Evaluation of Mobile Phase Conditions on the Resolution of Monoclonal Antibody Monomer from its Protein Variants Separated on a TSKgel SuperSW mAb HR SEC Column

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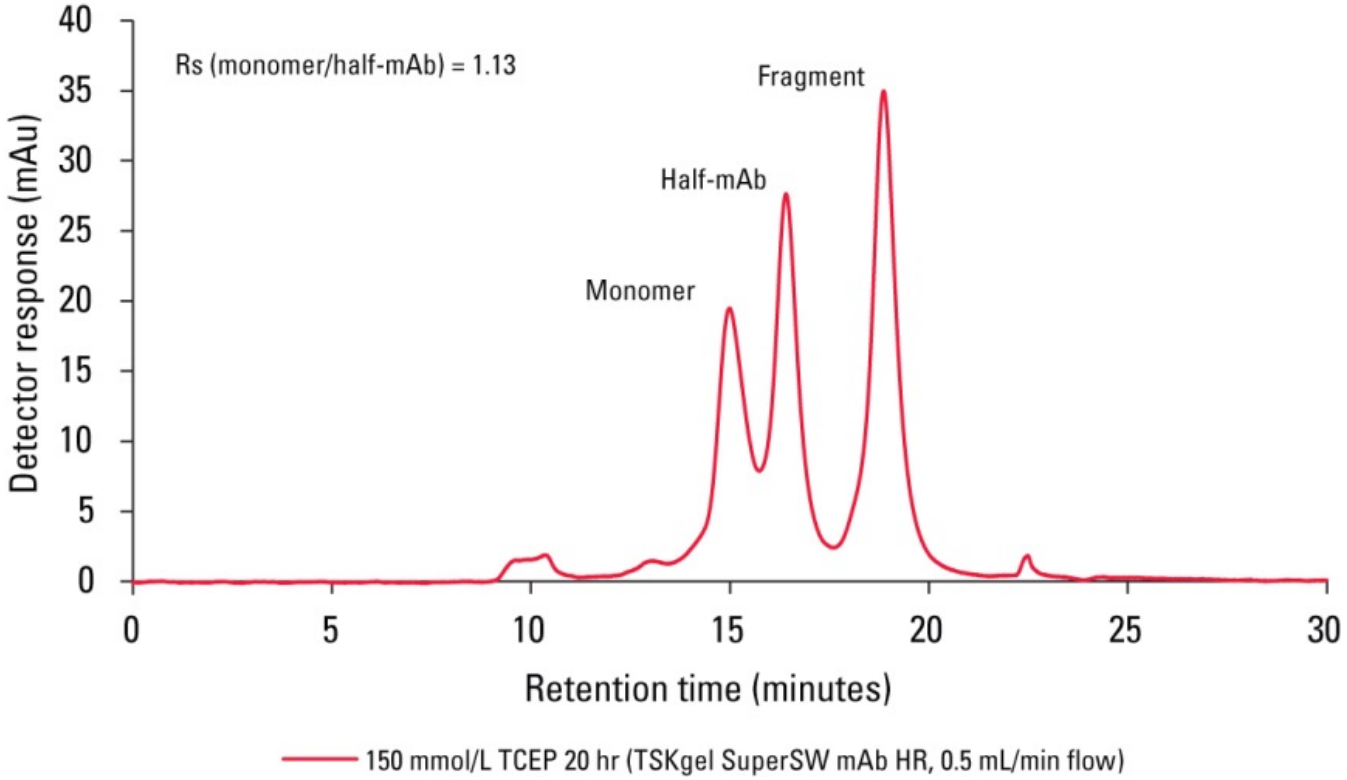
Introduction

- Monoclonal antibodies (mAbs) are multi-domain proteins that are extensively used as a research tool in molecular biology and in the development of therapeutics in medicine.
- Frequently mAbs are present as multiple variants which may have formed as a result of processing or storage conditions during the product development process.
- The presence of such variants can lead to deleterious effects on the efficacy and stability of the final product and must be removed from the protein sample in order to comply with strict regulatory guidelines and to maintain product quality. These impurities can have immunogenic reactions.

Introduction

- Size Exclusion Chromatography (SEC) is often used to separate protein variants differing on the basis of molecular mass, allowing for purification of the desired protein species.
- Figure 1 shows a representative chromatogram of mAb variants obtained by the reduction of the intact mAb using TCEP (tris (2-carboxyethyl) phosphine) separated by size exclusion chromatography.
- Modifications of mobile phase ionic strength and composition, as well as flow rate, can have a profound effect on the separation of the mAb variants when using SEC.

Figure 1: Separation of mAb 01 Monomer From its Half-mAb and Low Molecular Weight Fragment



- The separation of the mAb monomer from its molar mass variants using Size Exclusion Chromatography (SEC) is shown in this figure.
- All mAb variants were confirmed using SDS-PAGE.

Material and Methods

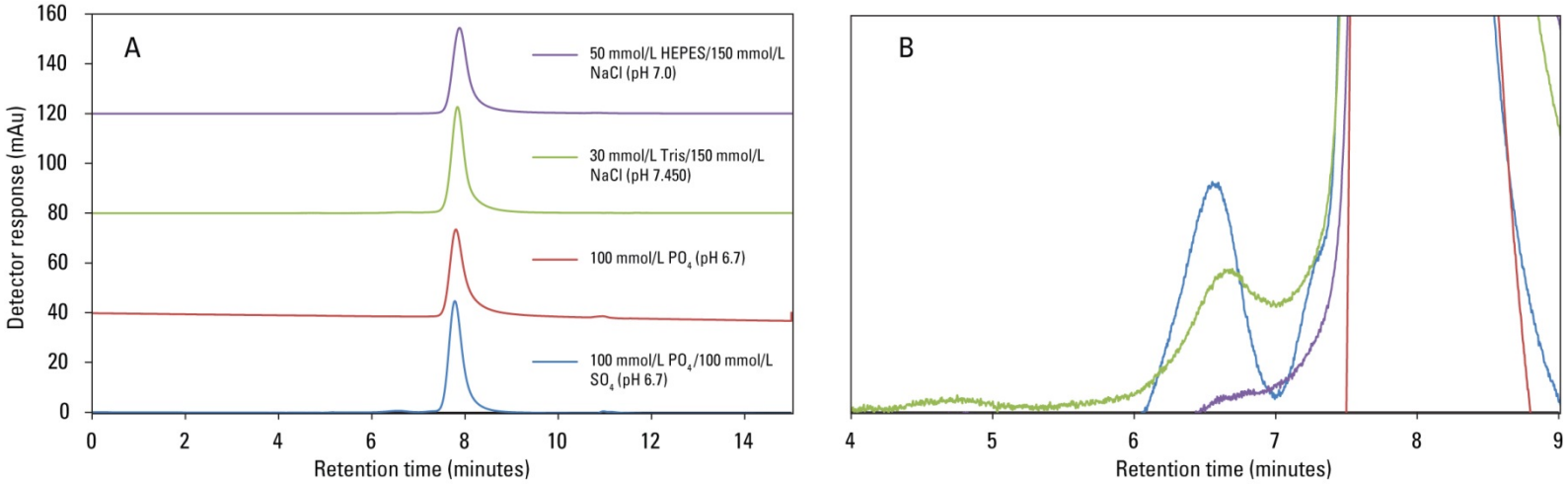
Column:	TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm
Instrument:	All analyses were carried out using an Agilent 1100 HPLC system run by Chemstation (ver B.04.02).
Mobile phase:	as noted
Flow rate:	1.0 mL/min, unless otherwise noted
Detection:	UV @ 280 nm
Temperature:	ambient
Injection vol.:	10 µL (approximately 46 µg of total protein content per injection)
Sample:	mAb 02, 4.7 mg/mL - a gift from Tosoh Bioscience GmbH

All the representative chromatograms shown in this presentation were verified by 3 consecutive injections.

Effect of Mobile Phase Compositions on the Separation of Native and Thermally Denatured mAb 02 Monomer using the TSKgel SuperSW mAb HR Column

- As SEC is a non-interaction form of chromatography, in which in an ideal situation the compounds are separated purely due to a sieving process without any interaction with the stationary phase, mobile phase conditions such as composition, ionic strength, and flow rate can have a large effect on the separation of the desired protein species from its molecular mass variants.
- The composition of the mobile phase can alter the properties of the SEC column or analyte of interest.
- Such effects may lead to a loss of resolution or yield unacceptable peak shapes.
- Here, four commonly used SEC mobile phase compositions were explored to evaluate the effect on the analysis of a monoclonal antibody in its native and thermally denatured form.

Figure 2: Effect of Mobile Phase Compositions on the Separation of Native mAb 02 Monomer using the TSKgel SuperSW mAb HR Column



- Panel A: Full scale chromatogram
- Panel B: Detailed view

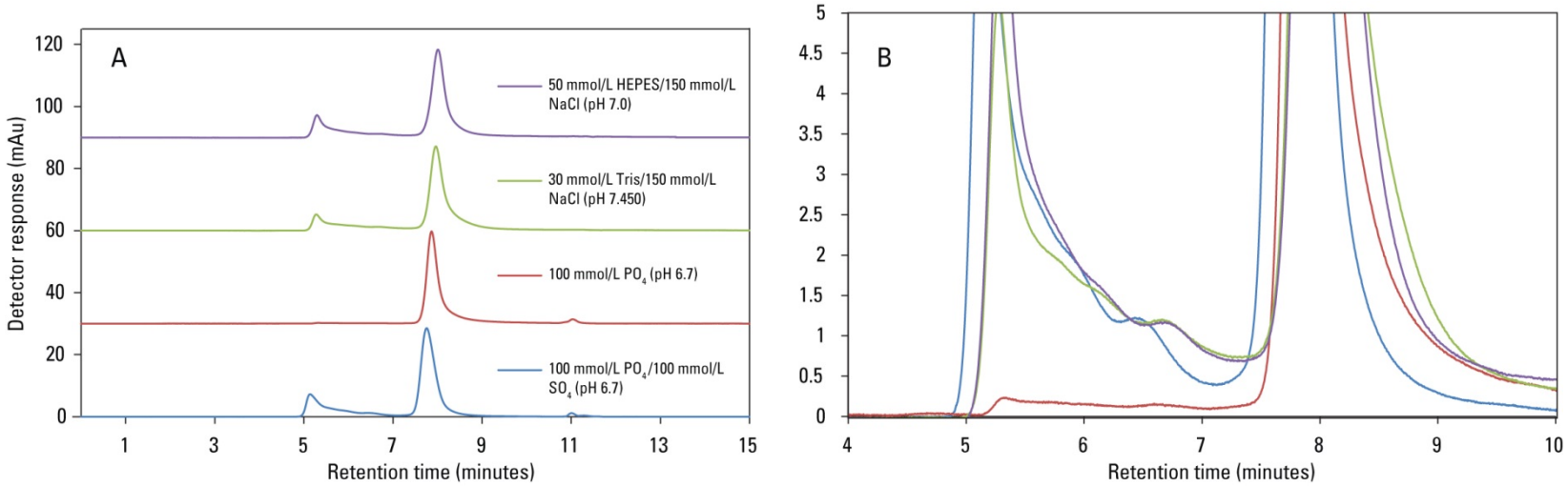
The result clearly shows that the use of 100 mmol/L sodium sulfate (blue trace) as a neutral salt additive to increase the ionic strength yields baseline separation of the mAb 02 dimer from the monomer.

Table 1: Effect of Mobile Phase Compositions on the Separation of Native mAb 02 Monomer using the TSKgel SuperSW mAb HR Column

Native mAb 02 monomer	Averaged Values (n = 3)			
Mobile phase	**Retention time**	**Area**	**As**	**N**
100 mmol/L PO_4 (pH 6.7)	7.810	902.648	1.911	3,097
100 mmol/L PO_4/100 mmol/L SO_4 (pH 6.7)	7.783	988.717	1.464	3,437
30 mmol/L Tris/150 mmol/L NaCl (pH 7.45)	7.831	962.641	1.463	3,754
50 mmol/L HEPES/150 mmol/L NaCl (pH 7.0)	7.884	834.569	1.485	2,898
Avg	7.827	922.144	1.581	3,296.667
Sd	0.037	59.417	0.191	326.903
%RSD	0.474	6.443	12.080	9.916

- mAb 02 monomer retention time remains consistent irrespective of the nature of the mobile phase composition.
- Addition of the neutral salt improved the peak asymmetry.

Figure 3: Effect of Mobile Phase Compositions on the Separation of Thermally Denatured mAb 02 Monomer using the TSKgel SuperSW mAb HR Column



- Panel A: Full scale chromatogram
- Panel B: Detailed view

Figure 3: Conclusions

- The result clearly shows that the use of 100 mmol/L sodium sulfate (blue trace) as a neutral salt additive to increase the ionic strength yielded better separation of the mAb 02 monomer from the dimer and higher order aggregates.
- The mAb 02 aggregate peaks could not be detected when no neutral salt was added to the 100 mmol/L sodium phosphate in the mobile phase (red trace).
- The use of 100 mmol/L sodium sulfate (blue trace) neutral salt additive yielded a sharper mAb 02 monomer peak compared to other mobile phase conditions.

Figure 4: Effect of Mobile Phase Compositions on the Separation of Thermally Denatured mAb 02 Monomer using the TSKgel SuperSW mAb HR Column: Peak Area

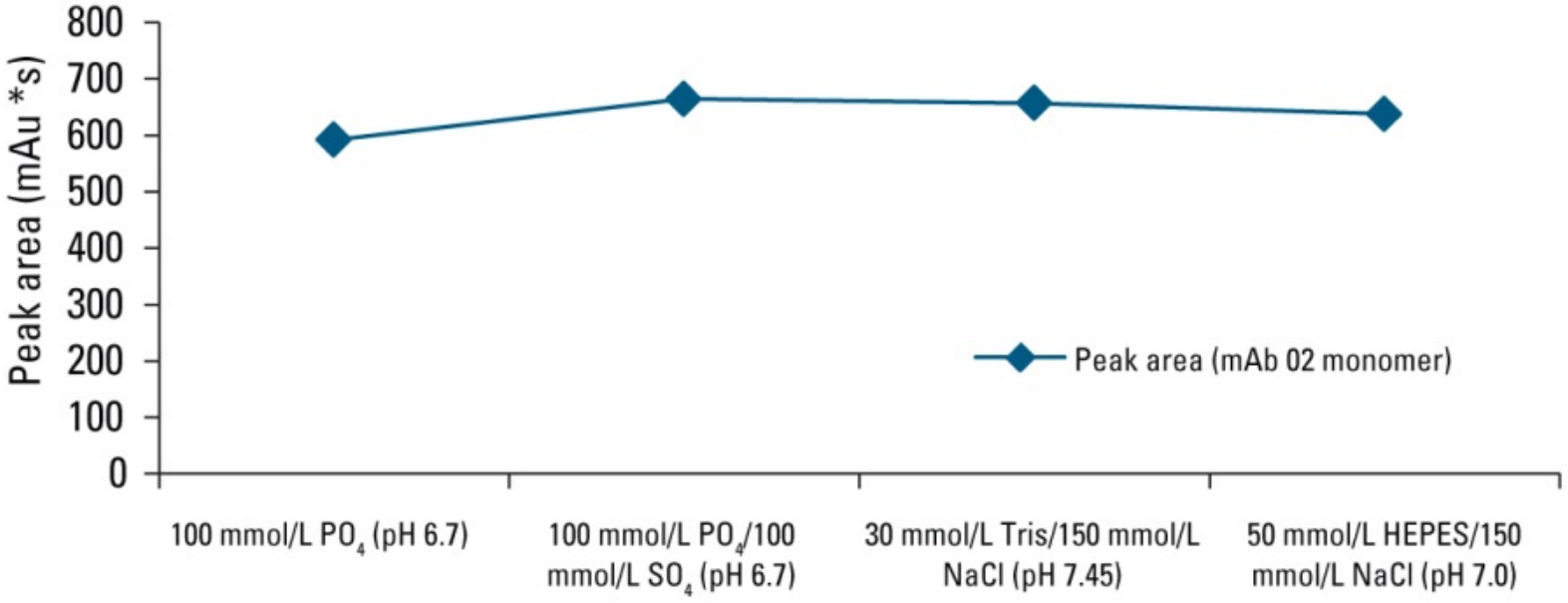


Figure 5: Effect of Mobile Phase Compositions on the Separation of Thermally Denatured mAb 02 Monomer using the TSKgel SuperSW mAb HR Column: Retention Time

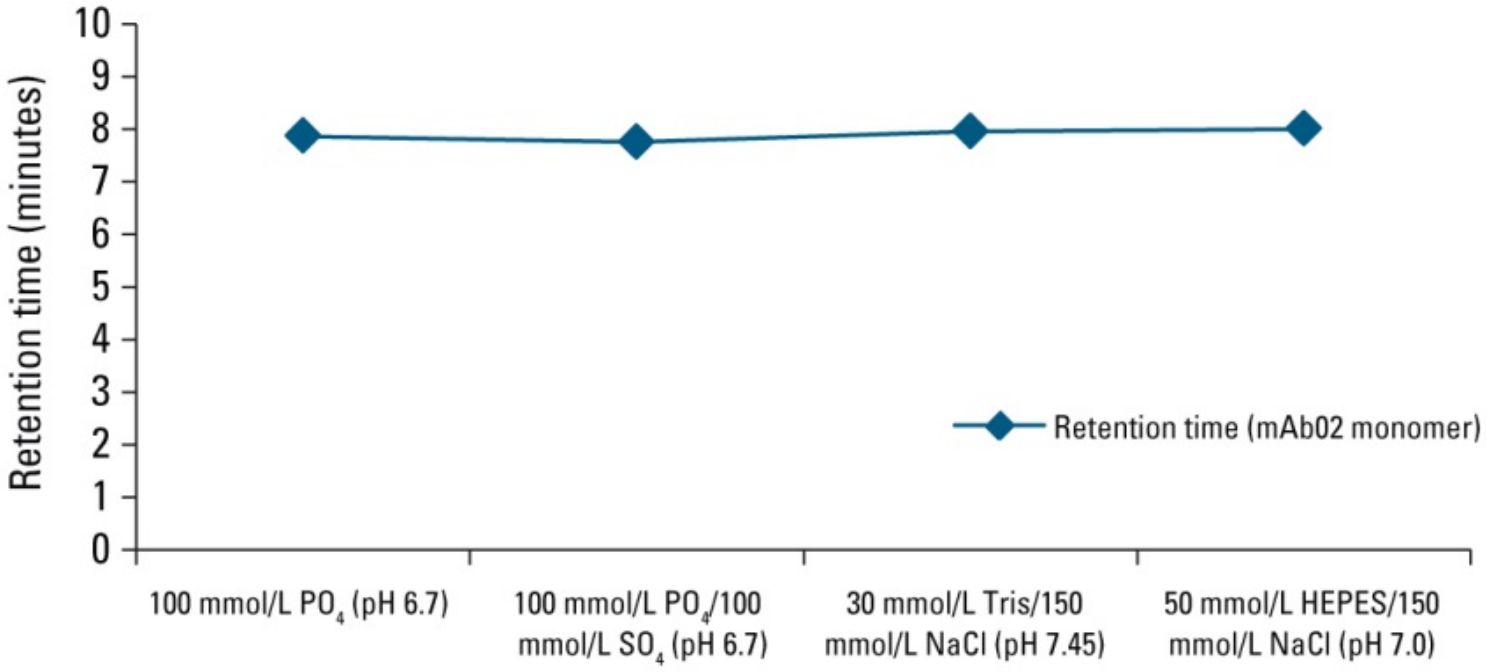
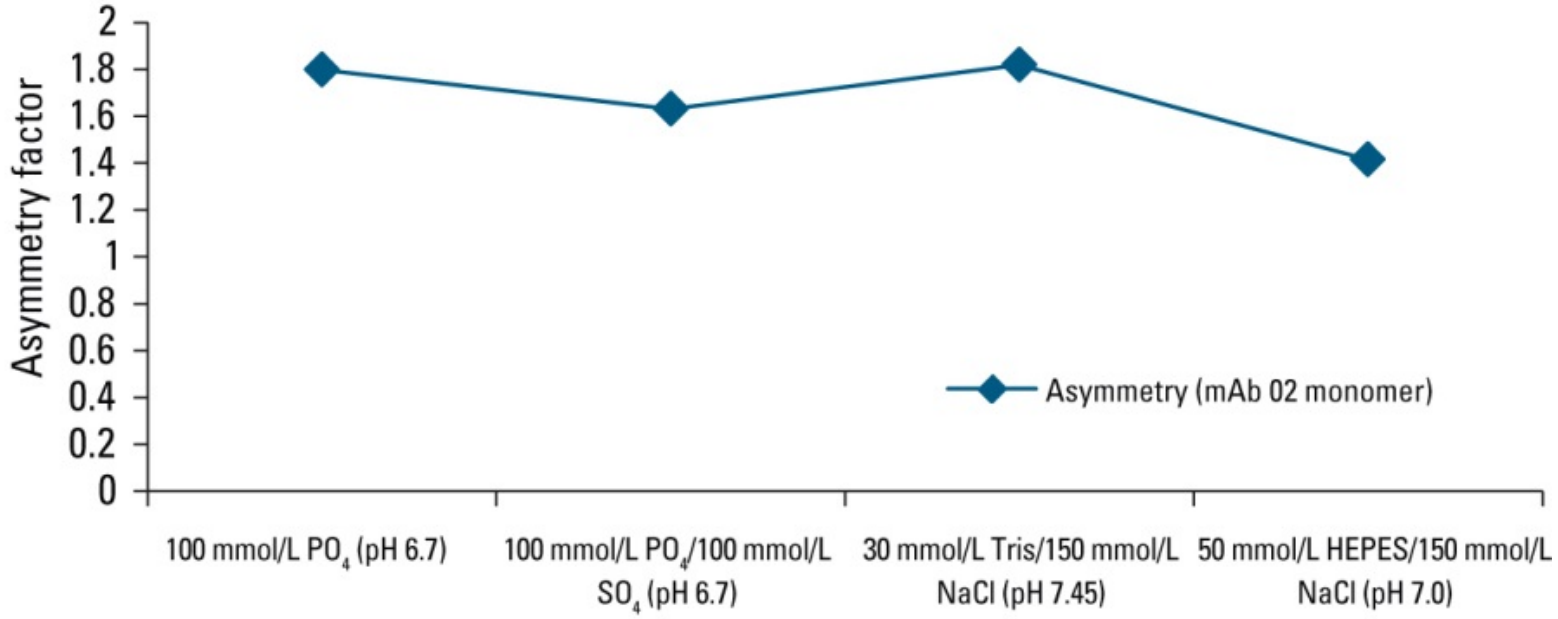


Figure 6: Effect of the mobile phase compositions on the Separation of Thermally Denatured mAb 02 Monomer using the TSKgel SuperSW mAb HR Column: Asymmetry



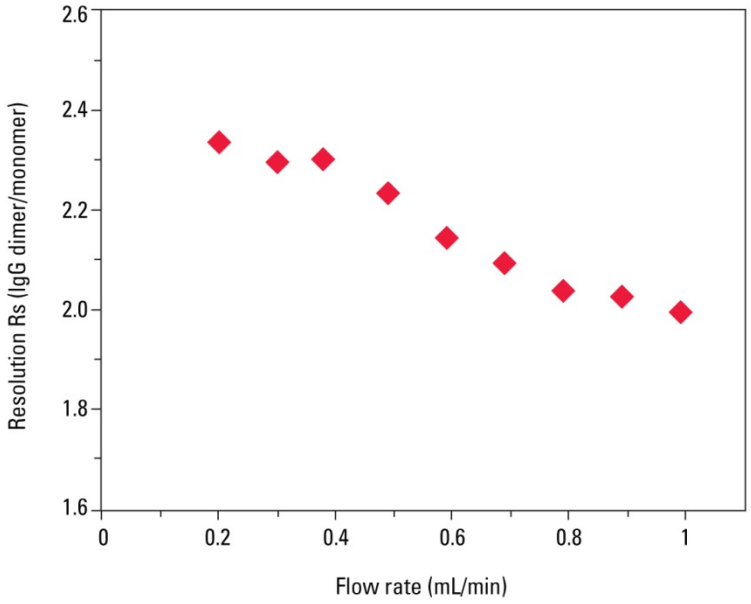
Figures 4, 5 and 6: Conclusion

The retention time, peak area, and peak asymmetry of the mAb 02 monomer peak remained relatively constant irrespective of the 4 mobile phase compositions evaluated

Effect of Flow Rate on the Resolution Between the IgG Monomer and Dimer

- Unlike changes in mobile phase composition, changes in flow rate do not affect the column or analyte during a separation.
- In contrast, altering the flow rate will directly affect band broadening of the analyte traveling through the SEC column.
- Such changes in band broadening directly affect column efficiency and resolution between molecular mass variants.
- Here, the effect of flow rate on monomer/dimer resolution of IgG was evaluated between 0.2 mL/min - 1.0 mL/min.

Figure 7: Effect of Flow Rate on the Resolution Between the IgG Monomer and Dimer



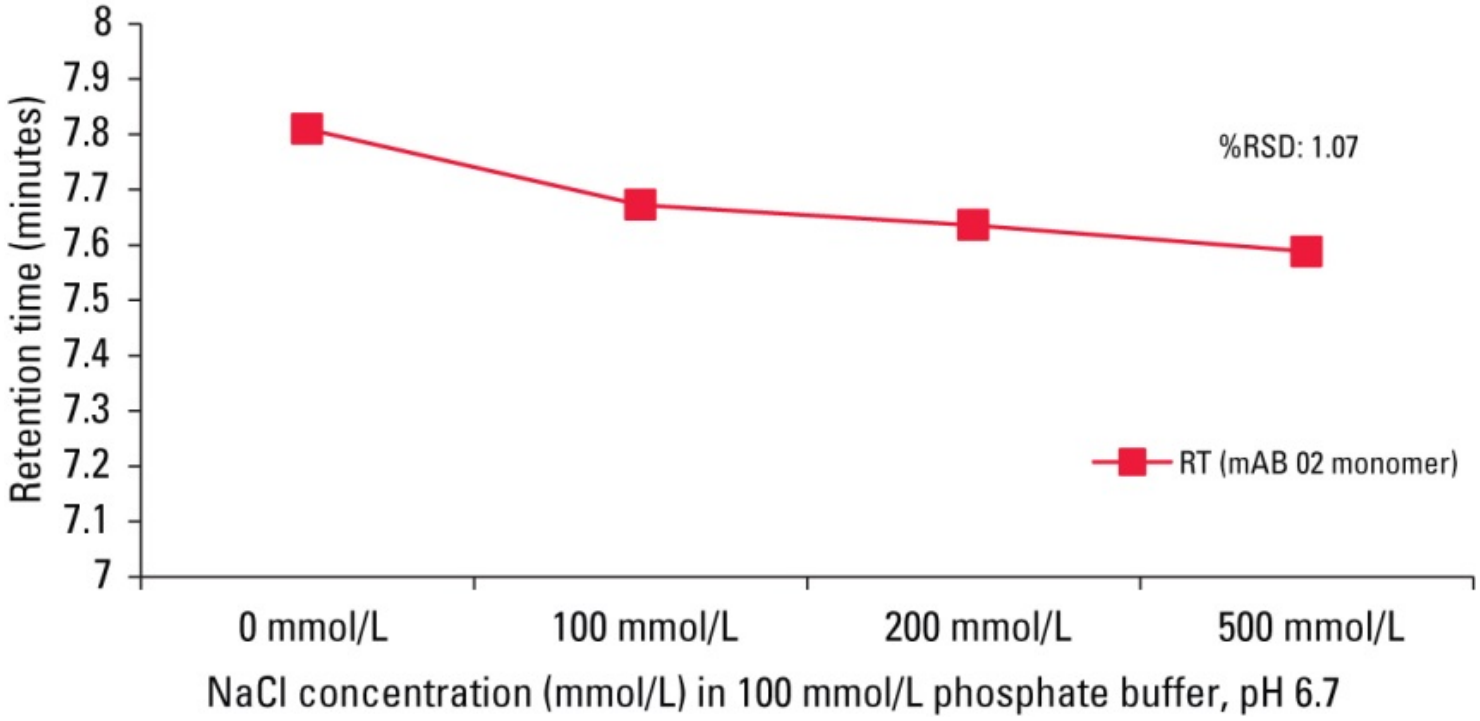
Column:	TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7, + 0.05% NaN_3
Injection vol.:	10 µL
Sample:	IgG preparation for intramuscular injection, 1.5 g/L

This result shows that even at 1.0 mL/min flow rate the resolution between the monomer and dimer is >1.5.

Effect of Mobile Phase Ionic Strength on Critical Peak Parameters

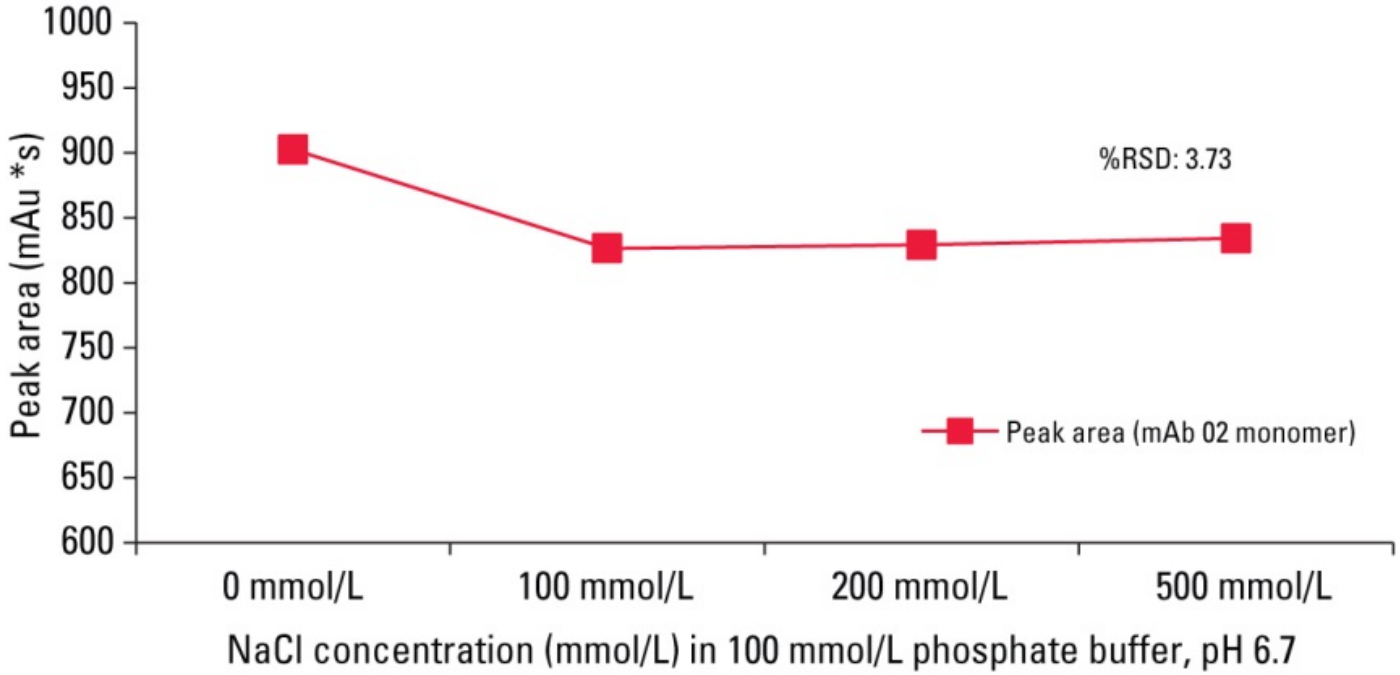
- Changes in the mobile phase ionic strength can lead to more hydrophobic or charge-related (ionic) interactions between the stationary phase and the analyte.
- Such changes can yield poor peak shape, irreproducible retention time, or resolution of molecular mass variants.
- Here, the ionic strength of the neutral salt in the mobile phase (NaCl) was varied from 0 mmol/L - 500 mmol/L.

Figure 8: Effect of Mobile Phase Ionic Strength on Retention Time



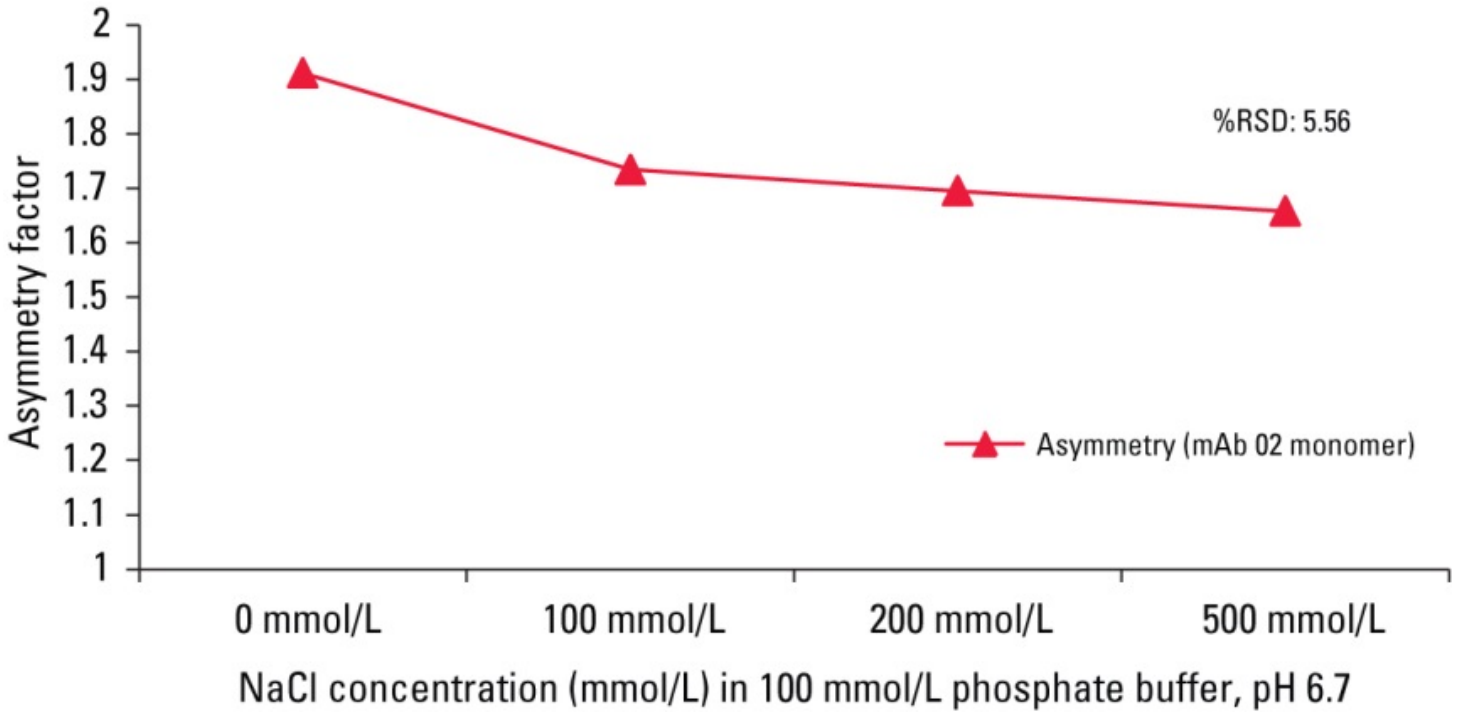
Retention time remained relatively constant with regards to changing the mobile phase ionic strength from 0 mmol/L to 500 mmol/L NaCl.

Figure 9: Effect of Mobile Phase Ionic Strength on Peak Area



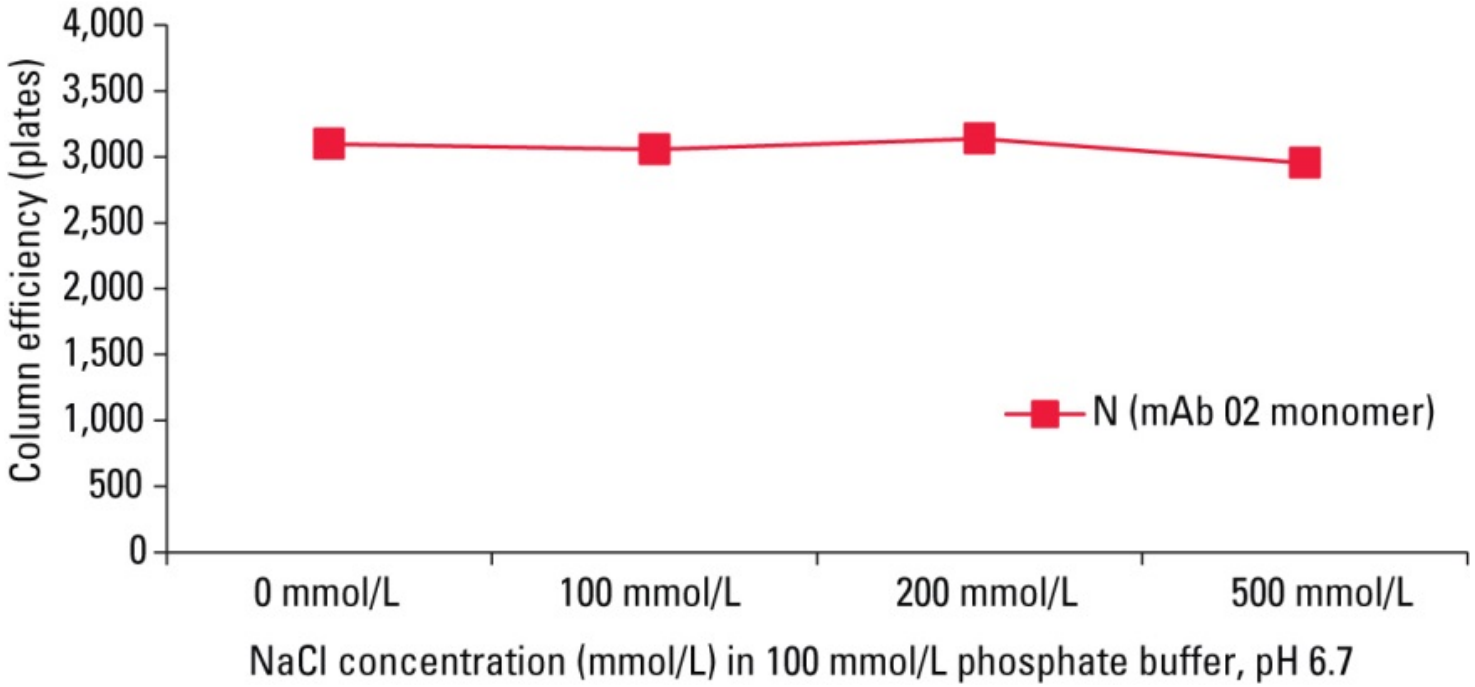
- Peak area remained nearly constant with regards to changing the mobile phase ionic strength from 100 mmol/L to 500 mmol/L NaCl.
- Monomer peak area at 0 mmol/L NaCl is larger than those observed at 100 - 500 mmol/L NaCl concentrations and is possibly due to co-elution, thereby increasing the overall %RSD value.
- Excluding the peak area value obtained at 0 mmol/L NaCl, %RSD from 100 mmol/L to 500 mmol/L NaCl is 0.38.

Figure 10: Effect of Mobile Phase Ionic Strength on Asymmetry



Peak asymmetry steadily improves with increasing ionic strength of the mobile phase.

Figure 11: Effect of Mobile Phase Ionic Strength on Column Efficiency



Column efficiency remained relatively constant with regards to changing the mobile phase ionic strength from 0 mmol/L to 500 mmol/L NaCl.

Conclusions

- Mobile phase parameters such as composition, ionic strength, and flow rate must be evaluated to obtain the best possible separation of the desired protein species from the undesired impurities and variants.
- Increased resolution between the protein monomer and dimer can be obtained by operating at lower flow rates.
- The TSKgel SuperSW mAb HR column is highly tolerant to mobile phase variations due to the proprietary diol coating which prevents unwanted secondary interactions such as ionic and hydrophobic interactions.