# Defining the Optimum Parameters for Efficient Size Separations of Proteins

Application Note

BioPharma


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## Introduction

The need for precise and accurate information relating to protein molecular weight, size, aggregation, and degradation fragmentation varies according to the application. You want speed and resolution for drug discovery and process monitoring, but in QA/QC of final product, accuracy of the quantitation is paramount. It is essential, when looking at column and method parameters, that you clearly define the objective of the separation and fully understand the parameters controlling the speed and resolution of the separation.

In this application note, we used a set of protein standards that span the typical range of characteristics of recombinant biopharmaceuticals, from antibodies to small globular proteins, to determine the influence of some parameters on resolution and data quality.

For size exclusion chromatography (SEC), it is essential that the separation mechanism is one of size and that there are no secondary interactions with the stationary phase. The materials used for SEC of proteins are normally based on a silica template, which is chemically modified to provide an inert hydrophilic surface. However, if this chemical modification is insufficient, underlying ion-exchange character will remain, and if too much polymer is introduced there is a possibility of hydrophobic interactions. For robust SEC separations, the column should exhibit size separations over a wide range of salt concentrations.

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## Materials and Methods

A mixture of standard proteins was used to determine the resolution characteristics of the columns. The test was performed prior to studying the effect of surface charge and hydrophobicity on retention and then after this experiment to look at changes in the separation characteristics.

We investigated surface charge and hydrophobicity by looking at retention times and peak shapes of four proteins, lysozyme, $\alpha$-chymotrypsinogen A, ovalbumin and myoglobin, at salt concentrations from 25 to 500 mM NaCl. We used three columns, the Agilent Bio SEC-3, Vendor A and Vendor B, as the literature suggests different particle chemistries may be used for the production of these columns.

A polyclonal mouse IgG was used to determine the effect of particle size, pore size, and chemistry on the resolution of the monomer, IgG, dimer, and degradation fragments.

### Conditions

| | |
|---|---|
| Columns | Agilent Bio SEC-3, 300Å, 4.6 x 300 mm, 3 µm |
| | Vendor A, 4.6 x 300 mm, 1.7 µm |
| | Vendor B, 4.6 x 300 mm, 4 µm |
| Instruments | Agilent 1290 Infinity or Agilent 1260 Infinity UHPLC |
| Eluent A | 150 mM phosphate buffer, pH 7 |
| Eluent B | 20 mM phosphate buffer + x mM NaCl, pH 7 |
| Flow rate | 0.35 mL/min |
| Detection | UV, 220 nm |
| Samples | 1. Bio-Rad Gel Filtration Standard (1 vial diluted with 5 mL of eluent) thyroglobulin, γ-globulin, ovalbumin, myoglobin, vitamin B12 |
| | 2. Lysozyme, $\alpha$-chymotrypsinogen A, ovalbumin, myoglobin |
| | 3. Mouse IgG |

## Results and Discussion

The chromatograms in Figure 1 show differences in the non-specific interactions of these three columns – all three columns exhibit ionic interactions in low salt conditions, as demonstrated by the retention of lysozyme, but Vendor A's column also shows non-specific interactions at the higher salt concentrations, as demonstrated by the poor peak shapes seen with the 500 mM NaCl eluent.

The change in efficiency and resolution of the protein standard separations after completing the evaluations of non-specific interactions using different salt concentrations is evident in Figure 2. The Agilent Bio SEC-3 column is more robust.

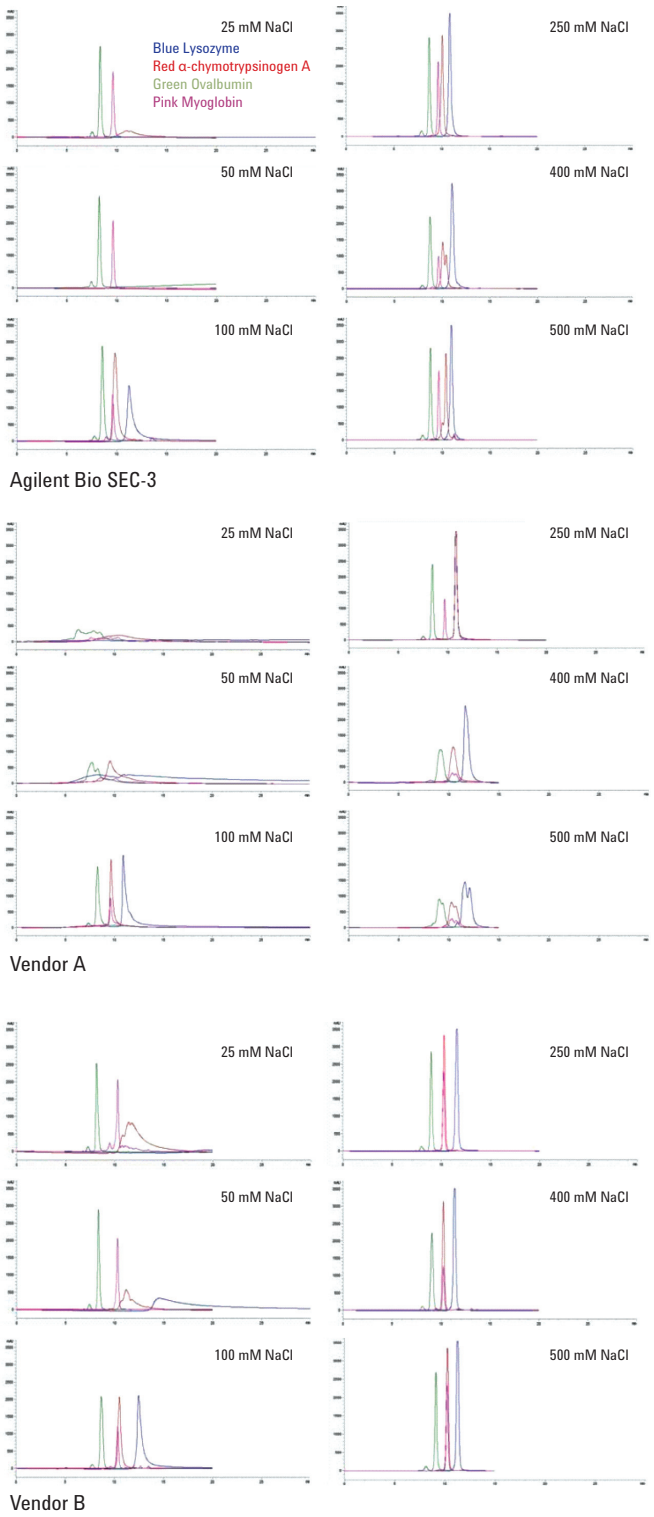


*Figure 1. The Agilent Bio SEC-3 column delivers lower non-specific interactions (eluent B). Chromatograms showing the dependency of retention times and peak shapes of the protein probes on the eluent salt concentration and column chemistry.*

When comparing the peak shapes, efficiency, and resolution before and after the investigation of retention time and peak shape dependency on salt concentration areas there was also a difference in the three columns (Figure 2). The column from Vendor A showed the biggest reduction, suggesting column fouling due to poor recovery or packed bed instability. This was confirmed by comparing the separation of the Bio-Rad protein test mix before and after the experiment. There was a reduction in column efficiency that was partially recovered by performing the recommended wash procedure.

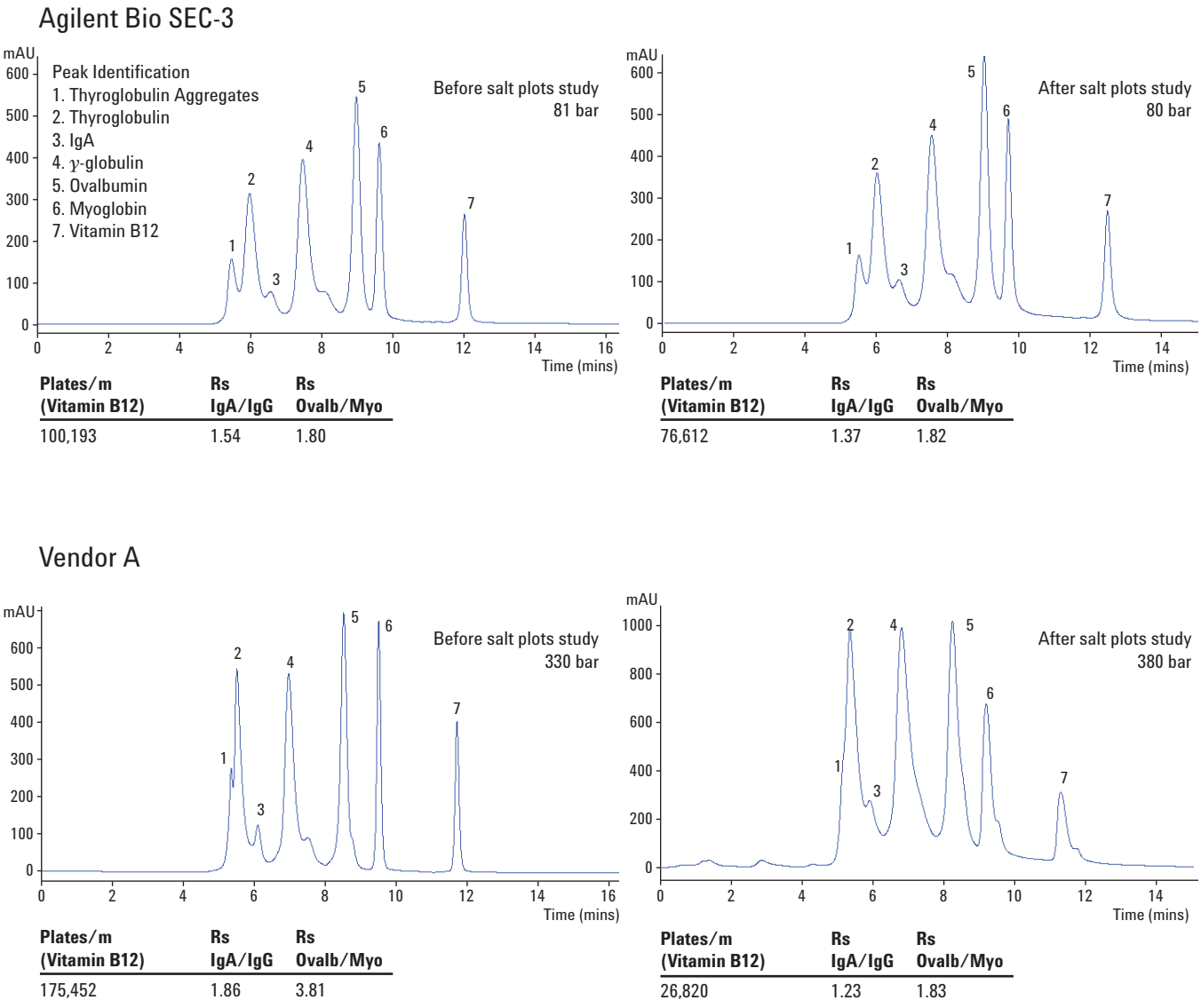


*Figure 2. Separation of protein standards (Bio-Rad Gel Filtration Standard) before and after the experiment to identify non-specific interaction (eluent A).*

## Separating Polyclonal Mouse Antibody

We further evaluated the effect of chemistry and particle size on separation with a polyclonal mouse IgG and Bio-Rad protein test mix. The efficiency was calculated using the vitamin B12 peak in the mix. The smaller particle sizes had higher plate counts, but the Agilent Bio SEC-3 had the best definition of the fragmentation pattern for the mouse IgG (Figure 3).

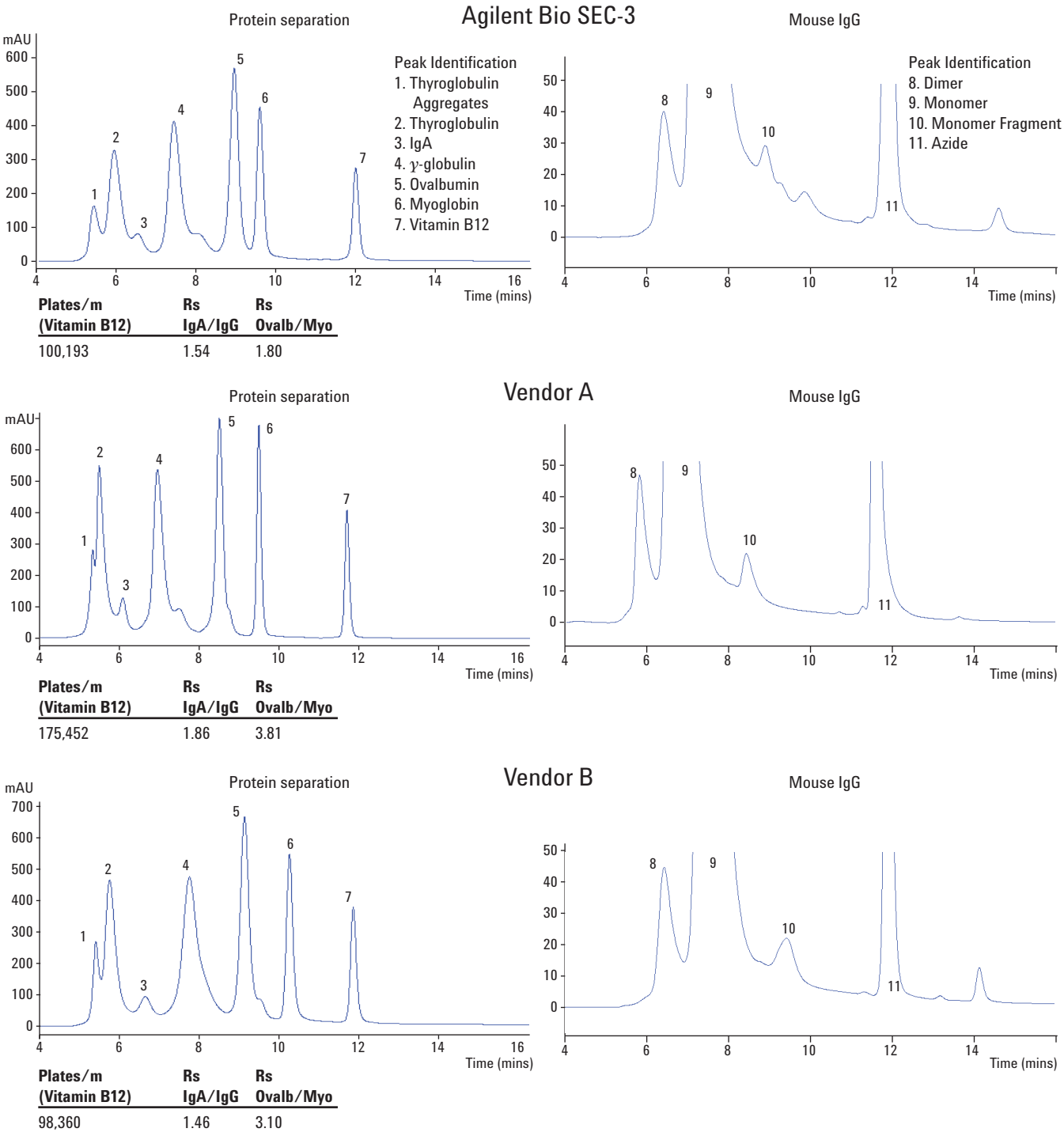


*Figure 3. The Agilent Bio SEC-3 column provides a better fragmentation pattern when separating protein text mix (left) and polyclonal antibody mouse IgG (right) (eluent A). Chromatograms showing the separation of the Bio-Rad protein test mix, left panel, and mouse IgG, right panel, obtained with the three test columns.*

## Conclusion

Particle and surface chemistry influence non-specific interactions in SEC, which can be ionic or hydrophobic. It is clear that the optimum eluent for the separation should be determined by the characteristics of the column stationary phase and the proteins to be analyzed, to minimize non-specific interactions. Sample recovery is also dependent on the stationary phase and the eluent. If attention is not paid to sample loss the column performance will deteriorate due to column fouling. Smaller particles provide higher efficiency but this does not always translate into improvements in the critical separation.

## For More Information

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Printed in the USA
September 13, 2011
5990-8895EN

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