

Agilent Application Solution

Development and partial validation of a SEC method for high-resolution separation and quantification of monoclonal antibodies

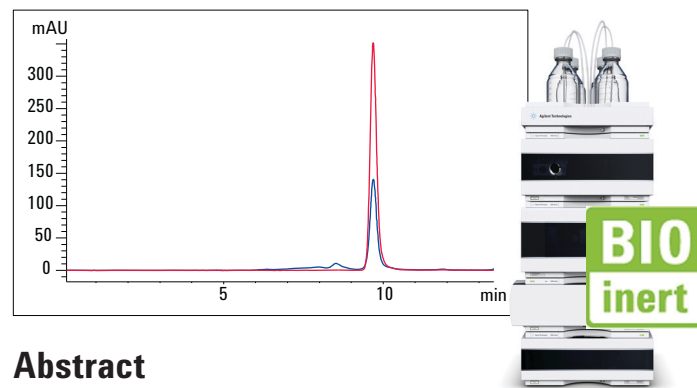
Agilent 1260 Bio-inert Infinity Quaternary LC System with an Agilent Bio SEC-3 column

Application Note

Biopharmaceuticals

Author

M. Sundaram Palaniswamy
Agilent Technologies, Inc.
Bangalore, India



Abstract

Size exclusion chromatography (SEC) is an important technique for the characterization of high molecular substances such as proteins or polymers. It is the standard method to determine and quantify aggregation levels for protein-based pharmaceuticals and is generally required for regulatory approval. This Application Note describes a simple and sensitive method for the quantitative analysis of monoclonal antibodies (mAbs) by SEC. Separation and quantification of mAbs was achieved by the Agilent 1260 Bio-inert Infinity Quaternary LC System using an Agilent Bio SEC-3 column. Bio SEC-3 columns are a breakthrough technology for size exclusion chromatography. They are packed with spherical, narrowly dispersed 3- μm silica particles coated with a proprietary hydrophilic layer. Calibration of the column was performed using protein molecular weight markers. Robustness of the method for routine analysis was established by validation studies using monoclonal antibody. Furthermore, the Bio SEC-3 column was able to separate and detect mAb aggregates. The bio-inertness and corrosion resistance of the instrument coupled with a simple and reproducible method is a practical solution for routine QA/QC analysis of monoclonal antibody for the biopharmaceutical industry.



Agilent Technologies

Introduction

Monoclonal antibodies (mAb) are glycoproteins of the immunoglobulin (Ig) family. They can be used as therapeutics for the treatment of several life-threatening conditions such as cancer, inflammatory diseases, complications arising after organ transplantation, infectious, and cardiovascular diseases. Like all other protein therapeutics, mAbs can undergo aggregation during production, storage and transportation. Aggregation of therapeutic proteins is undesirable since it can lead to activity loss, decreased solubility, and enhanced immunogenicity¹. SEC has been widely used to characterize protein aggregates present in human IgGs². It is the standard method for aggregation analysis and is required for submission of protein therapeutics for regulatory approval³. Agilent Bio SEC-3 columns are available in a variety of pore sizes, and are well suited for protein analysis – especially when determining the presence of dimers and aggregates in therapeutic biomolecules.

This Application Note demonstrates the suitability of the Agilent 1260 Bio-inert Quaternary LC System for quantification, separating and monitoring the integrity of IgG1 by SEC on an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3-µm column. Method validation of an optimized SEC method for quantification of IgG1 at low concentrations (using peak area) is described. We also demonstrate the suitability of the 1260 Bio-inert Quaternary LC System for separating and monitoring mAb aggregates with Bio SEC-3 columns.

Equipment

Instruments

A completely 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 Bio SEC-3, 300Å, 7.8 × 300 mm packed with 3-µm particles (p/n 5190-2511)

The complete sample flow path is free of any metal components such that the sample never gets in contact with metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Parameters	Conditions
Mobile phase A	150 mM sodium phosphate (pH = 7.0) containing 150 mM sodium chloride (mobile phase)
TCC temperature	Ambient
Isocratic run	Mobile phase A
Injection volume	5 µL
Flow rate	0.8 mL/min
UV detection	220 and 280 nm

Table 1
Chromatographic parameters used for SEC HPLC.

Software

Agilent ChemStation B.04.02
(or higher)

SEC Parameters

Chromatographic parameters for SEC using a 1260 Bio-inert LC System are shown in Table 1.

Reagents, samples and materials

Human monoclonal antibody IgG1 was a proprietary pharmaceutical molecule. DL-Dithiothreitol (DTT), sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, sodium chloride, hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma Aldrich. All the 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 Bio SEC-3 column

The Bio SEC-3 column was calibrated by measuring the elution volumes of several standards (protein aggregates, thyroglobulin (670 KDa), γ -globulin (158 KDa), ovalbumin (44 KDa), myoglobin (17 KDa), and vitamin B12 (1.35 KDa). The distribution coefficient (K) values of the standards were plotted against the log of their molecular weight.

Linearity and range

The calibration curve was constructed with eight standard concentrations of IgG1 in the range 12.5 to 2,000 $\mu\text{g}/\text{mL}$.

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

The IgG1 concentration that provides a signal-to-noise ratio (S/N) of > 3 was considered as LOD and $S/N > 10$ was considered as LOQ.

Procedure

A 5- μL solution of mobile phase A was injected as blank, followed by individual linearity level in six replicates. Area and retention time (RT) of each level were used to calculate standard deviation (SD) and relative standard deviation (RSD%) values. LOD and LOQ were established from the lower linearity level injections. Average area of each linearity level was plotted against the concentration of IgG1 to determine the linearity curve. Four critical method parameters were changed to evaluate the robustness of the method.

Robustness study

Four critical method parameters listed below were varied to validate the SEC procedure.

- Variation of injection volume ($\pm 10\%$)
- Variation of buffer pH ($\pm 10\%$)
- Variation of flow rate ($\pm 5\%$)
- Variation of buffer composition ($\pm 150 \text{ mM NaCl}$)

For each robustness parameter, 5 μL of IgG1 was injected, six times to calculate average area and RT. The percentage deviation (% accuracy) of area/retention time (RT) was calculated from the original method.

Preparation of protein aggregates

IgG1 aggregates were prepared by diluting monoclonal antibody in mobile phase to a final concentration of 1 mg/mL . Three methods for generating aggregates were evaluated:

1. Freeze-thaw stress:
 - a. The IgG1 solution was subjected to freeze-thaw cycles.
 - b. One freeze-thaw cycle consists of freezing IgG1 at $-20 \text{ }^\circ\text{C}$ for 60 minutes followed by thawing at $4 \text{ }^\circ\text{C}$ for 30 minutes.
 - c. An aliquot after each freeze-thaw cycle was analyzed by SEC.

2. Temperature stress:

- a. The IgG1 solution was incubated at $70 \text{ }^\circ\text{C}$.
- b. Samples were aliquoted in 10-minute intervals and were analyzed by SEC to observe for aggregates.

3. pH stress:

pH stress was carried out as described earlier¹. Briefly, 1 M HCl was slowly added drop-wise with a pipette to the IgG1 solution to change the pH from 6.0 to 1.0. Then, 1 M NaOH was added to adjust the pH to 10.0. Finally, 1 M HCl was added again to adjust the pH back to 6.0. There was an approximately 1-minute waiting time between the pH shifts, while constant stirring at 500 rpm.

Results and discussion

Separation and detection

The Agilent Bio SEC-3 column was calibrated using a series of standard proteins with known molecular weight (Figure 1). The distribution coefficient, K , was calculated for each standard protein using the formula:

$$K = (V_e - V_0) / (V_t - V_0)$$

V_e = elution volume

V_t = column volume

V_0 = void volume.

Standard Protein aggregates (void peak) in the protein marker were used to calculate the void volume which elutes at 6.973 minutes on the Bio SEC-3 column, corresponding to $V_0 = 5.578$ (mL). The calibration curve for biomolecules separated on the Bio SEC-3 column shows a linear relationship and defines the exclusion limit (670 KDa) for the protein range (1.3–670 KDa) analyzed (Figure 2). The molecular weight of an unknown protein can then be determined from its elution volume using this plot.

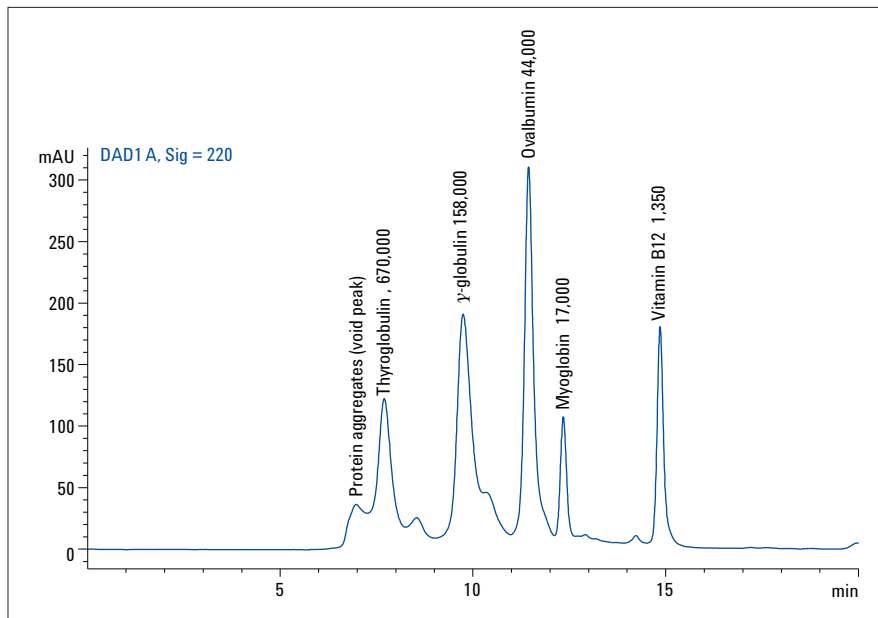


Figure 1
Separation of standards on an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3-µm column.

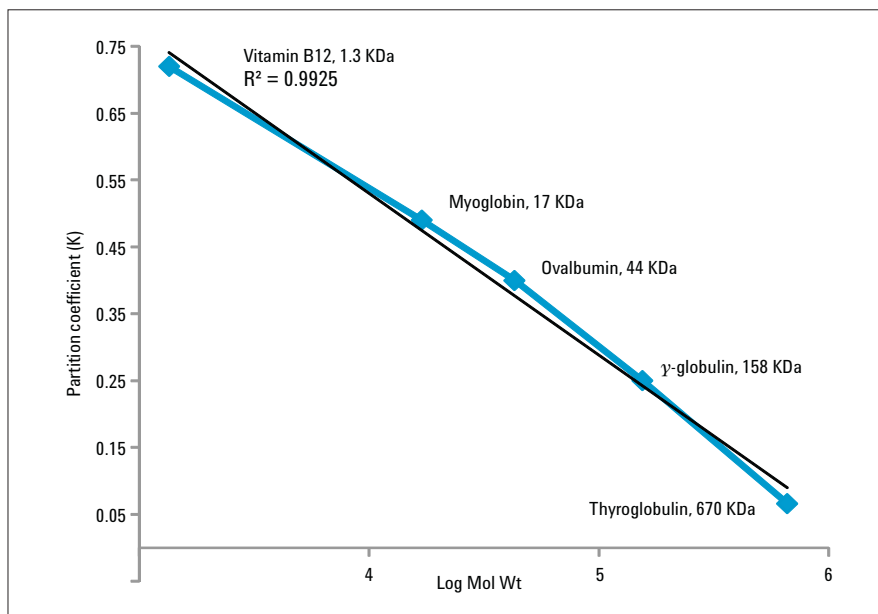


Figure 2
Calibration curve for standards on an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3-µm column.

Figure 3 demonstrates the excellent separation of intact IgG1 in 30 minutes using an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3-µm column under the chromatographic conditions. The absence of an early or late eluting peak in Figure 3 suggests that the IgG1 preparation is homogenous without any indication of aggregation or degradation.

Precision of retention time and area

Table 2 shows the average retention times and area RSDs from six replicates of an IgG1 injection. The retention time and peak area RSDs were 0.008% and 0.639% respectively, which demonstrates excellent reproducibility of the method and thus the precision of the system.

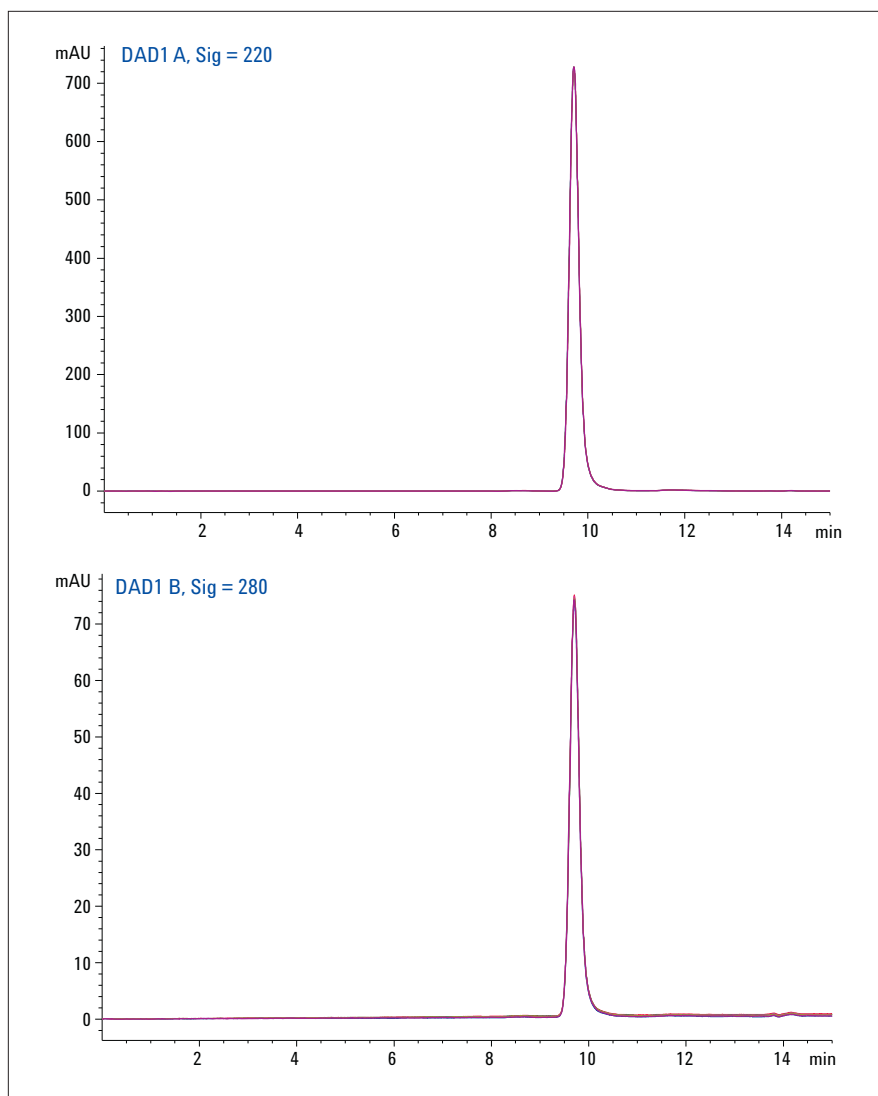


Figure 3
Agilent Bio SEC-3 profile of intact IgG1 on an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3-µm column (overlay of six replicates).

Retention time		Peak area	
Mean (min)	RSD	Mean (mAU/min)	RSD
9.708	0.008	10815	0.693

Table 2
Retention time and peak area precision (n = 6).

Robustness

To evaluate the robustness of the method, four critical method parameters of the original method were varied (Table 3). Allowed deviations for retention time and area RSD were set to $\pm 3.0\%$ and $\pm 5\%$ respectively. The red numbers indicate where the result exceeded the allowed deviation. The impact of injection volume, buffer pH and buffer composition on RT was within the acceptable limits. A variation in flow rate by $\pm 10\%$ compared to the actual method causes the RT RSD to deviate significantly, however, this deviation was expected due to isocratic elution on a SEC column. The area RSD of the IgG1 peak was within the acceptable limit. When the pH of the mobile phase was varied by $+10\%$, the area RSD deviated more than the allowed limit. There were no further significant changes in the chromatographic pattern when deliberate variations were made in experimental conditions, thus showing the method is robust. Our results show that the method is reliable for routine QA/QC application. However, some parameters like the buffer pH are critical and must be carefully controlled.

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

The LOD and LOQ were found to be $12.5 \mu\text{g/mL}$ and $25 \mu\text{g/mL}$, respectively, indicating that the method is sensitive. Observed LOD and LOQ values of IgG1 are tabulated in Table 4 and the overlay of LOD and LOQ chromatograms of IgG1 with blank is shown in Figure 4.

Parameters	Variations	RT deviation (limit $\pm 3.0\%$)	Area deviation (limit $\pm 5.0\%$)
Variation in injection volume ($5 \mu\text{L} \pm 10\%$)	- 10%	0.015	0.295
	+10%	0.056	1.229
Variation in buffer pH ($7.0 \pm 10\%$)	- 10%	0.169	-2.71
	+10%	0.159	6.870
Variation in flow rate ($0.8 \pm 5\%$)	- 5%	-5.227	3.10
	+5%	4.763	3.42
Variation in buffer composition ($\pm 150 \text{ mM NaCl}$)	-150 mM NaCl	0.169	3.10
	+150 mM NaCl	0.169	3.42

Table 3
Robustness (RT and Area RSD %) n = 6.

Concentration of IgG1 ($\mu\text{g/mL}$)	S/N = Signal-to-noise ratio	Average area
6.25	1.8	2.7
12.5 (LOD)	6.8	21
25 (LOQ)	19.6	71
50	76.66	197
100	185	454

Table 4
LOD, LOQ and S/N results (n = 6).

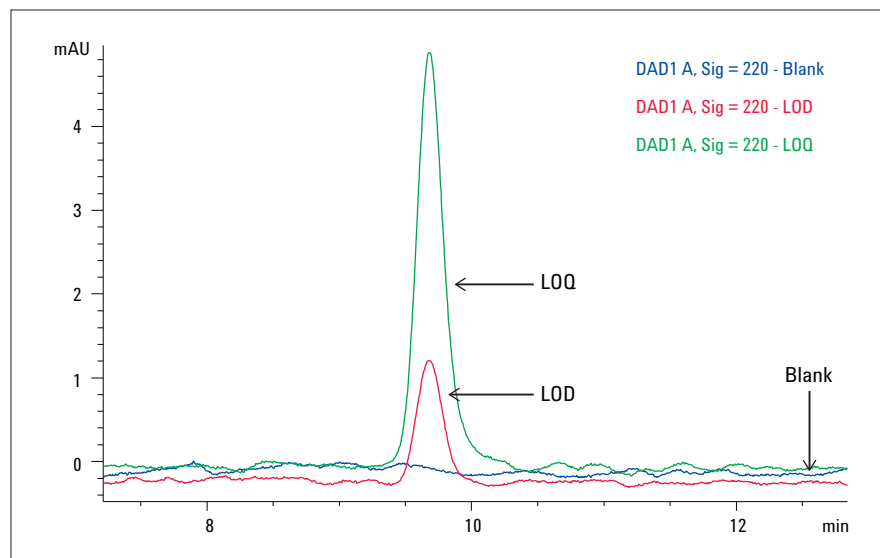


Figure 4
LOD ($12.5 \mu\text{g/mL}$) and LOQ ($25 \mu\text{g/mL}$) chromatograms of IgG1 overlaid with blank.

Linearity

Linearity curves for IgG1 were constructed from the LOQ level to a highest concentration level in the study using area response and concentration of IgG1. The accuracy results are shown in Table 5. The observed accuracy values of 82–112%, representing the range of 100–2,000 µg/mL, indicate that SEC method is quantitative and accurate. The linearity curve for IgG1 in the concentration range 12.5 to 2,000 µg is shown in Figure 5.

Concentration of IgG1 (µg/mL)	Avg area	Standard deviation area	Back calculation using the standard line equation	% Accuracy	%CV
12.5	21	2	-4.87	-39	7.27
25	71	3	5.57	22	4.27
50	197	3	31.74	63	1.46
100	438	1	81.58	82	0.26
200	921	1	181.75	91	0.06
400	1,959	3	397.01	99	0.16
1,000	5,275	10	1,084.76	108	0.18
2,000	10,815	0	2,233.66	112	0

Table 5
Summary of linearity range (n = 6).

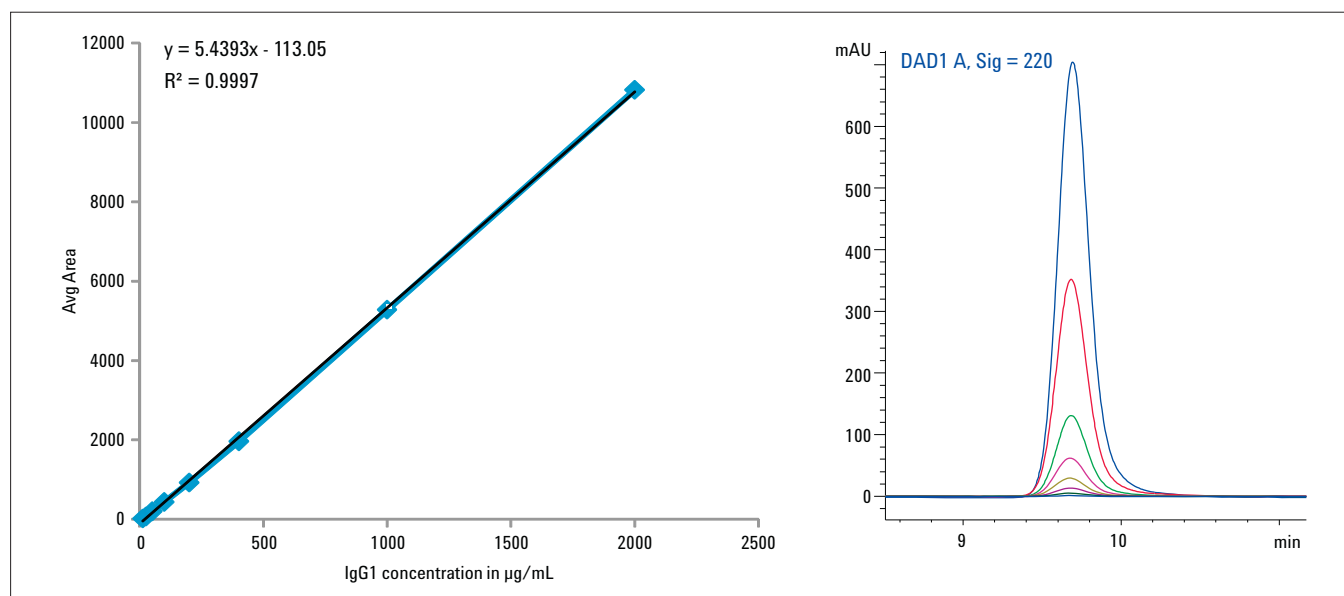


Figure 5
Linearity curve with eight standard concentrations of IgG1 ranging from 12.5 to 2,000 µg/mL showing excellent coefficient value. Also shown, chromatogram overlay for the linearity range.

Analysis of IgG aggregates

Aggregation is a major problem in the development of protein-based drugs because of a possible loss of potency and visual appearance of the product. Although aggregates are present in extremely low concentration, they may have a big impact on the quality of the product. SEC is most commonly

used to monitor aggregation, since it enables size-based separation and uses particles which are designed to have minimum interaction with biomolecules. We compared the intact and stressed (freeze-thaw and temperature) IgG1 samples by SEC HPLC. For the determination of aggregates, any peak from the chromatographic run, which was eluted before (left) the

intact IgG1 is defined as aggregates^{4,5}. It is evident that an applied stress on IgG1 resulted in conformational change (Figure 6). The intact IgG1 was predominantly monomeric. There was also a significant decrease in the peak height as a result of freeze-thaw and temperature stress, perhaps due to partial unfolding of these molecules.

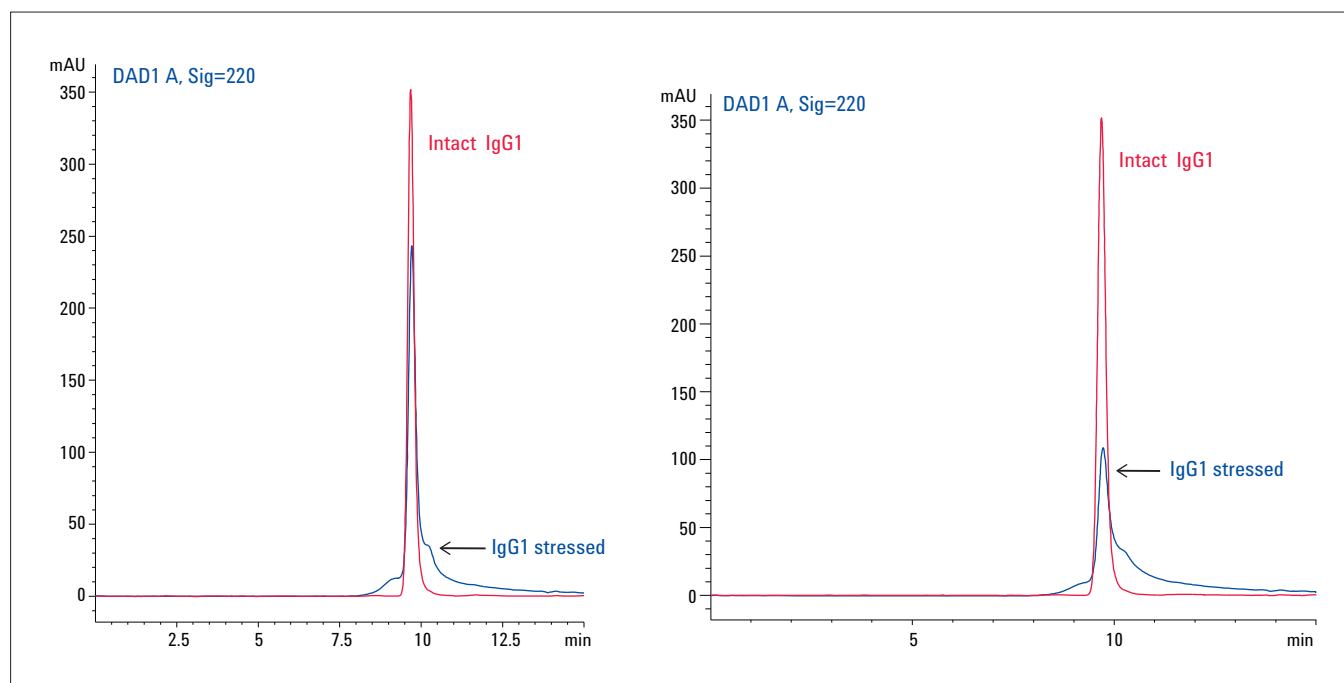


Figure 6
Agilent Bio SEC-3 chromatogram of IgG1 with freeze-thaw and temperature stress indicating conformational change.

The chromatogram of pH induced aggregates shown in Figure 7 indicates that the method was able to separate and detect aggregates as well as intact IgG1. Intact IgG1 and aggregates are distinctly separated from each other and eluted at 9.69 minutes and 8.5 minutes respectively.

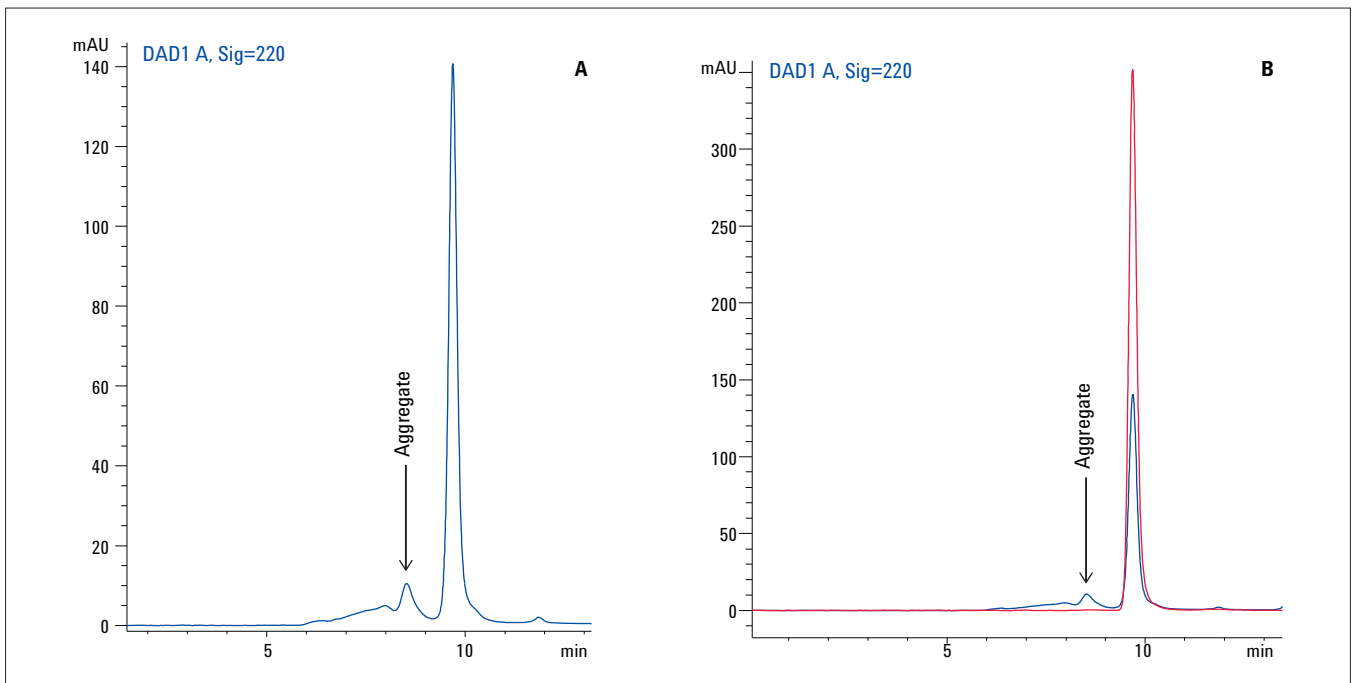


Figure 7
Agilent Bio SEC-3 chromatogram of IgG1 with pH-stress induced aggregates (A) and overlay with intact IgG1 (B).

Conclusion

Size-exclusion chromatography is commonly used for measuring and separating protein aggregates according to their size. Here, we show how the Agilent 1260 Infinity Bio-Inert LC System and an Agilent Bio SEC-3, 300Å, 3-µm column can be used to perform reproducible and high resolution analysis of monoclonal antibodies for biopharma process development and process monitoring. A simple and sensitive SEC method for identification and quantification of IgG1 was developed. There were no significant changes in the chromatographic pattern when the modifications were made in experimental conditions, thus showing the method to be robust. A linearity curve with eight standard concentrations of IgG1 ranging from 12.5 to 2,000 µg/mL shows an excellent coefficient of linearity value indicating that SEC method is quantitative and accurate. The LOD and LOQ were found to be 12.5 µg/mL and 25 µg/mL, respectively, indicating that the method is sensitive. The stress studies demonstrated that the SEC on Bio SEC-3 column was able to separate and detect aggregates and conformation changes in IgG1. The bio-inertness and corrosion resistance of the instrument coupled with simple and reproducible method make this solution particularly suitable for the QA/QC analysis of monoclonal antibody for the biopharmaceutical industry.

References

1. Basak Kükre & Vasco Filipe & Esther van Duijn & Piotr T. Kasper & Rob J. Vreeken & Albert J. R. Heck & Wim Jiskoot, *Pharm Res.* 27:2197–2204, **2010**.
2. Arakawa, T., Philo, J. S., Ejima, D., Tsumoto, K., Arisaka, F, *Aggregation analysis of therapeutic proteins, part 1. General aspects and techniques for assessment.* *BioProcess International*, Vol. 4, No. 10, 32-42, **2006**.
3. A. Buchacher, G. Iberer, A. Jungbauer, H. Schwinn, D. Josic, *Biotechnol. Prog.* 17 140, **2001**.
4. Skoog, D.A.; Holler, F.J.; Nieman, T.A. *Principles of Instrumental Analysis*, 5th Ed.; Harcourt Brace and Company, **1998**.
5. Rodriguez-Diaz, R.; Wehr, T. *Use of Size Exclusion Chromatography in Biopharmaceutical Development*, in *Analytical Techniques for Biopharmaceutical Development*; Rodriguez-Diaz, R.; Wehr, T.; Tuck, S.; Eds.; Informahealthcare: New York, **2005**.

**[www.agilent.com/chem/
bio-inert](http://www.agilent.com/chem/bio-inert)**

© Agilent Technologies, Inc., 2012
Published in the USA, August 1, 2012
5991-0835EN

