# Analysis of Aggregates and Degradants of mAb and Antibody Drug Conjugate Using Size Exclusion 300Å, 2.7µm Column With an Aqueous Mobile Phase

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## Introduction

Determination of aggregate content in biotherapeutic proteins is a Critical Quality Attribute, identified as a potential cause of immunogenic response. For quantification of dimers, higher aggregates and also for lower molecular weight degradants, size exclusion chromatography is the preferred analytical method.

In recent years antibody drug conjugates (ADCs) are being developed and manufactured making use of the specificity of a monoclonal antibody to deliver a small molecule cytotoxic drug to the desired location.

The incorporation of several small drug molecules on the surface of a monoclonal antibody, for example through conjugation via lysine side chains or through linkages to partially reduced disulphide bridges, causes a notable change in the characteristics of the mAb. Aggregation is still a major concern, however the molecule may no longer behave in an ideal manner during size exclusion chromatography (SEC). Consequently many examples of SEC separation of ADCs require the use of organic modifiers in the mobile phase in order to overcome non-specific interactions. This in turn makes method development much more complex. In this presentation we describe a unique size exclusion chromatography sorbent engineered to provide the optimum pore size and particle size, but with a proprietary hydrophilic polymer coating designed to overcome many of the drawbacks seen with certain mAbs and ADCs that exhibit undesirable secondary interactions seen with many commercially available SEC columns.

Initial comparisons were made using a simple protein standard on a selection of popular commercially available SEC columns, Figure 1. When comparing columns from different suppliers it is useful to use a protein mixture that covers the molecular weight range of interest. The mixture should ideally reveal information regarding the exclusion limit of the column, the void volume (retention time at point of exclusion), and the total permeation limit. This will enable comparisons to be made regarding available pore volum for a separation. Of particular interest is the shape of the individual protein peaks too; this helps to identify columns that may exhibit non-specific interactions that may cause peaks to become broad or tailing.

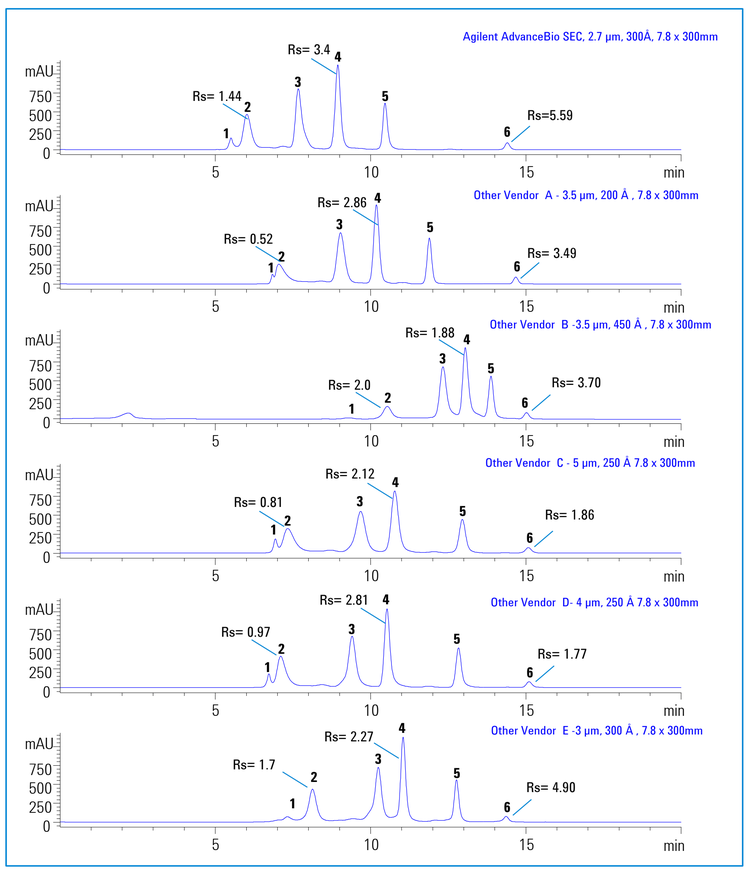


Figure 1 Separation of a protein standard

Key:
1 Thyroglobulin aggregates
2 Thyroglobulin
3 γ-Globulin
4 BSA
5 Myoglobin
6 Uracil

| Columns (7.8 x 300 mm) | Pore Volume (mL) |
|---|---|
| Agilent AdvanceBio SEC 300Å, 2.7 µm | 7.11 |
| Other vendor A 200Å, 3.5 µm | 6.25 |
| Other vendor B 450Å, 3.5 µm | 4.57 |
| Other vendor C 250Å, 5 µm | 6.51 |
| Other vendor D 250Å, 4 µm | 6.70 |
| Other vendor E 300Å, 3 µm | 5.62 |

Table 1 Pore Volume Comparison

## Experimental

Experimental details are highlighted in the Table below:

| Parameters | |
|---|---|
| Columns | 7.8 x 300-mm SEC columns |
| Flow rate | 0.8 mL/min |
| Mobile phase | 150 mM phosphate buffer, pH 7.0 |
| Instrument | Agilent 1260 Infinity Bio-inert Quaternary LC |
| Injection volume | 5 µL |
| Temperature | Ambient |
| Detection | UV, 220 nm |
| Samples | Protein molecular weight markers (thyroglobulin aggregate, thyroglobulin, IgG, bovine serum albumin, myoglobin), and uracil total permeation marker<br>Therapeutic monoclonal antibody (trastuzumab)<br>Therapeutic antibody drug conjugate |

## Results and Discussion

As can been seen from Figure 1 and Table 1, the performance of an SEC column can best be compared using the separation of a protein mixture. However monoclonal antibodies may behave differently. Some mAbs require considerable additional method development in order to optimize the separation and provide a robust separation. In the examples shown we have used 150 mM sodium phosphate pH 7.0 as mobile phase as, in our hands, this has consistently provided good separations with very few molecules needing additional refinement. The importance of both the pore volume and peak shape on resolution can be observed in order to maximise resolution and provide greater accuracy for quantification of small amounts of protein aggregate (Figure 2):.

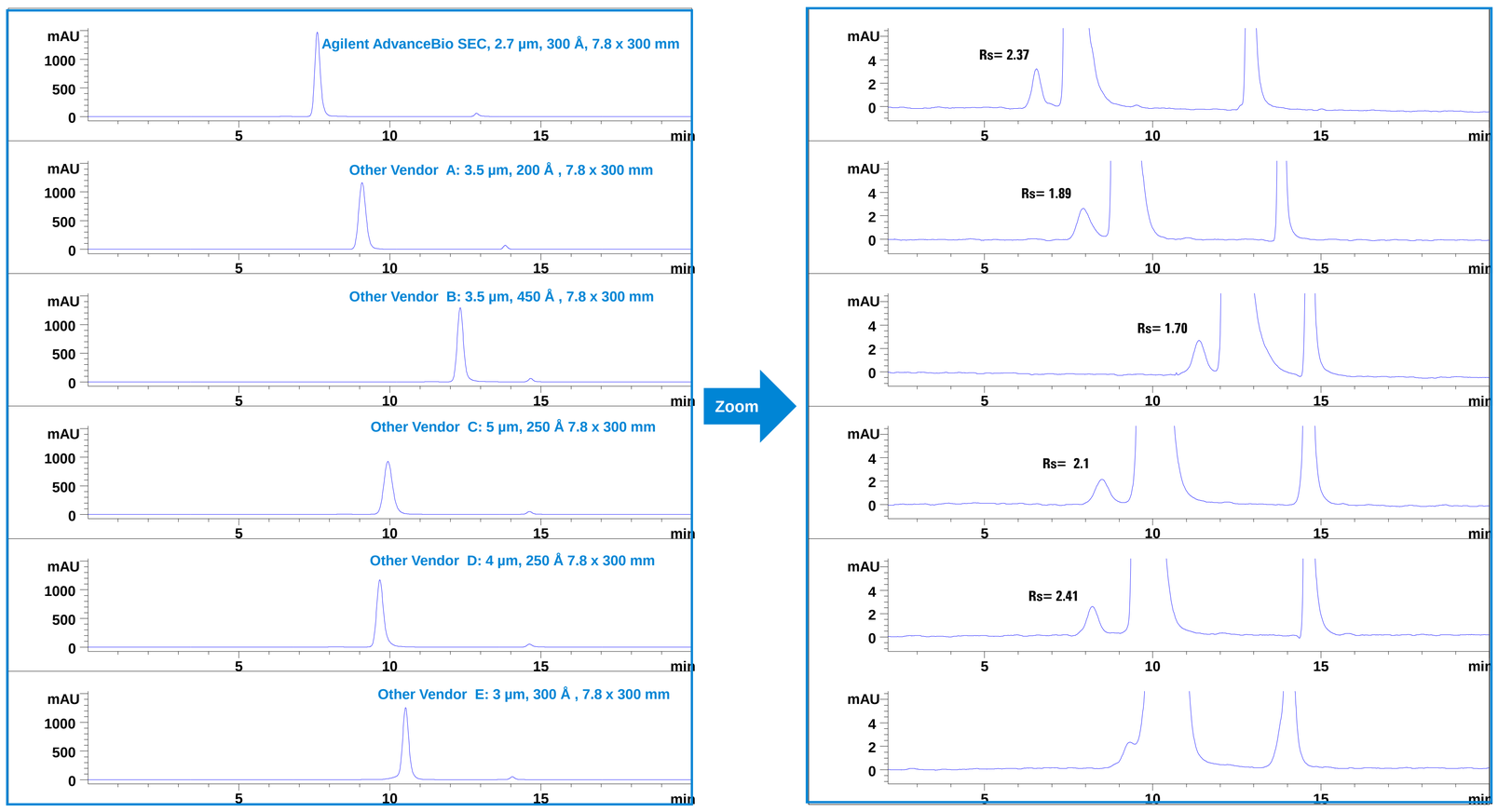


Figure 2 Separation of trastuzumab
Left hand side shows full scale chromatograms, right hand side is close up of baseline region showing resolution of a small amount of dimer.

The inclusion of a small molecule cytotoxic drug on the surface of an mAb in the case of ADCs can significantly alter the elution behaviour. Often the retention time increases and the peak shape deteriorates noticeably. It may be possible to improve the peak shape and retention time by incorporating organic modifier in the mobile phase, however in the case of the new AdvanceBio SEC column this may not be necessary (Figure 3):

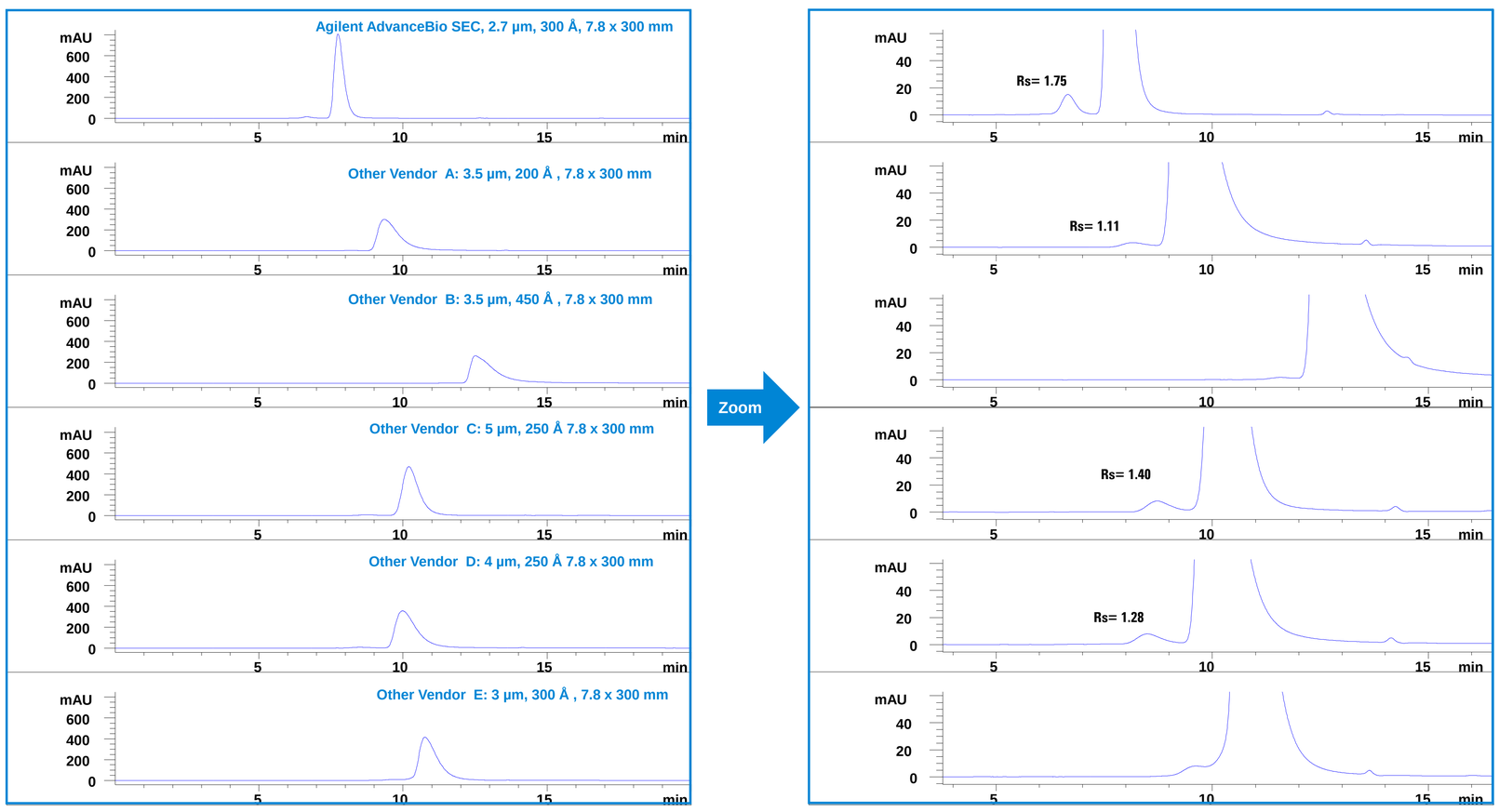


Figure 3 Separation of an ADC
Left hand side shows full scale chromatograms, right hand side is close up of baseline region showing resolution of a small amount of dimer.

The new AdvanceBio SEC columns have been designed specifically to overcome some of the common issues found in size exclusion chromatography of protein aggregates, particularly biotherapeutics such as mAbs and ADCs.

The 2.7 µm particle size ensures very high efficiency separations but does not restrict the operation to UHPLC instrumentation: the AdvanceBio SEC columns will operate on legacy 400 bar instruments through to high end UHPLC systems. The pore size has been optimized for the separation of protein aggregates and the pore volume has been increased to provide maximum resolution but without compromising column lifetime. Finally the novel proprietary hydrophilic polymer coating improves peak shape, not only for proteins but also more challenging ADCs without the need to resort to including organic modifier in the mobile phase.

The columns tested in this poster have all been 7.8 x 300 mm dimensions in order to make such direct comparisons possible. The AdvanceBio SEC product range includes shorter 15 cm columns for higher throughput applications and use at higher flow rates, and 4.6 mm ID for situations where sample availability may be limited and higher sensitivity is needed.

## References


- Mahler, H-C et al., J. Pharm. Sci., 2009, 98(9), 2909-2934
- Cromwell, M E M et al., The AAPS Journal 2006, 8(3), E572-E579


## Conclusions

- The accurate and reliable quantification of protein aggregates relies on a combination of column performance and pore volume.
- To date, many vendors have been unable to provide an optimized column for SEC separation that works well for proteins, mAbs and ADCs.
- The new AdvanceBio SEC column range has been designed specifically to overcome these deficiencies and to provide excellent results for a diverse range of biotherapeutics.

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