAb standard. Additionally, the LMWS4 has improved S/N on the XBridge Premier Protein SEC Column, potentially allowing for improved quantification as well. Full details for the separation of the NISTmAb RM 6871 are shown in Table 1.

Peak	Attribute	XBridge Premier Protein SEC 250 Å, 2.5 µm			Competitive dSEC-2 200 Å 2.5 µm		
		Kadcyla standard	Waters mAb standard	NIST RM6871	Kadcyla standard	Waters mAb standard	NIST RM6871
Main	% Area	98.28	96.34	96.84	98.49	97.12	97.62
	USP res (HH)	2.68	3.47	3.44	1.71	2.53	2.59
	5-Sigma	7934	19995	19998	2229	12273	12363
	USP tailing	1.45	1.13	1.13	1.70	-	1.10
HMWS	% Area	1.72	2.47	2.73	1.51	2.28	2.20
LMWS 1 and 2 (LMWS 1 only - NIST)	Start P/V ratio	-	4.15	1.46	-	-	-
	USP res (HH)	-	2.63	-	-	-	-
	% Area	-	0.74	0.26	-	-	-
LMWS 3 and 4	% Area	-	0.45	0.17	-	0.61	0.18

*Table 1. Tabular data for the separation of all three standards on the XBridge Premier Protein SEC 250 Å and the competitive silica dSEC-2 column. All values except start p/v ratio were calculated by Empower CDS. Start p/v ratio was calculated by taking peak height at apex and dividing by height of the valley between the LMWS 1 peak and the main peak. 5-Sigma is the plate count determined at 4.4% peak height.*

For most relevant parameters, the XBridge Premier Protein SEC Column achieved better and accurate results within expected ranges for all three samples. The competitive column falls outside the expected percent peak area for HMWS for the ADC sample and is just within the expected ranges for the NIST mAb and waters size variant standard. The peak area percentages for the HMWS are higher on the XBridge Premier Protein SEC Column for each standard by up to 0.53% of the total area of the separation. Reliable quantitation and recovery of the HMWS is critical for manufacturing and quality control of biopharmaceutical mAbs. Although the percent area for HMWS was within parameters for the standard, the lower percent area suggests an inaccurate representation of the species in the final product. Critical aspect is the separation of the LMWS1&2 from the main peak. The XBridge Premier Protein SEC Column can more effectively separate the LMWS1&2 from the main peak allowing for potentially more reliable characterization of the sample. Without good separation of this peak, the



percent peak area for the fragment peak would be lower than it should be, leading to improper characterization and poor overall results.

---

## Conclusion

SEC analysis of biopharmaceutical compounds is a useful technique for accurate characterization and process monitoring of biotherapeutic proteins but can be susceptible to undesired secondary interactions that can confound results. The Waters XBridge and ACQUITY Premier Protein SEC 250 Å columns, available in two particle sizes, are specifically designed to mitigate secondary interactions by employing a proprietary column chemistry as well as hydrophilic MaxPeak Premier HPS hardware optimized for protein separations. The novel design allows for better separation of HMWS and LMWS compared to a competitive column in the analysis of three different standards. Consistent characterization of biopharmaceutical compounds can be achieved using the XBridge and ACQUITY Premier Protein SEC 250 Å columns, providing crucial information and confidence in the results for critical assays.

---

## References

1. 50 of 2020's Best-selling Pharmaceuticals. *Drug Discovery and Development*. Accessed 3-Jan-2021. <https://www.drugdiscoverytrends.com/50-of-2020s-best-selling-pharmaceuticals/> <  
<https://www.drugdiscoverytrends.com/50-of-2020s-best-selling-pharmaceuticals/>>
2. Novel Drug Approvals for 2021. FDA website. Accessed 4-Jan-2021. <https://www.fda.gov/drugs/new-drugs-fda-cders-new-molecular-entities-and-new-therapeutic-biological-products/novel-drug-approvals-2021> <  
<https://www.fda.gov/drugs/new-drugs-fda-cders-new-molecular-entities-and-new-therapeutic-biological-products/novel-drug-approvals-2021>>
3. Wheaton RM, Bauman WC. Non-Ionic Separations with Ion Exchange Resins. *Annals of the New York Academy of Sciences*. 1953. 159-176.
4. Kellett J, Birdsall RE, Yu YQ. Increasing Recovery of Phosphorylated Peptides Using ACQUITY Premier

Technology Featuring MaxPeak High Performance Surfaces. Waters Application Note [720007198EN](#), 2021.

5. DeLano M, Walter TH, Lauber M, Gilar M, Jung MC, Nguyen J, Boissel C, Patel AV, Bates-Harrison A, Wyndham K. Using Hybrid Organic-Inorganic Surface Technology to Mitigate Analyte Interactions with Metal Surfaces in UHPLC. *Analytical Chemistry* 2021 5773–5781.
6. Jung M, Lauber M. Demonstrating Improved Sensitivity and Dynamic Range with MaxPeak High Performance Surface (HPS) Technology: A Case Study in the Detection of Nucleotides. Waters Application Note. [720007053EN](#), 2021.
7. Kizekai L, Lauber M. Waters ACQUITY and XBridge Premier Protein SEC 250 Å Columns: A New Benchmark in Inert SEC Column Design. Waters Application Note. [720007493EN](#), 2022.
8. Waters mAb Size Variant Standard Care and Use Manual, [720006811EN](#) <<https://www.waters.com/waters/support.htm?lid=135068415&lcid=135068414&type=USRM>> , 2022.

## Acknowledgements

Pamela Irenata for development of test parameters.

Mark Trahan and Andrew Steere for allowing access and assisting in setting up the instrument.

---

## Featured Products

[Empower Chromatography Data System <https://www.waters.com/10190669>](https://www.waters.com/10190669)

720007528, February 2022

© 2022 Waters Corporation. All Rights Reserved.