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Abstract

High-throughput and rapid size-exclusion chromatography (SEC) methods can be useful to support biotherapeutic process and formulation development. In addition, a rapid SEC method will be more effective for real-time purity analyses to support a production process. In these applications, SEC can be used to monitor self-associated or aggregated protein impurities or protein fragmentation that can impact product safety or efficacy.

In a previous application note, it was demonstrated that effective separations and extended column lifetimes could be achieved using mobile phases at physiological pH (7.4) and ionic strength using Waters™ MaxPeak Premier Protein SEC Columns. Consequently, the evaluation of the performance and column lifetime of both UPLC and HPLC versions of these recently introduced columns were evaluated while operating near their specified maximum pressures, resulting in analysis times from 2.1 to 3.0 minutes.

For this study, the lifetimes of a Waters ACQUITY™ Premier Protein SEC (250 Å, 1.7 µm, 4.6 x 150 mm) Column and two XBridge™ Premier Protein SEC (250 Å, 2.5 µm) Columns with dimensions of 4.6 x 150 mm and 7.8 x 150 mm were successfully evaluated over the course of 500 analyses. The separation performance was evaluated for both self-associated and selected fragmented size variants of four marketed monoclonal antibody biosimilars.

Benefits

- Rapid SEC analyses (2.1 to 3.0 minutes), SEC analyses of mAb size variants on UPLC and HPLC systems
 - Column lifetimes greater than 500 analyses near maximum specified column pressures
 - Reproducible determinations of both HMWS and LMWS impurity levels throughout lifetime study
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Introduction

High-throughput and rapid SEC methods can be useful to monitor aggregated protein impurities or protein fragmentation, which can impact product safety or efficacy, while decreased analysis times can potentially help to accelerate manufacturing process and formulation development.^{1,2} In addition, a rapid SEC method will also be more effective for real-time purity analyses to potentially support a registered production process.³ In a previous study, Waters XBridge Premier Protein SEC 250 Å, 2.5 µm and ACQUITY Premier Protein SEC 250 Å, 1.7 µm Columns with lengths of 300 mm had been demonstrated to provide high resolution separations and long-term stability at physiological pH (7.4) and ionic strength, due in large part to the use of both pH stable ethylene-bridged hybrid (BEH) SEC particles bonded with hydrophilic hydroxy-terminated polyethylene oxide (PEO) and PEO modified column hardware to reduce protein-surface interactions.⁴ Consequently, the rapid and high-throughput SEC analysis capabilities, and column lifetimes of both UPLC and HPLC versions of MaxPeak Premier SEC Columns at lengths of 150 mm were evaluated while running at operating pressures near their respective specified maximums, resulting in analysis times from 2.1 to 3.0 minutes.

For this study, an ACQUITY Premier Protein SEC (250 Å pore size, 1.7 µm particle size) 4.6 x 150 mm Column and two XBridge Premier (250 Å, 2.5 µm) 150 mm length Columns with internal diameters of 4.6 mm and 7.8 mm were evaluated over the course of 500 injections for the analysis of the four marketed monoclonal antibody (mAb) drug products currently available as biosimilars in the USA. The samples of bevacizumab, infliximab, and rituximab were biosimilar drug products and the sample of trastuzumab was the originator drug product. The separation performance was evaluated for both self-associated and selected fragmented mAb size variants.

Experimental

Sample Description

Biosimilar mAbs were bevacizumab (Mvasi, 25 mg/mL), infliximab (Avsola, 10 mg/mL), rituximab (Ruxience, 10 mg/mL), and trastuzumab was the originator biologic (Herceptin, 21 mg/mL). All samples were analyzed neat following one or more freeze-thaw cycles.

LC Conditions

LC system:	ACQUITY UPLC H-Class Bio with CH-A Column Heater
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell
Wavelength:	280 nm
Vials:	Polypropylene 12 x 32 mm Screw Neck, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639)
Column(s):	ACQUITY Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm, plus mAb Size Variant Standard (p/n: 176004783) XBridge Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm, plus mAb Size Variant Standard (p/n: 176004781) XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 x 150 mm, plus mAb Size Variant Standard (p/n: 176004779)
Column temp.:	Ambient
Sample temp.:	6 °C
Injection volume:	Infliximab and rituximab: 5 mL for 7.8 x 150 mm column and 2 mL for 4.6 x 150 mm columns Bevacizumab and trastuzumab: 2.5 mL for 7.8 x 300 mm column and 1 mL for 4.6 x 150 mm columns

Flow rate:	0.75–2.0 mL/min
Mobile phase A:	Phosphate-buffered saline (DPBS, 10X), Dulbecco's formula 10X (Alfa Aesar, J61917) (0.1 µm sterile filtered)
Mobile phase B:	Milli-Q 18 MΩ water (0.1 µm sterile filtered)

Data Management

Chromatography software:	Empower™ 3 Chromatographic Data System
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Results and Discussion

The high-throughput SEC separations of quantifiable high molecular weight species (HMWS) and low molecular weight species (LMWS) impurities of four different mAb samples were evaluated over 500 runs on the ACQUITY Premier Protein (4.6 x 150 mm), XBridge Premier Protein (4.6 x 150 mm), and XBridge Premier Protein (7.8 x 150 mm) SEC Columns. A thorough study on the impact of these four therapeutic mAb samples on ACQUITY and XBridge Premier SEC Column lifetimes has been previously demonstrated.⁴ Therefore, for this study, mAb samples were only evaluated at the reported time points (0 or initial, 100, 200, 300, 400, 500 injections) while blank (water) 5 µL intermediate injections were made. Also, 10X DPBS concentrate was mixed with water by the LC to produce 1X PBS mobile phase. In addition, the 10X DPBS concentrate and Milli-Q 18 MΩ water were 0.1 µm sterile filtered prior to use. Injection volumes for bevacizumab and trastuzumab were 1.0 µL for the 4.6 x 150 mm columns and 3.0 µL for the 7.8 x 150 mm columns. While for infliximab and rituximab these injection volumes were 2.0 µL and 6.0 µL, respectively.

The flow rates and resultant pressures used for this study are shown in Table 1. The flow rates used were selected to test column lifetimes at or near the recommended maximum column pressures of the columns. The recommended column pressures represent the pressure due only to the column which is determined by subtracting the LC system pressure observed for the system with a zero dead volume union (p/n: [289000439](#) <

<https://www.waters.com/nextgen/global/shop/service-parts--kits/289000439-union-zero-dead-volume.html>) in place of the column from the system pressure observed with the installed column. For all three columns, the flow rates exceeded the recommended maximums. At these flow rates the column pressures for the 4.6 x 150 mm ACQUITY Premier and XBridge Premier Columns were close to the recommended maximums. The 7.8 x 150 mm XBridge Premier Column was run at the maximum flow rate of the UPLC System (2.00 mL/min), which was twice the recommended flow rate for the column, resulting in a column pressure well below the recommended maximum. For the 4.6 x 150 mm columns a 0.0025" (64 µm) PEEK connector (p/n: 700009971 <<https://www.waters.com/nextgen/global/shop/service-parts--kits/700009971-assy-tube-inlet-0025-id-peek-nut-85.html>>) was used between the column and TUV detector while for the 7.8 x 150 mm column a 0.004" (100 µm) PEEK connector (p/n: 700009972 <<https://www.waters.com/nextgen/global/shop/service-parts--kits/700009972-tubing-assembly-detector-inlet-peek-0004-id-with-peek-nut-85-lon.html>>) was used.

Column	Specified maximum		Tested		
	Flow rate (mL/min)	Column pressure (PSI, [bar])	Flow rate (mL/min)	Total pressure (PSI, [bar])	Column pressure (PSI, [bar])
ACQUITY Premier SEC (4.6 x 150 mm)	0.50	7000, [483]	0.75	8720, [601]	6700, [462]
XBridge Premier SEC (4.6 x 150 mm)	0.50	4500, [310]	1.00	7010, [483]	4270, [294]
XBridge Premier SEC (7.8 x 150 mm)	1.00	4500, [310]	2.00	6900, [476]	3540, [244]

Table 1. The flow rates and resultant pressures.

Expanded-scale chromatograms for the mAbs separated on the ACQUITY Premier and two XBridge Premier Columns are presented in Figure 1. All three columns produced similar overall chromatographic profiles with respect to the trace levels of HMWS and LMWS impurities. The 4.6 x 150 mm XBridge Column produced predictably lower resolutions in comparison to the other two columns due in part to the higher linear velocities used. In addition, the particle size of the 4.6 x 150 mm XBridge Column (2.5 µm) is also approximately 50% larger than that of the 4.6 x 150 mm ACQUITY Column (1.7 µm) and the larger internal diameter of 7.8 x 150 mm XBridge Column is less impacted by LC system dispersion.⁵ The 5-sigma (5σ) system dispersion of the LC system used for this study was measured to be 7.9 µL.⁶ In comparing the ACQUITY Column to the 7.8 x 150 mm XBridge Column we generally observe more resolution of the HMWS variants for the ACQUITY Column due to its smaller particle size while LMWS2 is generally more separated from the peak tail of the monomer for the 7.8 x 150 mm XBridge Column due to LC system dispersion effects.

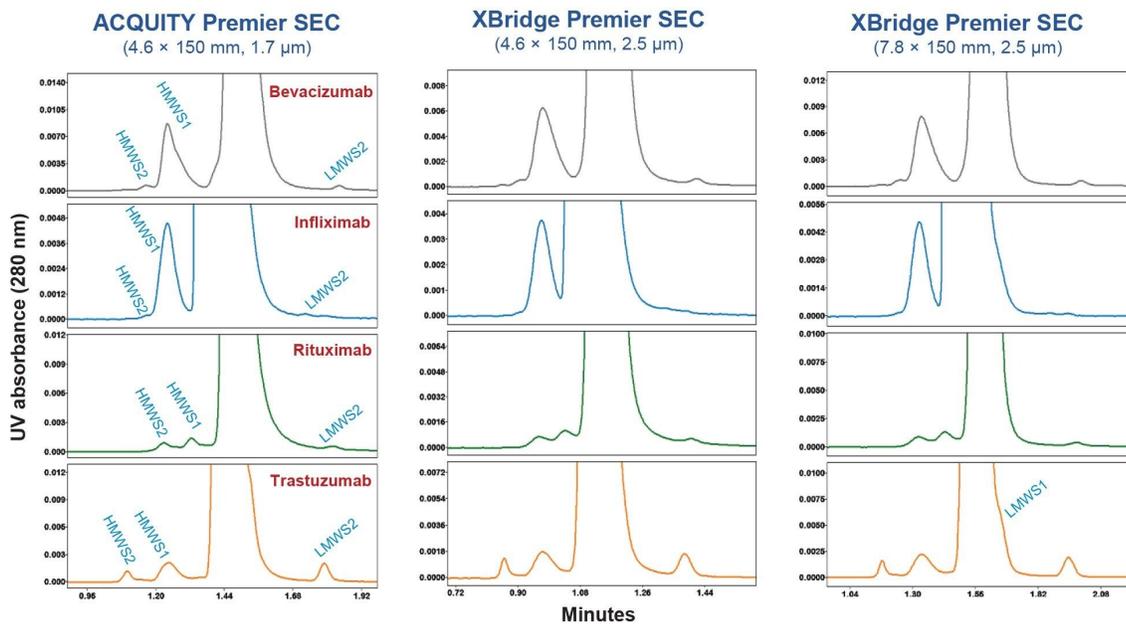


Figure 1. Shown is a comparison of high-throughput biosimilar mAb sample SEC separations using 150 mm length MaxPeak Premier SEC Columns. The flow rates used were 0.75 mL/min for the 4.6 x 150 mm ACQUITY Premier SEC Column, 1.0 mL/min for the 4.6 x 150 mm XBridge Premier SEC Column, and 2.0 mL/min for the 7.8 x 150 mm XBridge Premier SEC Column, resulting in analysis times of 2.8 min, 2.1 min, and 3.0 min, respectively. Additional experimental conditions and peak descriptions are provided in text.

For these samples, HMWS2 and HMWS1 are presumed to predominately represent multimeric forms of the mAb and dimeric self-associated forms of the mAb. Antibody fragmentation can also be observed in these samples as LMWS1 and LMWS2. Under these conditions, LMWS1, which is presumed to be primarily the result of a single cleavage in the mAb hinge region yielding an approximately 100 KDa fragment comprised of a covalent Fc domain and a single Fab domain, is only observed as a slight trailing shoulder on the main (monomer) peak for trastuzumab with the 7.8 x 150 mm column and was therefore not quantified. LMWS2, which is principally comprised of single Fab and Fc domains resulting from the cleavage of both heavy chains in the hinge region, was quantified for all the antibody samples except for infliximab where it was not reported due to its consistently low abundance ($\leq 0.02\%$). Since LMWS2 is generally generated through the same degradation pathway as LMWS1 it may be reasonable to quantify only LMWS2 in order to monitor that degradation pathway for selected samples.

Expanded views of representative chromatograms from the three column lifetime studies are presented in Figures 2 through 4. We observe that the overall chromatographic profiles appear consistent with respect to retention times and the separations of HMWS and the LMWS2 size variants including their partially resolved shoulder peaks.

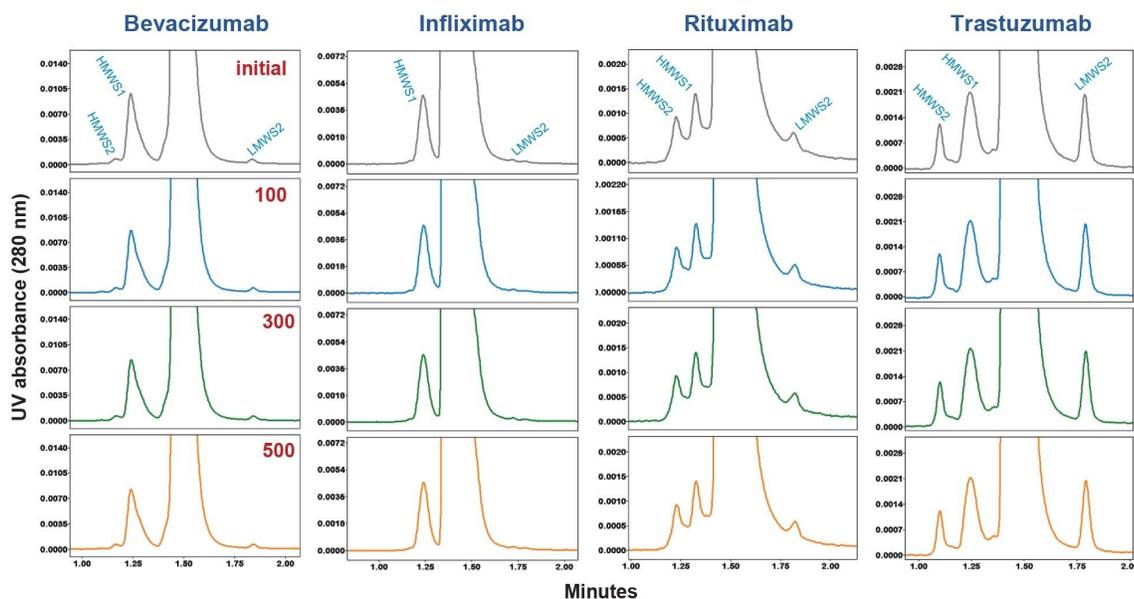


Figure 2. ACQUITY Premier SEC (4.6 x 150 mm) expanded-scale chromatograms of biosimilar mAb samples from high-throughput column lifetime study. Approximate initial injection and injection numbers 100, 300, and 500 are shown. DPBS was used as a mobile phase and the flow rate was 0.75 mL/min with an analysis time of 2.8 min. Additional experimental conditions and peak descriptions are provided in text.

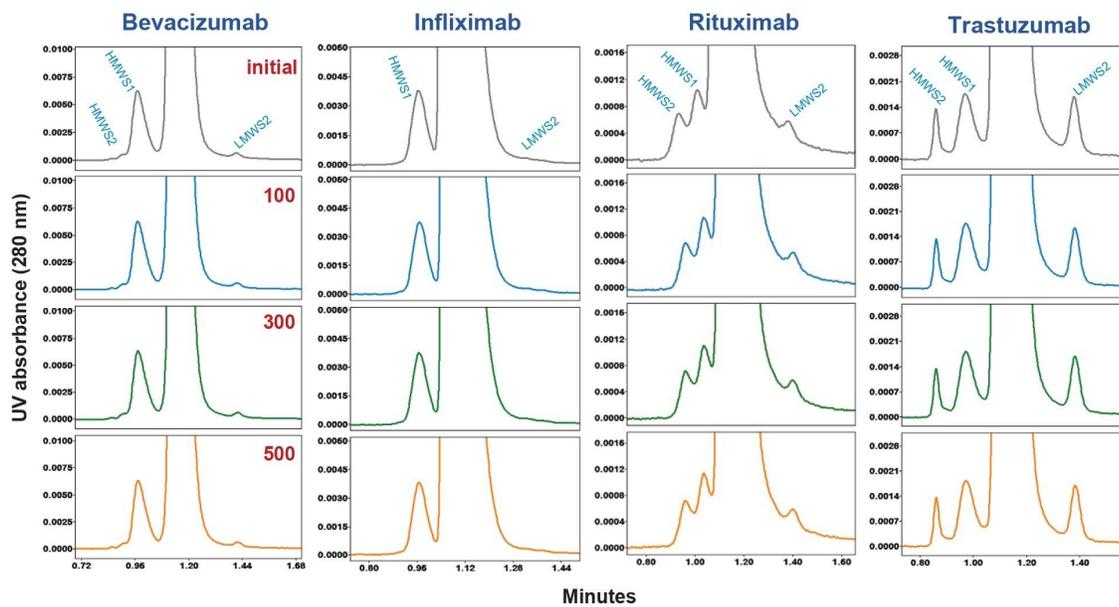


Figure 3. XBridge Premier SEC (4.6 x 150 mm) expanded-scale chromatograms of biosimilar mAb samples from high-throughput column lifetime study. Approximate initial injection and injection numbers 100, 300, and 500 are shown. DPBS was used as a mobile phase and the flow rate was 1.00 mL/min with an analysis time of 2.1 min. Additional experimental conditions and peak descriptions are provided in text.

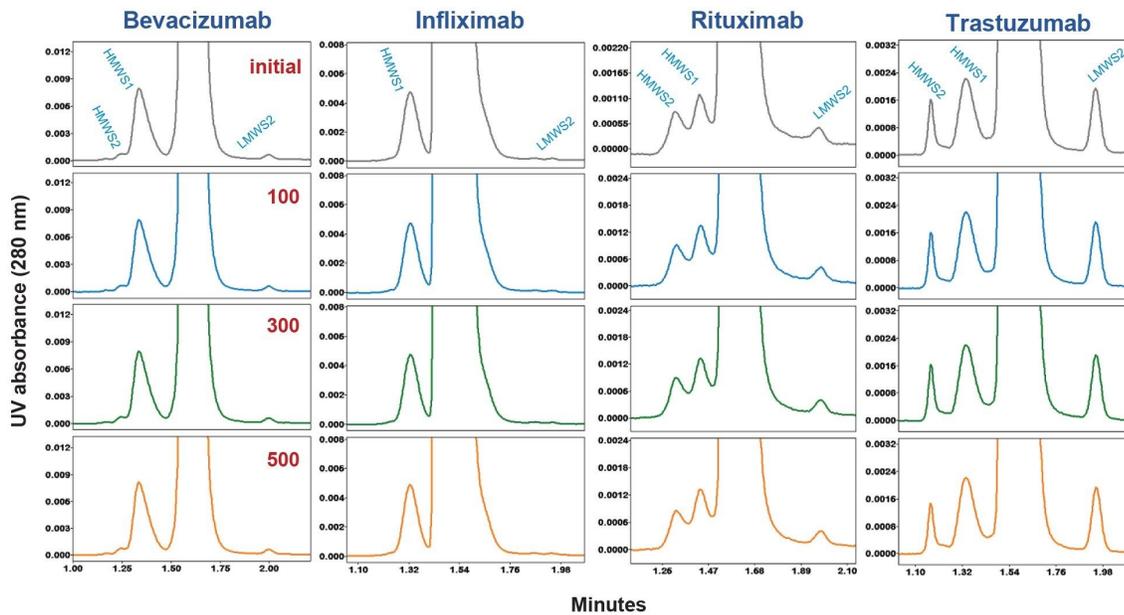


Figure 4. XBridge Premier SEC (7.8 x 150 mm) expanded-scale chromatograms of biosimilar mAb samples from high-throughput column lifetime study. Approximate initial injection and injection numbers 100, 300, and 500 are shown. DPBS was used as a mobile phase and the flow rate was 2.00 mL/min with an analysis time of 3.0 min. Additional experimental conditions and peak descriptions are provided in text.

Changes in the relative peak areas of the measurable HMWS and LMWS size variants identified in the chromatograms are presented in Figures 5 through 7. In addition, for all samples the resolution of HMWS1 was evaluated as an assessment of functional column efficiency changes. Throughout the over 500 injections the relative quantitative measurements of HMWS and LMWS impurities, and the resolution of HMWS1 remained relatively unchanged for all three columns indicating that these columns are capable of sustained performance when operated above the recommended flow rates. However, it is predicted that column lifetimes will be shortened when used at these higher flow rates in comparison to the use of flow rates within the specified guidelines.

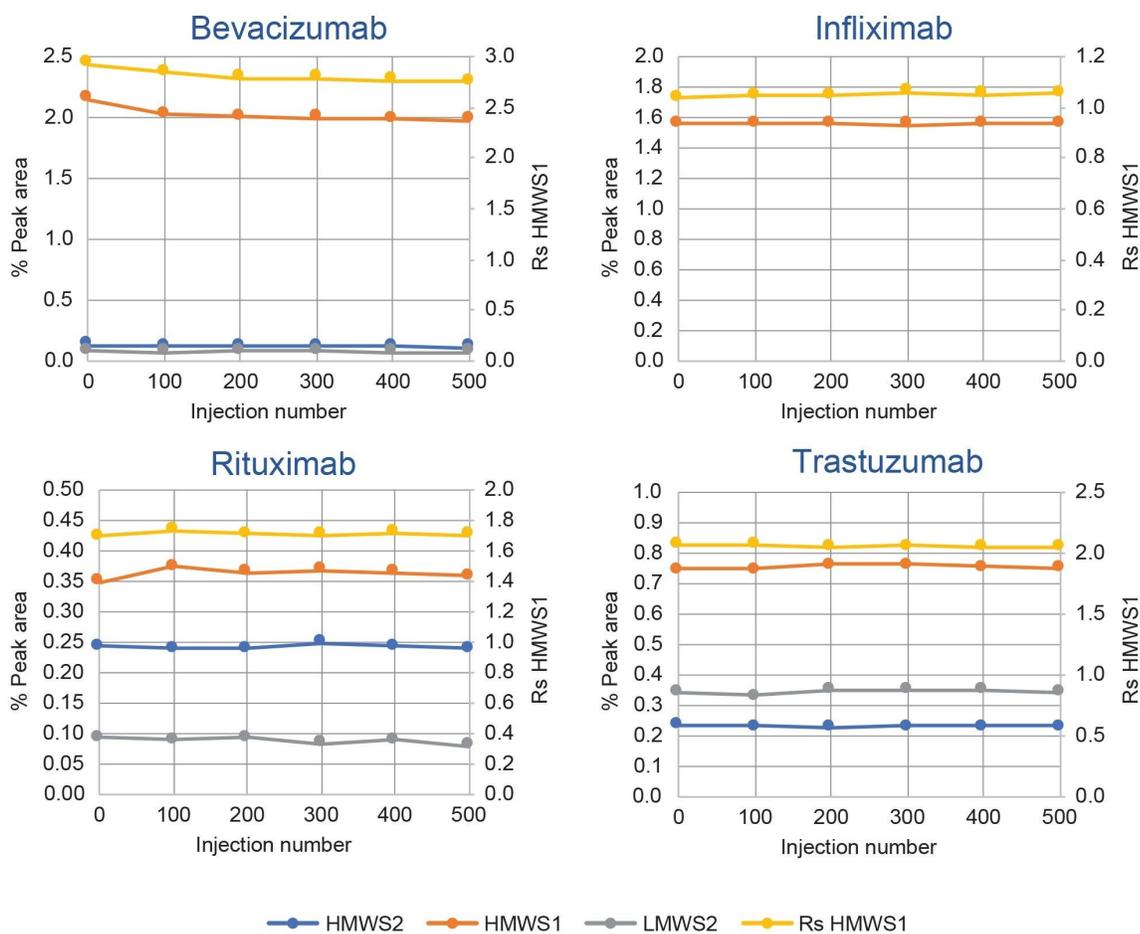


Figure 5. ACQUITY Premier Protein SEC (4.6 x 150 mm) Column high-throughput lifetime study (Figure 2) quantitative results are presented for the biosimilar mAb samples. HMWS and LMWS2 relative abundances (left axis) and the resolution (Rs, USP peak at half-height) values for HMWS1 (right axis) are plotted. Timepoints analyzed in duplicate. Approximate initial injection and injection numbers 50, 100, 200, 300, 400, and 500 are shown. Additional experimental conditions and peak descriptions are provided in text.

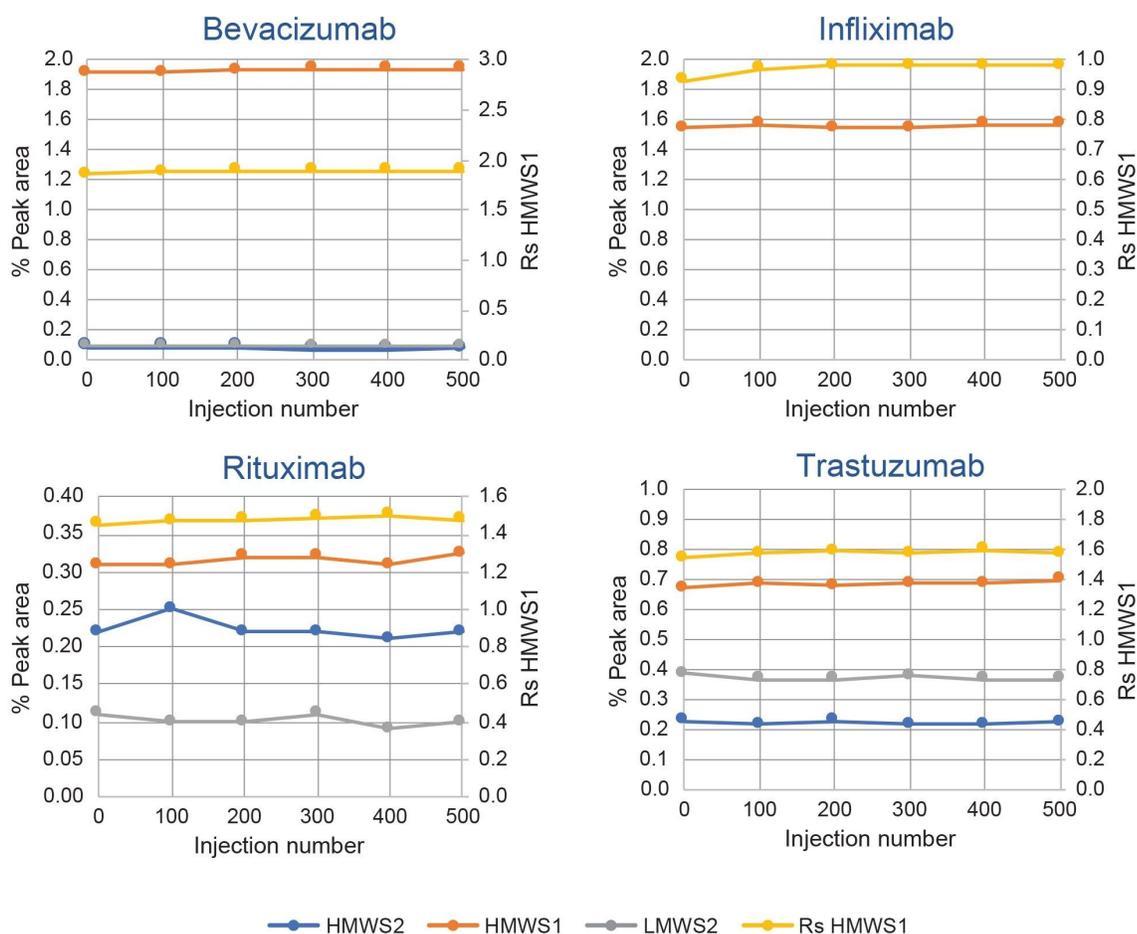


Figure 6. XBridge Premier Protein SEC (4.6 x 150 mm) Column high-throughput lifetime study (Figure 3) quantitative results are presented for the biosimilar mAb samples. HMWS and LMWS2 relative abundances (left axis) and the resolution (Rs, USP peak at half-height) values for HMWS1 (right axis) are plotted. Timepoints for only initial and final timepoints analyzed in duplicate. Approximate initial injection and injection numbers 50, 100, 200, 300, 400, and 500 are shown. Additional experimental conditions and peak descriptions are provided in text.

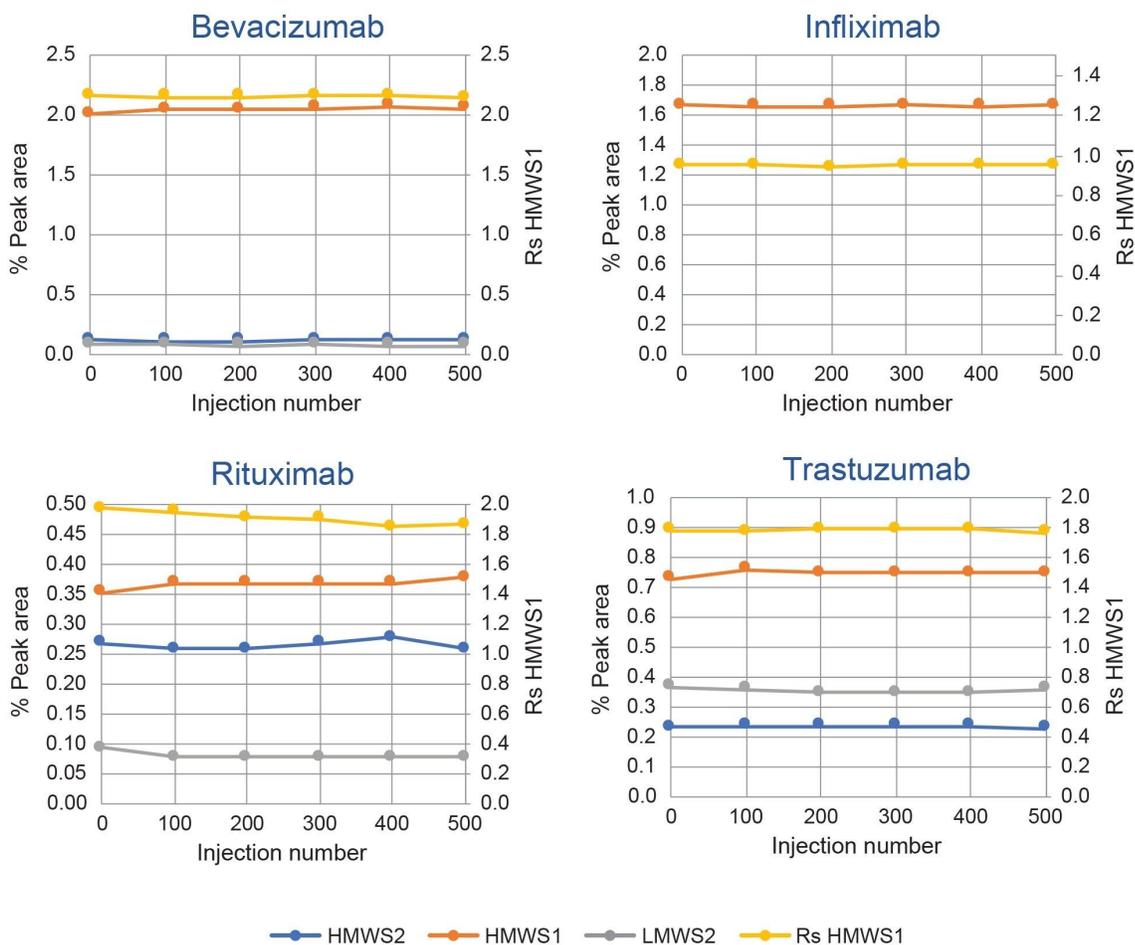


Figure 7. XBridge Premier Protein SEC (7.8 x 150 mm) Column high-throughput lifetime study (Figure 2) quantitative results are presented for the biosimilar mAb samples. HMWS and LMWS2 relative abundances (left axis) and the resolution (Rs, USP peak at half-height) values for HMWS1 (right axis) are plotted. Timepoints for only initial and final timepoints analyzed in duplicate. Approximate initial injection and injection numbers 50, 100, 200, 300, 400, and 500 are shown. Additional experimental conditions and peak descriptions are provided in text.

Conclusion

It was previously demonstrated that the technological advancements in both the column hardware and packed particle chemistry of the ACQUITY and XBridge Premier 250 Å SEC Columns provide extended column lifetimes when using physiological pH mobile phases.⁴ While this previous column lifetime study focused on high resolution separations with 300 mm length columns at flow rates below the recommended maximums, this study evaluated rapid and high-throughput separations using 150 mm columns. In addition to shorter column lengths, higher flow rates exceeding the recommended maximums were also studied resulting in analysis times between 2.1 and 3.0 minutes.

In this study, UPLC compatible 4.6 x 150 mm ACQUITY Premier SEC and XBridge Premier SEC Columns, and the HPLC compatible 7.8 x 150 XBridge Premier SEC Column were evaluated on a low dispersion UPLC (5 σ system dispersion of 7.9 μ L). While HPLC systems with adequate pressure limits (*e.g.*, Waters Arc™ HPLC System, pressure limit 9500 PSI, 655 bar) are capable of running the 4.6 x 150 mm columns at the noted flow rates, the larger system dispersion volumes of HPLC systems may degrade separation performance. Therefore, it is recommended that the larger internal diameter 7.8 x 150 XBridge Premier SEC Column be used with HPLC systems. Demonstrated consistent performance with respect to HMWS2, HMWS1, and LMWS2 quantification and the resolution between the HMWS1 (predominantly dimeric) size variant and the main peak (monomer) over the course of more than 500 injections. Performance was evaluated for a series of four antibody drug products currently marketed as biosimilars.

For this study, the mobile phase was 0.1 μ m sterile filtered and properly formulated drug product samples that are not expected to contain appreciable levels of subvisible or larger particulates (≥ 0.1 μ m) were analyzed. These two factors greatly reduce the probability of column fouling. However, if development samples that may contain larger quantities of subvisible or larger particles are being analyzed, it is recommended that a guard column (MaxPeak Premier Protein SEC Guard, p/n: [186009969](https://www.waters.com/nextgen/global/shop/columns/186009969-maxpeak-premier-protein-sec-guard-250a-25--m-46-x-30-mm-1-pk.html) <
<https://www.waters.com/nextgen/global/shop/columns/186009969-maxpeak-premier-protein-sec-guard-250a-25--m-46-x-30-mm-1-pk.html>>) or sample pre-treatment such as centrifugation be used.⁶

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