High-throughput and Sensitive Size Exclusion Chromatography (SEC) of Biologics Using Agilent AdvanceBio SEC Columns

Agilent AdvanceBio SEC 300 Å, 2.7 µm columns

Application note

Bio-Pharmaceutical

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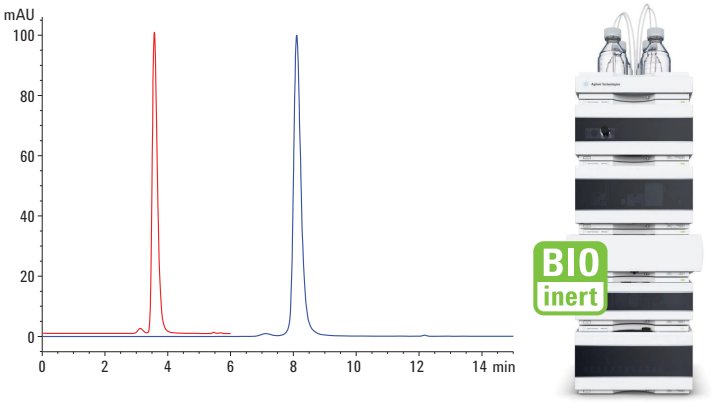
Abstract

Monoclonal antibody (mAb) aggregation can arise due to multiple mechanisms during cell culture, harvest, purification, storage, and filling. Size Exclusion Chromatography (SEC) is a standard method for size-based separation of mAbs. It is considered as a reference and powerful technique both for qualitative and quantitative assessment of aggregates. SEC analysis of multiple in-process samples not only takes several hours to complete but can also critically influence aggregation status as the samples are held in an unfavorable environment. This study demonstrates the use of shorter and narrower Agilent AdvanceBio SEC columns for fast, high-resolution, sensitive, and reproducible separation and quantitation of mAbs and Antibody Drug Conjugate (ADC). Separation and quantitation was achieved in less than 4 minutes, and more importantly, this method was able to monitor and detect aggregates created due to stress.



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Introduction

Proteins frequently aggregate when exposed to stress conditions such as changes in pH, temperature, or concentration. Aggregation can occur at many different stages of the production process: upstream, downstream, or simply during storage. Size Exclusion Chromatography (SEC) is a method for monitoring and characterizing aggregates of monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs). However, SEC separations are usually carried out with large columns operated at comparatively low flow rates, and analysis times are often prolonged. More recently, Ultra High Performance Liquid Chromatography (UHPLC) using sub-2 µm columns has been employed to overcome these challenges, demonstrating much shorter analysis times. However, when applying very fine particles and high flow rates, thermal and shearing forces can become critical for temperature or pressure-sensitive proteins [1]. Furthermore, SEC analysis of ADCs using aqueous mobile phase provides poor peak shape and unacceptable resolution between aggregates and monomers. These problems may be explained by nonspecific interactions between hydrophobic cytotoxic drugs and the stationary phase. To solve this problem and improve peak shape, various organic modifiers are added to the SEC mobile phase. However, these organic modifiers could potentially damage the protein and compromise column lifetime.

Agilent has made several advancements in SEC to improve the quality of information that can be obtained. Among them, is the development of shorter (150 mm) and narrower (4.6 mm) columns having an optimum pore volume and pore size, combined with a unique hydrophilic polymer coating. These developments ensure that peaks are well resolved and sharp without the need to add any organic modifiers to the aqueous mobile phase. This application note demonstrates the application of shorter and narrow Agilent AdvanceBio SEC columns for fast high-throughput SEC analysis of mAbs and ADCs. This study also shows the usage of these columns for the quantitation of these molecules.

Materials and Methods

Instruments, columns, and standards

A biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC system with a maximum pressure of 600 bar consisting of the following modules was used:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity Diode Array Detector with 60 mm Max-Light high sensitivity flow cell (G4212B option 33)
- Agilent AdvanceBio SEC, 300 Å, 7.8 × 150 mm packed with 2.7 µm particles (p/n PL1180-3301)
- Agilent AdvanceBio SEC, 300 Å, 4.6 × 150 mm packed with 2.7 µm particles (p/n PL1580-3301)
- AdvanceBio SEC 300 Å Protein Standard, lyophilized. 1.5 mL (p/n 5190–9417)

Software

Agilent ChemStation B.04.03 (or higher)

SEC parameters

Table 1 shows the chromatographic parameters for SEC using an Agilent 1260 Bio-inert LC System.

Table 1. Chromatographic parameters used for SEC HPLC.

Parameter	Conditions
Mobile phase	150 mM sodium phosphate, pH 7.0 (mobile phase A)
TCC Temperature	Ambient
Isocratic run	Mobile phase A
Injection volume	5 µL for 7.8 × 150 mm column and 2 µL for 4.6 × 150 mm column
Flow rate	1 mL/min for 7.8 × 150 mm column 0.35 mL/min for 4.6 × 150 mm column
UV detection	220 and 280 nm

Reagents, samples, and materials

Innovator and biosimilar rituximab, Herceptin, and ADC were purchased from local pharmacy and stored according to the manufacturer's instruction. Monobasic and dibasic sodium hydrogen phosphate, hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich. All chemicals and solvents used were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used.

Calibration of Agilent AdvanceBio SEC columns

AdvanceBio SEC columns were calibrated by measuring the elution volumes of Agilent 300 Å Protein standards (thyroglobulin (670 KDa), γ-globulin (158 KDa), ovalbumin (44 KDa), myoglobin (17 KDa), and angiotensin II (1,000 Da). The log molecular weight (logMW) values of the AdvanceBio SEC protein standards were plotted against the elution volumes to determine the exclusion limit.

Limit of quantitation (LOQ) and limit of detection (LOD)

As an example, Herceptin and ADC were used for LOD and LOQ measurements. The protein concentration that provided a signal-to-noise ratio (S/N) > 3 was considered the LOD, and S/N > 10 was considered the LOQ.

Procedure

To calculate area and retention time (RT) deviation, 5 µL and 2 µL of mobile phase was injected as blank, followed by six replicates of intact and stressed mAbs.

Preparation of rituximab aggregates

Innovator rituximab and ADC were used for aggregate analysis as a representative example of mAbs and ADC. Aggregates were induced as described earlier with slight modification [2].

1. 1 M HCl was slowly added drop-wise to the sample solution (2 mg/mL) to change the pH from 6.0 to 1.0.
2. 1 M NaOH was added to adjust the pH to 10.0.
3. 1 M HCl was added again to adjust the pH back to 6.0.

There was a waiting time of approximately 1 minute between the pH shifts, while stirring constantly at 500 rpm. The resulting solution was incubated at 60 °C for 60 minutes.

Results and Discussion

Separation and detection

The AdvanceBio SEC columns were calibrated using a series of Agilent 300Å protein standards with known molecular weight. Thyroglobulin aggregates (void peak) in the protein marker were used to calculate the void volume. These aggregates elute at 2.56 minutes on an AdvanceBio SEC, 7.8 × 150 mm column, with a void volume (V_0) = 2.56 mL, and 2.35 minutes on 4.6 × 150 mm column, corresponding to a V_0 = 0.805 mL, respectively. The calibration curve for proteins separated on the AdvanceBio SEC column shows a linear relationship, and defines the exclusion limit (670 kDa) and total permeation limit (1,000 Da) for the protein range analyzed. The molecular weight of an unknown protein can then be determined from its elution volume using this plot (Figure 1).

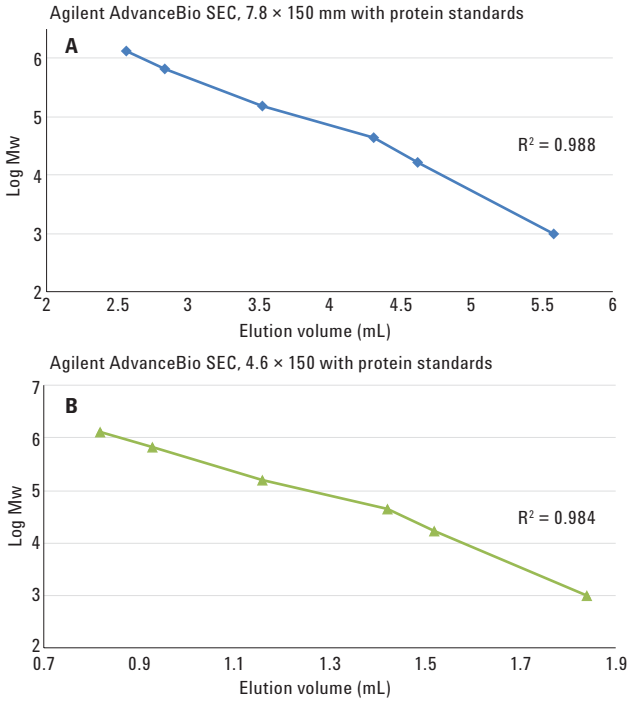

Figure 1. Calibration curve for protein standards on 7.8 × 300 mm (A) and 4.6 × 150 mm (B) Agilent AdvanceBio SEC 300Å columns.

The objective was to improve the analysis throughput by reducing the run time. The flow rate determines run time in SEC for a given column dimension, however, at higher flow rates, resolution could be compromised. To achieve faster run time, the ratio of column void volume to the flow rate needs to be decreased. Reducing the column length and increasing the flow rate is the straightforward approach for faster SEC [2,3]. Figure 2 shows the SEC chromatographic profiles of rituximab biosimilar and innovator, Herceptin, and ADC on an AdvanceBio SEC, 7.8 × 150 mm column. These chromatograms demonstrate excellent separation of the monomer in less than 4 minutes under chromatographic conditions.

To achieve better analytical sensitivity, the separation was carried out on an AdvanceBio SEC, 4.6 × 150 mm column. Figure 3 shows that this resulted in superior separation performance. In both the cases, the absence of an early or late eluting peak suggests that the marketed mAb preparation is homogenous without any indication of aggregation or degradation. Analysis of hydrophobic ADC with aqueous mobile phase using both these columns resulted in a symmetrical peak, indicating no secondary interactions of the hydrophobic payload with the stationary support. The shorter AdvanceBio SEC columns were able to separate and resolve ADC aggregates. This separation indicates its suitability to characterize ADC with sufficient information to support development, lot release, and stability studies.

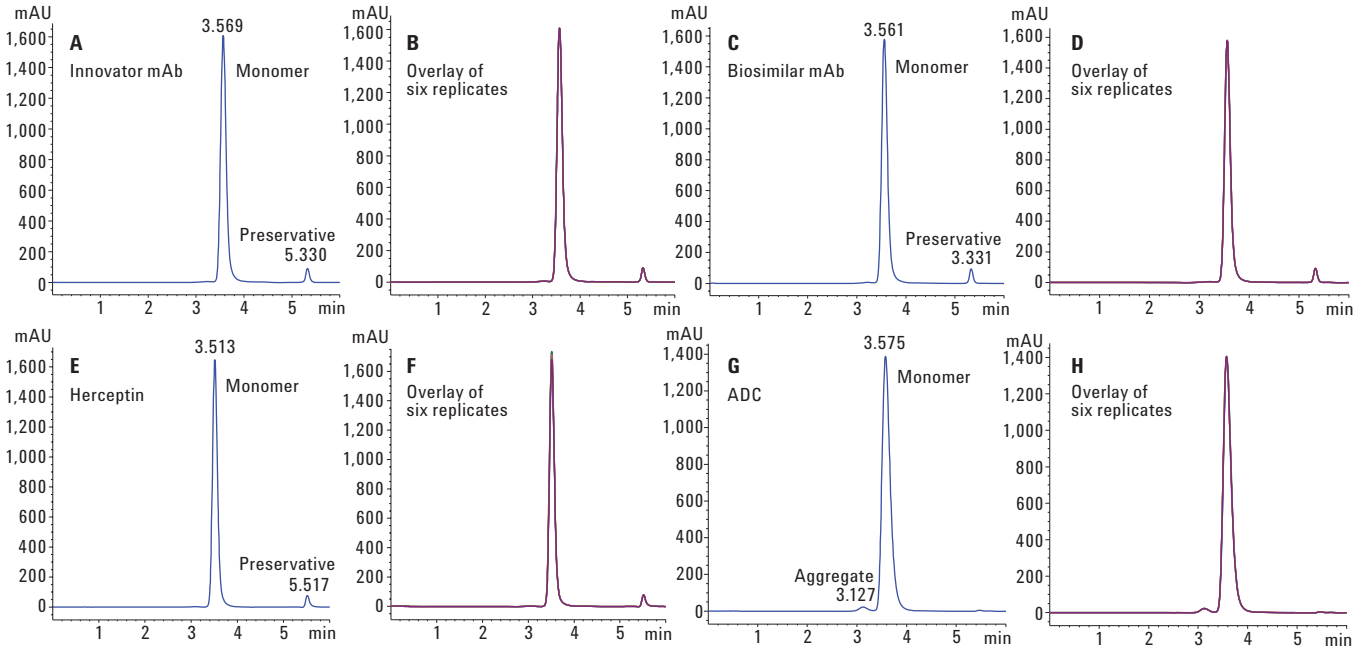

Figure 2. SEC chromatographic profiles of native rituximab innovator and biosimilar, Herceptin, and ADC on an Agilent AdvanceBio SEC, 300Å, 7.8 × 150 mm, 2.7 µm column.

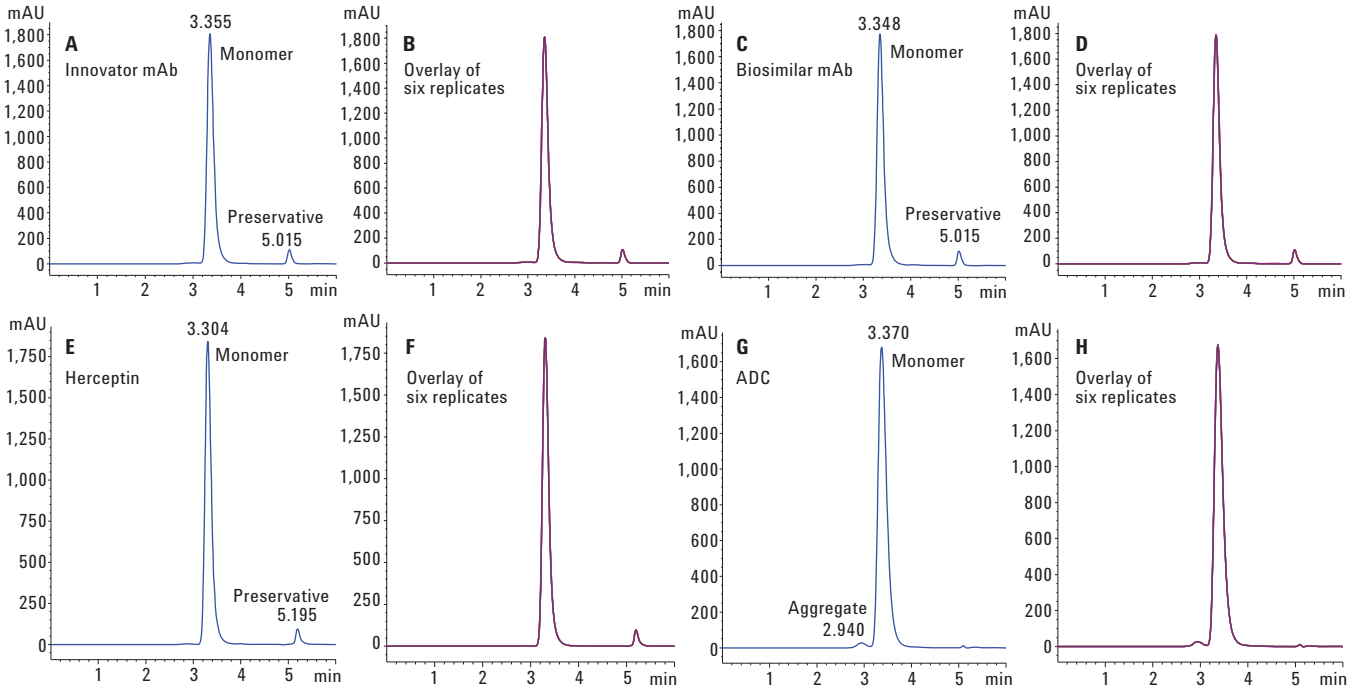

Figure 3. SEC chromatographic profiles of native rituximab innovator and biosimilar, Herceptin, and ADC on an Agilent AdvanceBio SEC, 300Å, 4.6 × 150 mm, 2.7 µm column.

Precision of RT and area

To establish the method precision, relative standard deviation (RSD) values for RT and area of all four biologics were calculated at a 10 µg on-column concentration for 7.8 × 150 mm, and 4 µg for 4.6 × 150 mm columns. Table 2 displays the average RTs and area RSDs from six replicates of samples. The highest observed area RSD value was 0.21%, and RT RSD was 0.02%. The area and retention time RSDs demonstrate excellent reproducibility of the method, and thus the precision of the system.

Table 2. RT and peak area precision (n = 6) of samples.

	Agilent AdvanceBio SEC, 300Å, 7.8 × 150 mm, 2.7 µm		Agilent AdvanceBio SEC, 300Å, 4.6 × 150 mm, 2.7 µm	
Sample	**RT RSD**	**Peak area RSD**	**RT RSD**	**Peak area RSD**
Rituximab innovator	0	0.15	0.02	0.02
Rituximab biosimilar	0	0.04	0.01	0.01
Herceptin	0	0.21	0.01	0.02
ADC	0	0.01	0	0.02

Quantification of Herceptin and ADC using shorter and narrow Agilent AdvanceBio SEC columns

LOD and LOQ

The on-column LOD and LOQ using 7.8 × 150 mm and 4.6 × 150 mm columns for Herceptin and ADC are summarized in Table 3. Figure 4 shows an overlay of LOD and LOQ chromatograms with the blank for the Herceptin and ADC samples. Note that the narrow 4.6 × 150 mm column provided a sensitive analysis of the biologics.

Table 3. LOD and LOQ values for samples.

	Agilent AdvanceBio SEC, 300Å, 7.8 × 150 mm, 2.7 µm		Agilent AdvanceBio SEC, 300Å, 4.6 × 150 mm, 2.7 µm	
Sample	**LOD**	**LOQ**	**LOD**	**LOQ**
Herceptin	78 ng	156 ng	31.2 ng	62.4 ng
ADC	78 ng	156 ng	31.2 ng	62.4 ng

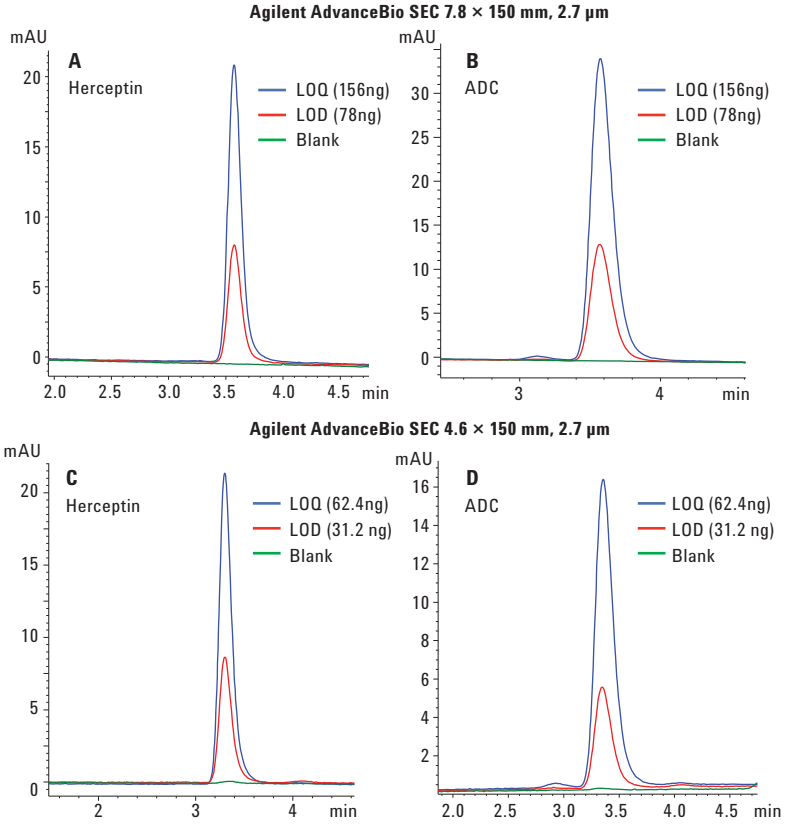

Figure 4. SEC chromatographic profiles for LOD and LOQ of Herceptin and ADC overlaid with the blank for the 7.8 × 150 mm and 4.6 × 150 mm Agilent AdvanceBio SEC 300Å columns.

Linearity

The observed linearity curves for Herceptin and ADC were constructed from the LOQ level to the highest concentration level in the study using area response and concentration of Herceptin/ADC. Figure 5 shows the linearity curve for Herceptin and ADC in the concentration range of 15.6 to 2,000 µg/mL on both columns. The observed regression coefficient (R^2) for the mAbs and ADC indicate that the method is quantitative in the range analyzed.

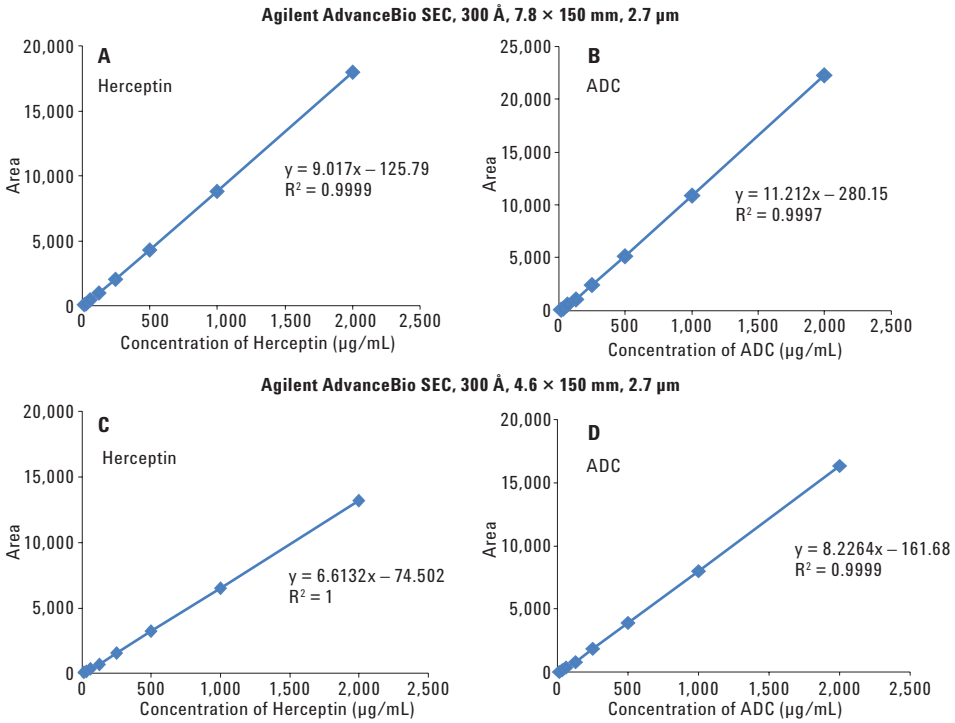

Figure 5. Linearity curve with eight standard concentrations of Herceptin and ADC on Agilent AdvanceBio SEC columns ranging from 15.6 to 2,000 µg/mL, showing excellent coefficient values.

Aggregation/degradation analysis

The control of protein aggregation is always a concern during the purification, formulation, and manufacture of protein products. We compared the native and forced-stress Herceptin and ADC between AdvanceBio SEC columns for monitoring aggregates and degradants. Any peaks from the chromatographic run eluting before the monomeric form were considered as aggregates, and any eluting later were considered as fragments/degradants.

The chromatograms of pH/heat-induced aggregates show that both of these AdvanceBio SEC columns were able to separate and detect aggregates and fragments. Monomer, aggregates, and degradants were distinctly and well separated from each other, as shown in Figures 6 and 7. There was also a significant decrease in the peak height of monomer as a result of stress (data not shown).

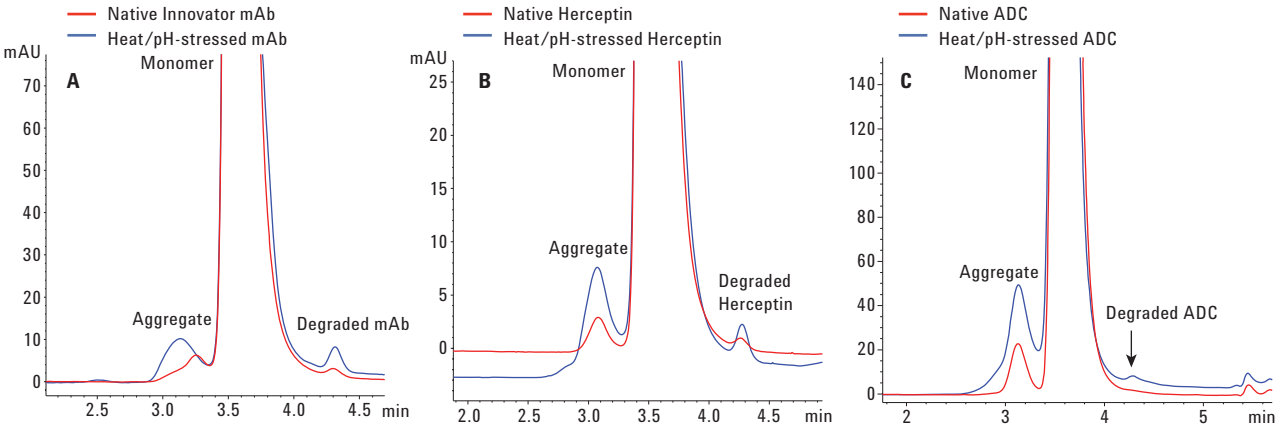

Figure 6. Chromatogram of native (control, red trace) innovator mAb, Herceptin, and ADC overlaid with heat/pH stressed (blue trace) using an Agilent AdvanceBio SEC 300 Å, 7.8 × 150 mm, 2.7 µm column.

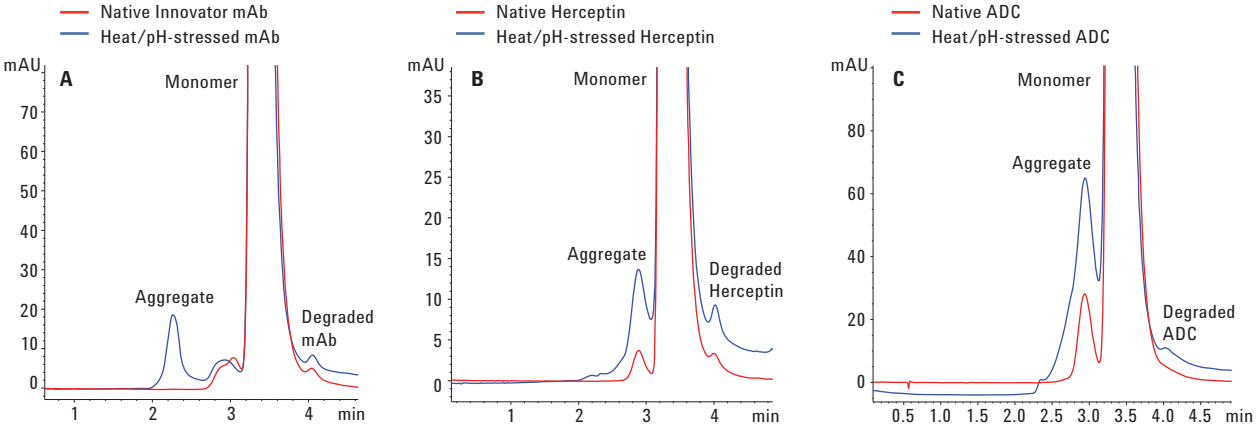

Figure 7. Chromatogram of native (control, red trace) innovator mAb, Herceptin, and ADC overlaid with heat/pH stressed (blue trace) using an Agilent AdvanceBio SEC 300 Å, 4.6 × 150 mm, 2.7 µm column.

Conclusion

SEC is widely used for the characterization of protein aggregates. Due to their potential immunogenicity, the aggregate levels have to be thoroughly controlled during production of recombinant proteins. This study demonstrates the use of shorter and narrower Agilent AdvanceBio SEC columns with 2.7 µm particles for the high-throughput separation of monomers, aggregates, and fragments in less than 4 minutes. AdvanceBio SEC columns were able to provide superior peak shapes of hydrophobic ADC without using organic modifiers in the mobile phase. Area and RT precision was excellent, and demonstrate the reliability of the method for routine use. The method was also quantitative and accurate, as determined by the coefficient of linearity values for the concentration range evaluated. Finally, the AdvanceBio SEC shorter and narrower columns provided certainty of monitoring aggregates and fragments based on forced-stress studies. These results indicate that these columns are suitable for applications where high-throughput and analytical sensitivity are required.

References

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