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Application Note

MaxPeak Premier Protein SEC 250 Å Column Lifetimes at Physiological pH for Polysorbate (Tween) Formulated Biosimilar Monoclonal Antibodies

Stephan M. Koza, Hua Yang, Ying Qing Yu

Waters Corporation

Abstract

Self-associated or aggregated protein impurities in therapeutics is a concern due to the potential of eliciting an immunogenic response. As a result, appropriate formulations often include polysorbate (Tween 20 or Tween 80) to prevent protein self-association and surface adsorption. As size exclusion chromatography (SEC) is often used to monitor these protein-related impurities, SEC column compatibility with the protein and its formulation buffer components is also of importance.

It was demonstrated in an earlier publication that effective separations could be achieved at or near physiological pH (7.4) and ionic strength using Waters Premier Protein SEC Columns. As these moderately basic pH conditions can be detrimental to the stability of silica-based packed particles the lifetimes of these recently introduced columns were evaluated at pH 7.4 for the analysis of four marketed mAb biosimilars formulated with either Tween 20 or Tween 80. Since it has been proposed in an earlier publication that these formulation surfactants may decrease the lifetime of the previous generation Waters BEH Protein SEC Columns, the lifetime one of these columns was reassessed to determine if the improvements in column lifetime could be realized with

the Waters Premier SEC™ Columns. For this study, Waters XBridge Premier™ (2.5 µm particle size, 250 Å pore size), ACQUITY Premier Protein SEC™ (250 Å, 1.7 µm), and ACQUITY Protein SEC™ (200 Å, 1.7 µm) Column performances were evaluated over the course of 500 analyses. The separation performance was evaluated for both self-associated and fragmented mAb size variants.

Benefits

- Stability to evaluate protein therapeutic size variants using SEC with buffers at or near physiological pH (~7.4) and ionic strength (~150 mM)
- Column lifetimes greater than 500 analyses for therapeutic mAb samples formulated with polysorbates (Tween 20 or Tween 80)
- Reproducible determinations of both HMWS and LMWS impurity levels throughout lifetime study

Introduction

Self-associated or aggregated protein impurities, generally referred to as high molecular weight species (HMWS), in parenteral protein therapeutics may elicit an immunogenic response in some patients.[1] As a result, appropriate formulations often include a non-ionic surfactant such as polysorbate (Tween 20 or Tween 80) to prevent protein self-association.[2] Size exclusion chromatography (SEC) is often used to monitor HMWS and in some cases therapeutic protein related fragments (low molecular weight species, LMWS). Consequently, SEC column compatibility with both the protein and its formulation buffer components, in addition to the mobile phase used, are key considerations when developing an SEC method.

Both the column hardware and packed particle chemistry of the Waters XBridge Premier Protein SEC 250 Å, 2.5 µm and ACQUITY Premier Protein SEC 250 Å, 1.7 µm columns have been optimized to reduce protein-surface interactions.[3] Specifically, for the SEC analysis of proteins formulated with polysorbate, bonding of the bridged ethyl hybrid (BEH) SEC particle with hydroxy-terminated polyethylene oxide (PEO) instead of diol to further minimize secondary hydrophobic interactions with proteins would also be predicted to reduce potential fouling of the column with surfactant.[4]

For this study, Waters XBridge Premier (2.5 µm particle size, 250 Å pore size), ACQUITY Premier Protein SEC (250 Å, 1.7 µm), and ACQUITY Protein SEC (200 Å, 1.7 µm) Column performances were evaluated over the course

of 500 analyses of the four different marketed mAb drug products currently available as biosimilars in the USA. The samples of bevacizumab, infliximab, and rituximaub were biosimilar drug products and the sample of trastuzumab was the originator drug product. The bevacizumab and trastuzumab drug products are formulated with Tween 20, and the infliximab and rituximab drug products are formulated with Tween 80.

In an earlier study we demonstrated the ability of the Waters Premier SEC columns to provide effective separations using Dulbecco's phosphate buffered saline (DPBS) at a mildly basic physiological pH (~7.4) and ionic strength (~150 mM) as a mobile phase.[3] Since basic pH conditions can reduce the stability of silica-based packed particles, the Waters Premier Column lifetimes were performed using DPBS as mobile phase while the ACQUITY Protein SEC Column required a 1.5X DPBS (pH 7.4, ionic strength ~225 mM) buffer to achieve an effective separations for all four mAbs.

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-30A APH Column Heater
Detection:	ACQUITY UPLC TUV Detector™ with 5 mm titanium flow cell, wavelengths: 280 nm and 214 nm
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n:

	186002639)
Column(s):	XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 x 300 mm, Column plus mAb Size Variant Standard (p/n: 176005070) ACQUITY Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 300 mm, Column plus mAb Size Variant Standard (p/n: 176005072) ACQUITY UPLC Protein BEH SEC Column, 250 Å, 1.7 µm, 4.6 x 300 mm, Column and BEH200 SEC Protein Standard Mix (p/n: 176003905)
Column temp.:	Ambient
Sample temp.:	6 °C
Injection volume:	Infliximab and rituximab: 10 µL for 7.8 x 300 mm column and 4 µL for 4.6 x 300 mm columns Bevacizumab and trastuzumab: 5 µL for 7.8 x 300 mm column and 2 µL for 4.6 x 300 mm columns
Flow rate:	0.2–0.5 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 (FR 4)

Results and Discussion

XBridge Premier, ACQUITY Premier, and ACQUITY BEH Column Lifetime Assessments

The separations of HMWS and LMWS impurities were evaluated for over 500 analyses of four different mAb samples (~125 analyses per mAb sample) using the XBridge Premier Protein (7.8 x 300 mm), ACQUITY Premier Protein (4.6 x 300 mm), and ACQUITY Protein BEH (4.6 x 300 mm) SEC columns. All four of the mab samples are formulated with surfactant above the critical micelle concentration (CMC). Bevacizumab (MVASI) and trastuzumab (Herceptin) are formulated with 0.04% and 0.009% Tween 20, which has a CMC of 0.006%.[5] Rituximab (Ruxience) and infliximab (AVSOLA) are formulated with 0.02% and 0.0.005% Tween 80, which has a CMC of 0.001%.[5] The SEC mobile phase used for the Premier Protein SEC columns was Dulbecco's phosphate buffered saline (DPBS) at a mildly basic physiological pH (~7.4) and ionic strength (~150 mM), and for the ACQUITY Protein SEC Column DPBS at a 1.5X concentration (~225 mM ionic strength) was used.[3] For this study, 10X DPBS concentrate was diluted to the targeted concentration by the LC. In addition, the 10X DPBS concentrate and Milli-Q 18 MΩ water were 0.1 µm sterile filtered prior to use.

The flow rate used for the XBridge Premier Column time-point analyses was 0.5 mL/min (25-minute run time). Intermediate timepoints were run at a flow rate of 0.80 mL/min with interlaced mAb injections (10-minute runtime). The ACQUITY Premier Column was run at 0.35 mL/min for intermediate runs and for the infliximab and trastuzumab time-point analyses. The flow rate was reduced to 0.20 mL/min for bevacizumab and rituximab time point analyses to improve upon the inadequate resolution observed for the LMWS1 fragment in those samples at 0.35 mL/min. The mAb LMWS1 peak elutes immediately after the main (monomer) peak and as a result is generally more susceptible to resolution loss due to extra-column dispersion at abundances of approximately 0.5% or less on an LC with a 5σ system dispersion greater than 10 µL.[6] The ACQUITY UPLC H-Class equipped with a CH-30A column heater with an active preheater (APH) used for the ACQUITY Premier column lifetime study had a 5σ system dispersion of 17 µL. The ACQUITY BEH SEC Column was run at a flow rate of 0.35

mL/min for all analyses on a comparably configured ACQUITY UPLC H-Class, and mAb injections were interlaced for the intermediate timepoints. All timepoints were analyzed in duplicate.

Full-scale chromatograms for the mAbs separated on the XBridge Premier, ACQUITY Premier, and ACQUITY Protein BEH columns are presented in Figure 1. All three columns produced comparable overall chromatographic profiles with trace levels of HMWS and LMWS impurities. Expanded views of representative chromatograms from the three column lifetime studies are presented in Figures 2 through 4. Upon visual scrutinization we observe that the overall chromatographic profiles appear consistent with respect to retention times and the separations of HMWS and LMWS size variants including their partially resolved shoulder peaks. 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 was also observed in these samples as LMWS1 and LMWS2. LMWS1 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, while LMWS2 is principally comprised of single Fab and Fc domains.

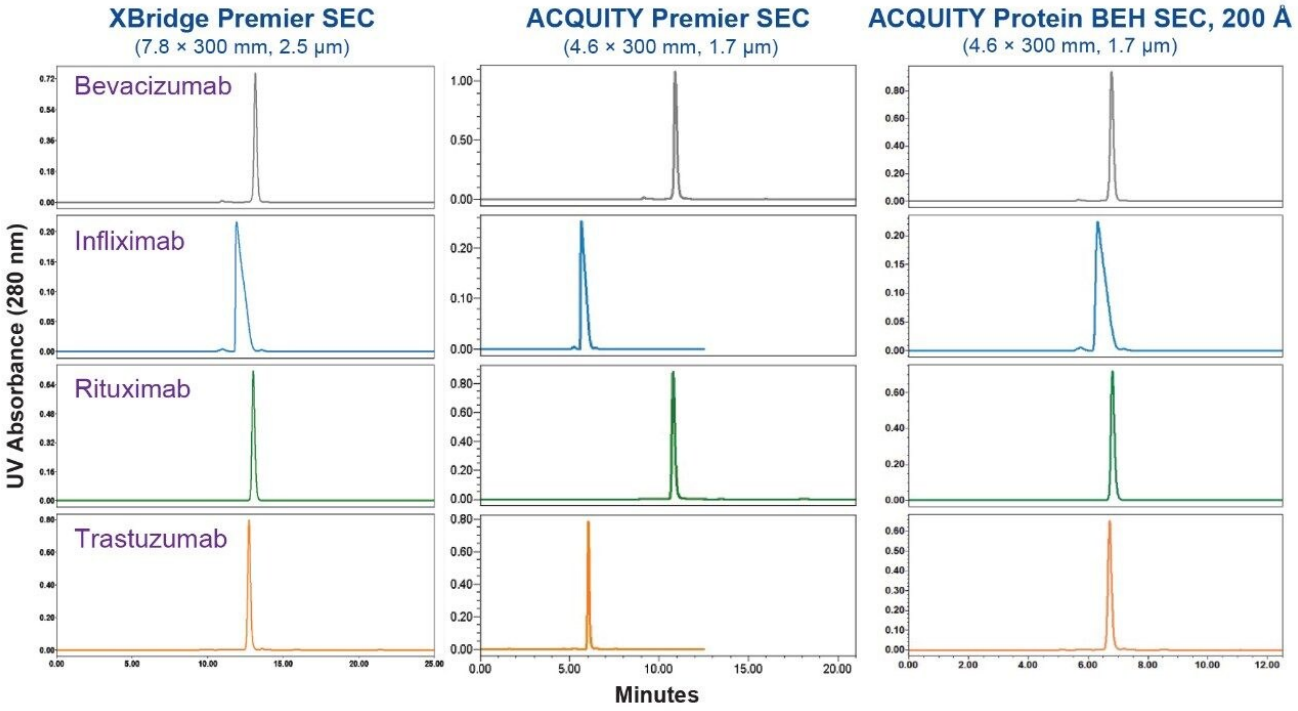

Figure 1. Full-scale chromatograms of biosimilar mAb sample SEC separations using Waters Premier SEC and Protein BEH SEC columns. DPBS was used as a mobile phase for the Waters Premier SEC Columns and DPBS at 1.5 times its specified concentration was used for the Protein BEH SEC Column. For the ACQUITY Premier SEC Column bevacizumab and rituximab were analyzed using a flow rate of 0.20 mL/min while infliximab and trastuzumab were analyzed at 0.35 mL/min. The flow rate used for the ACQUITY Protein BEH SEC Column was 0.35 mL/min and 0.50 mL/min was used for the XBridge Premier SEC Column. Additional experimental conditions provided in text.

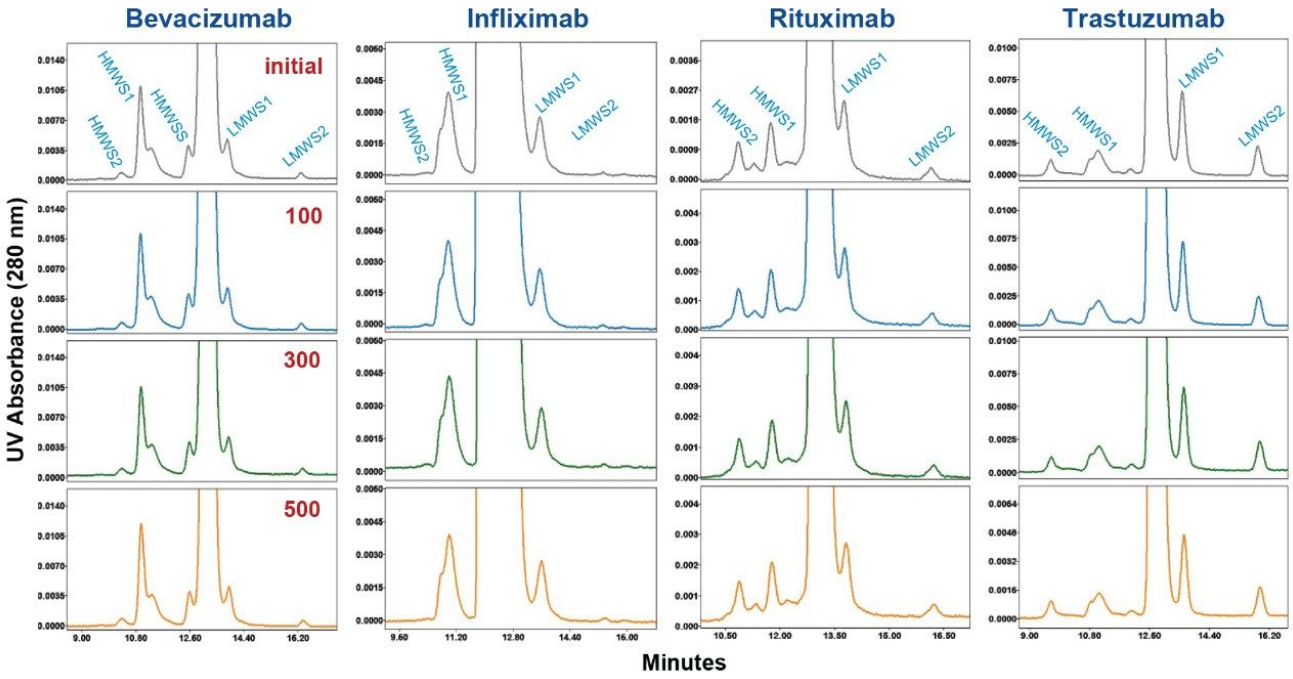

Figure 2. XBridge Premier 250 Å SEC Column expanded-scale chromatograms of biosimilar mAb sample SEC separations from lifetime study. Approximate initial injection and injection numbers 100, 300, and 500 are shown. DPBS was used as a mobile phase and the flow rate was 0.50 mL/min. Additional experimental conditions and peak descriptions are provided in text.

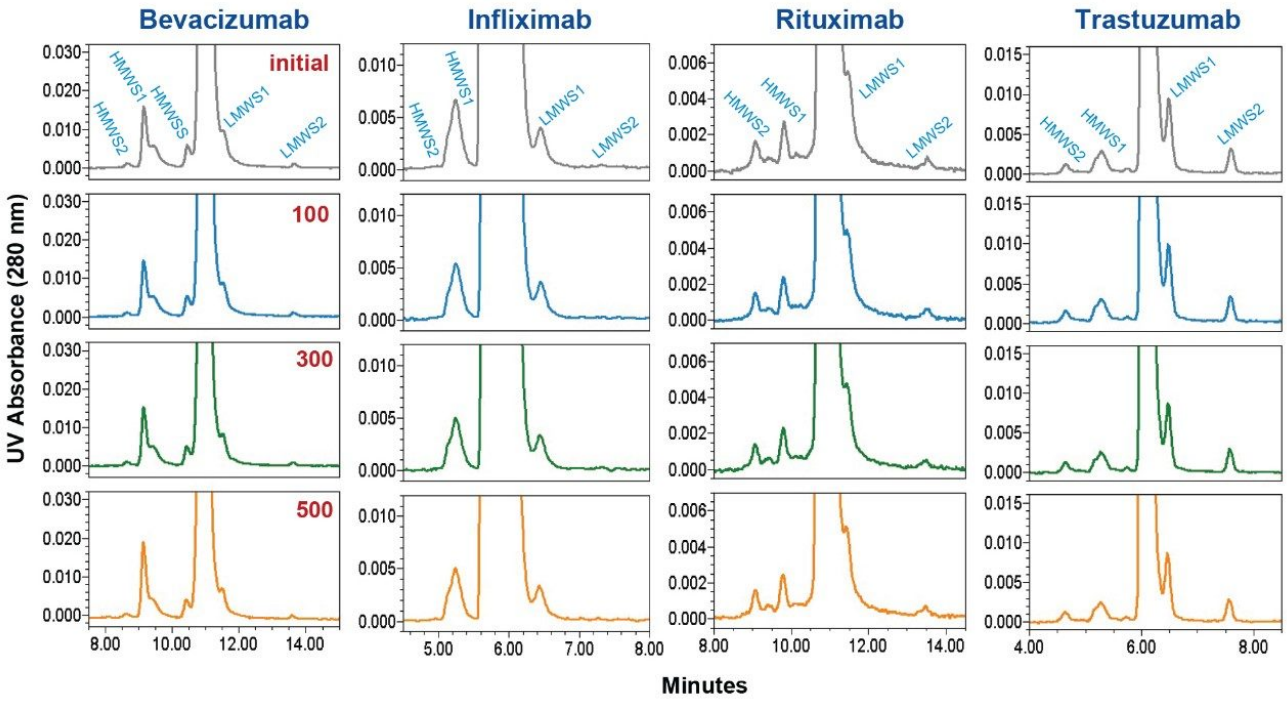

Figure 3. ACQUITY Premier 250 Å SEC Column expanded-scale chromatograms of biosimilar mAb sample SEC separations from lifetime study are presented. Approximate initial injection and injection numbers 100, 300, and 500 are shown. DPBS was used as a mobile phase. Bevacizumab and rituximab were analyzed using a flow rate of 0.20 mL/min while infliximab and trastuzumab were analyzed at 0.35 mL/min. Additional experimental conditions and peak descriptions are provided in text.

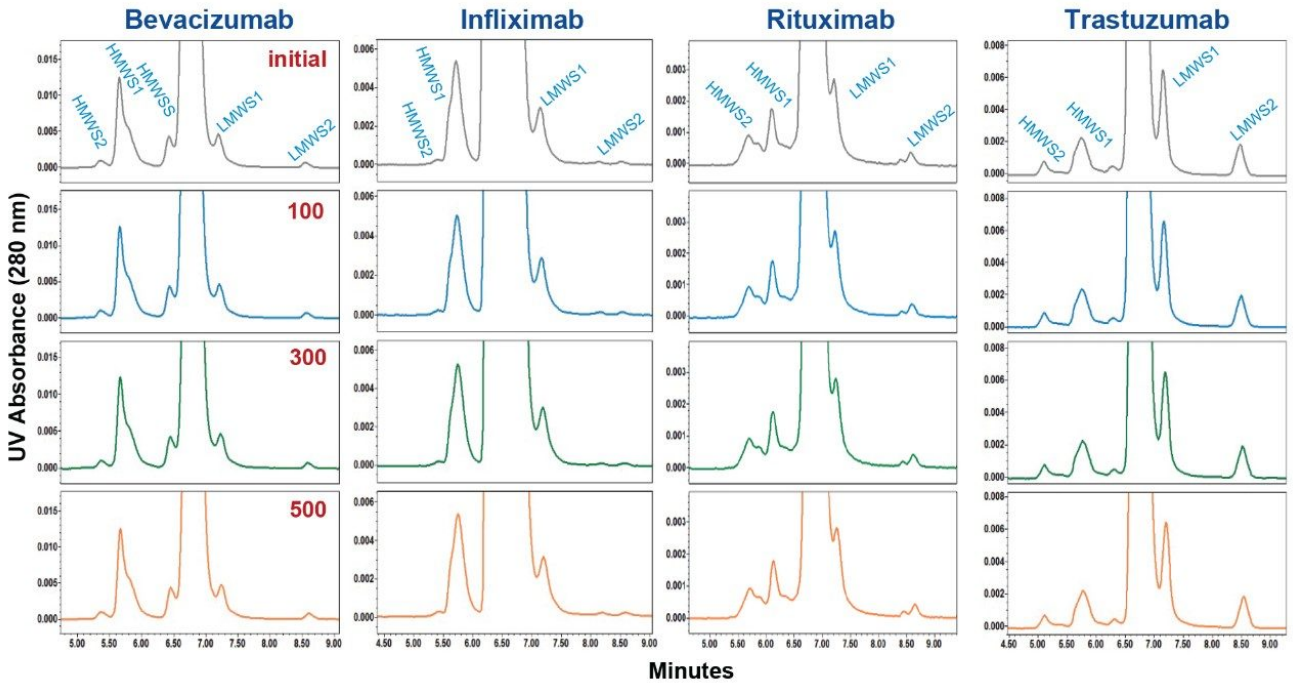

Figure 4. ACQUITY Protein BEH 200 Å SEC expanded-scale chromatograms of biosimilar mAb sample SEC separations from column lifetime study are presented. Approximate initial injection and injection numbers 100, 300, and 500 are shown. DPBS was used as a mobile phase and the flow rate was 0.35 mL/min. Additional 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. The relative peak areas for a HMWS shoulder (HMWSS) observed in bevacizumab was also monitored, while results for the LMWS2 of infliximab was not reported due to its consistently low abundance ≤0.02%) In addition, for all samples the partial resolution of LMWS1 was evaluated using peak-to-valley ratio (P/V, USP Chapter <621>). Due to its low abundance and elution position in the tail of the predominant monomer peak the P/V of LMWS1 acts as a bellwether for functional column efficiency loss.

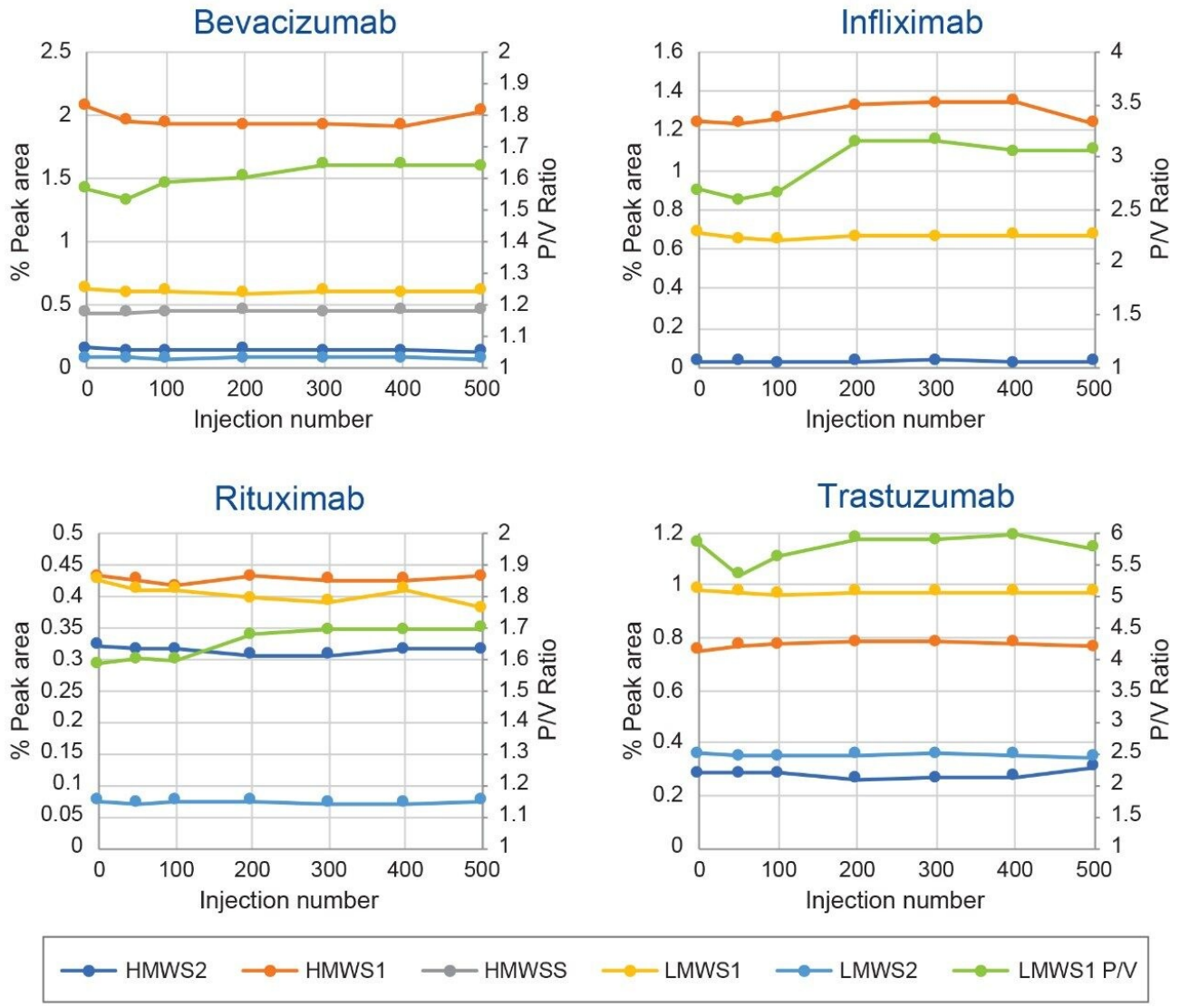

Figure 5. Shown are the XBridge Premier Protein SEC, 250 Å SEC Column lifetime study quantitative results (Figure 2). Included are the HMWS and LMWS relative abundances (left axis) and the P/V values for LMWS1 (right axis) for the biosimilar mAb samples evaluated. Timepoints were analyzed in duplicate. 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.

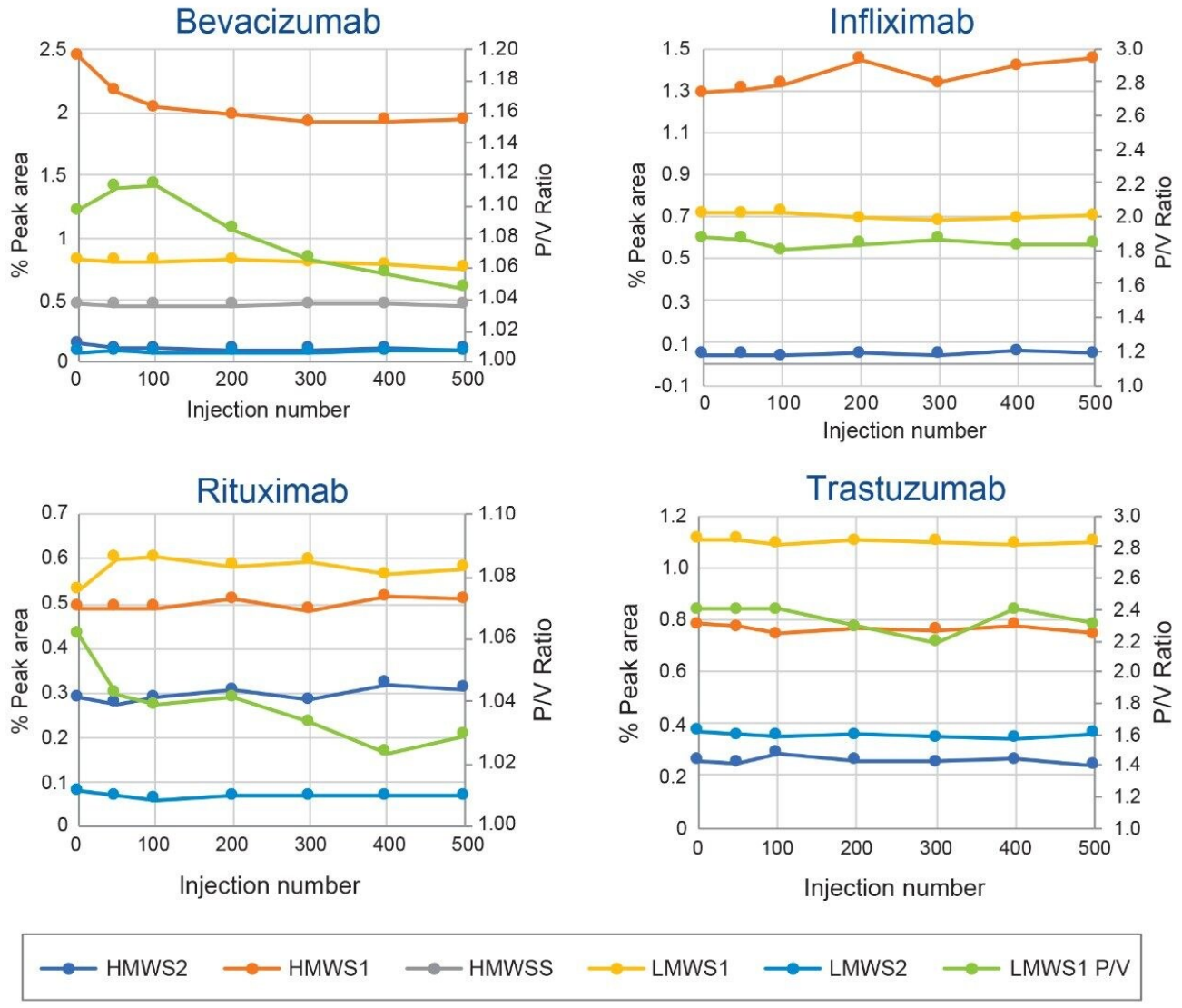

Figure 6. Shown are the ACQUITY Premier Protein SEC, 250 Å SEC Column lifetime study quantitative results (Figure 3). Included are the HMWS and LMWS relative abundances (left axis) and the P/V values for LMWS1 (right axis) for the biosimilar mAb samples evaluated. Timepoints were analyzed in duplicate. 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.

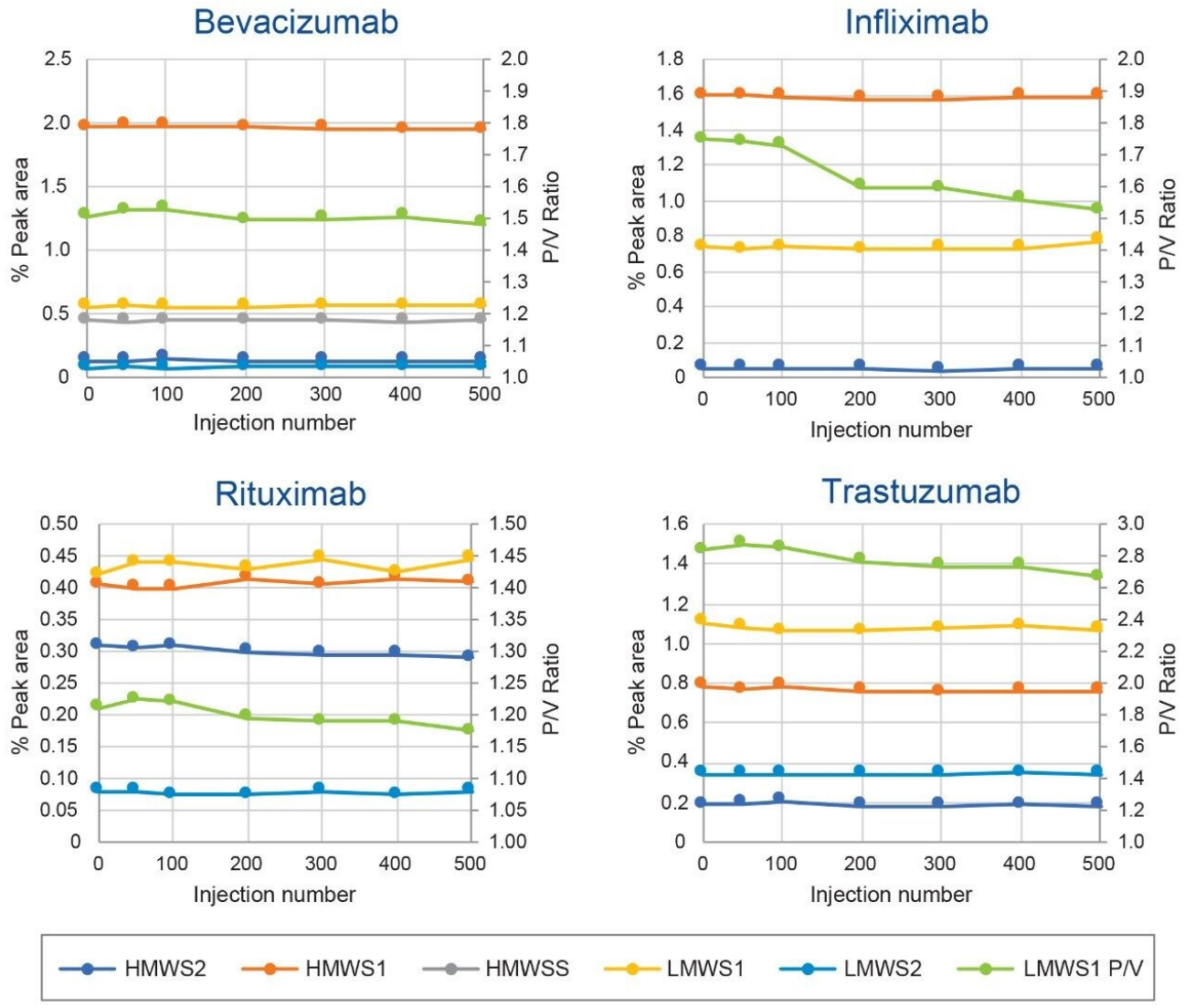

Figure 7. Shown are the ACQUITY Protein BEH 200 Å SEC Column lifetime study quantitative results (Figure 4). Included are the HMWS and LMWS relative abundances (left axis) and the P/V values for LMWS1 (right axis) for the biosimilar mAb samples evaluated. The timepoints were analyzed in duplicate. Approximate initial injection and injection numbers 50, 100, 200, 300, 400, and 500 are shown. Additional experimental conditions and peak descriptions are provided in text.

Throughout the over 500 mAb drug product injections the chromatographic profiles, relative quantitative measurements of HMWS and LMWS impurities, and P/V of LMWS1 remained relatively unchanged for all three

columns. The most notable HMWS variations observed were for the ACQUITY Premier Column where higher levels of HMWS1 were observed for the early timepoints of bevacizumab. Since no major change in separation of the HMWS1 related sub-species were observed this difference is presumed to be related to sample handling and aliquoting. In addition, minor losses in the resolution of LMWS1 were observed for some mAb samples with the ACQUITY Premier Column and the ACQUITY Protein BEH Column, while P/V values were consistent for the XBridge Premier Column. This is likely due to the lower initial P/V values observed for the two ACQUITY columns being more greatly impacted by general losses in efficiency and the greater propensity for columns packed with smaller particle sizes to be impacted by particulates in the sample or mobile phase. Of note, is that the quantifications of LMWS1 was generally unchanged throughout all three lifetime studies.

As previously noted, bonding of the bridged ethyl hybrid (BEH) SEC particle with hydroxy-terminated polyethylene oxide (PEO) instead of diol was predicted to reduce potential fouling of the column with surfactant. However, in this study the diol-bonded ACQUITY Protein BEH exhibited effective performance after more than 500 analyses of mAb samples formulated with Tween 20 or Tween 80 in contrast to a previous study.[4] Since the ionic strength and pH of the phosphate and sodium chloride mobile phase used for that initial study (~200 mM ionic strength, pH 6.8) is similar to that of 1.5X DPBS (~225 mM ionic strength, pH 7.4), it is likely that the loss in ACQUITY Protein BEH Column performance observed in the earlier study was due to sample or mobile phase particulates.

Conclusion

It was previously demonstrated that the technological advancements in both the column hardware and packed particle chemistry of the XBridge and ACQUITY Premier 250 Å SEC columns provide low levels of unspecific protein-column interactions thereby allowing for the analysis of therapeutic protein HMWS and LMWS with DPBS buffers at or near a mildly basic physiological pH (about 7.4) and ionic strength (about 150 mM). Using DPBS buffers, we evaluated the column lifetime performance of these columns, along with a previous generation ACQUITY Protein BEH Column (200 Å), all of which are specified for long term usage at an upper pH of 8.0. The therapeutic drug product mAb samples used for these lifetime studies are formulated with Tween 20 or Tween 80 and were injected without prior dilution.

The effective column performance with respect to HMWS and LMWS separation and quantification was maintained on all three columns for over more than 500 analyses of therapeutic mAb samples. These results

demonstrate that extended column lifetimes with a physiological pH mobile phase and for protein samples formulated with polysorbate surfactant can be achieved on the XBridge Premier SEC, ACQUITY Premier SEC, and ACQUITY Protein BEH columns. Additional considerations to improve the lifetime performance of these columns packed with either 1.7 µm or 2.5 µm particle sizes include 0.1 µm sterile filtration of the mobile phases and ensuring that samples do not contain appreciable levels of subvisible or larger particulates (≥0.1 µm). In the event that development samples may contain larger quantities of subvisible or larger particles 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>) or sample pre-treatment such as centrifugation be used.[7]

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