TOSOH

APPLICATION NOTE

INFLUENCE OF HPLC-SYSTEM DEAD VOLUME ON THE PERFORMANCE OF (U)HPLC COLUMNS

UHPLC columns show worse results than HPLC columns on non-optimized HPLC Systems

INTRODUCTION

Recent trends in HPLC column and particle technology have facilitated faster, more efficient separations by utilizing smaller particle size solid supports and reducing column geometry. Optimization of these column parameters yields improvements in sensitivity and chromatographic resolution, which results in more accurate quantitation, identification, and characterization of analytes. However, to benefit from these optimized columns it is necessary to use a likewise optimized HPLC-System.

Size exclusion chromatography (SEC) is the standard method for aggregate and fragment analysis of monoclonal antibodies (mAbs) in biopharmaceutical quality control (QC). Ideally, in SEC there is no interaction of the sample with the column and the separation solely occurs by diffusion of the sample in and out of the pores. Because of the absence of interaction with the stationary phase, SEC methods are generally faster than adsorptive methods and are more sensitive to increased dead volume of the system.

This application note compares the performance of a TSKgel UP-SW3000 and TSKgel SuperSW mAb HR column on a non-optimized HPLC system, an optimized HPLC system and a state-of-the-art UHPLC system. Comparisons between columns and instruments were made to isolate and understand the impact of each variable on the chromatographic separation.

The dead volume of the non-optimized HPLC was especially increased by using larger than normal tubings to emphasize the effect. But, due to the use of salt containing mobile phases, the inner diameter of the tubings is often chosen to be larger to counteract salt precipitation.

EXPERIMENTAL CONDITIONS

Columns:
1. TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm L
2. TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 30 cm L

Instruments:
1. Thermo Fisher Dionex Ultimate® 3000 (Fitted with Peek Tubings)
2. Thermo Fisher Dionex Ultimate 3000 (Fitted with Viper Tubings)
3. Thermo Fisher Vanquish (Fitted with Viper Tubings)

Mobile phase: 100 mmol/L $NaH_2PO_4Na_2HPO_4$, pH 6.7, 100 mmol/L, Na_2SO_4, 0.05 % NaN_3
Gradient: isocratic
Flow rate: UHPLC: 0.35 mL/min; HPLC: 1.0 mL/min
Detection: UV @ 280 nm and 20 Hz, 2.5 µL flow cell, 7 mm pathlength
Temp.: 25 °C
Injection vol.: UHPLC: 10 µL; HPLC: 20 µL

Samples:
1. Protein Standard Mix 15 - 600 kDa (69385 Sigma-Aldrich)
2. TBG mAb 01 (stressed), 3.8 mg/mL in mobile phase, 4 °C

(U)HPLC Instrument	Injector to Column	Column to Detector	Extra column dead volume
System 1	0.13 mm ID x 350 mm 0.76 mm ID x 240 mm	0.76 mm ID x 240 mm 0.13 mm ID x 600 mm	232 µL
System 2	0.1 mm ID x 350 mm 0.1 mm ID x 250 mm	0.1 mm ID x 250 mm 0.13 mm ID x 600 mm	15 µL
System 3	0.1 mm ID x 350 mm	0.1 mm ID x 440 mm	7 µL

Table 1

Extra column dead volume of the UHPLC and HPLC systems used.

RESULTS AND DISCUSSION

Figure 1 shows the resolution of the 15 kDa – 600 kDa Protein Standard with TSKgel SuperSW mAb HR and TSKgel UP-SW3000 on the differently optimized systems. Table 2 shows the asymmetry and theoretical plate count for the last peak in the used standard mix. There is a drastic loss in column performance when using the TSKgel UP-SW3000 on a the non-optimized HPLC system.

The amount of theoretical plates drops from 51.023 on System 3, to 44.853 on System 2 and to just 4.152 plates per column on System 1. Due to the larger inner diameter and higher flow rate when using the TSKgel SuperSW mAb HR, the analyte spends less time in the extra column dead volume of the system. Therefore the peak broadening due to longitudinal diffusion in the capillaries is decreased and the number of theoretical plates of the 4 µm TSKgel SuperSW mAb HR is greater compared to the TSKgel UP-SW3000.

When comparing the stressed mAb injections (Figure 2) all resolution of aggregates and fragments is lost when using the TSKgel UP-SW3000 on the non-optimized System 1.

By reducing the extra column dead volume, the resolution of monomer, dimer, trimer and fragments becomes better on the TSKgel UP-SW3000 compared to the TSKgel SuperSW mAb HR due to the smaller particle size. Table 2 shows the results for the non-stressed mAb sample.

CONCLUSIONS

The data clearly shows, that the extra column dead volume drastically influences column performance. When using a not optimized system it is recommended to use a larger inner diameter column. This way the time the analyte spends in the extra column volume is decreased and the extra column volume compared to the column volume becomes smaller. Smaller particle size and narrower column ID increase efficiency values resulting in sharper, taller peaks, which translates to a better resolution for QC, but only if the correct HPLC system is chosen. Instrument dispersion volume has a direct effect on column performance in SEC; instrument optimization is key to improving separation quality. An optimized UHPLC method provides the best quality separation, yielding to higher resolution and sensitivity.

PROTEIN STANDARDS FOR TSKgel UP-SW300 AND SuperSW mAb HR

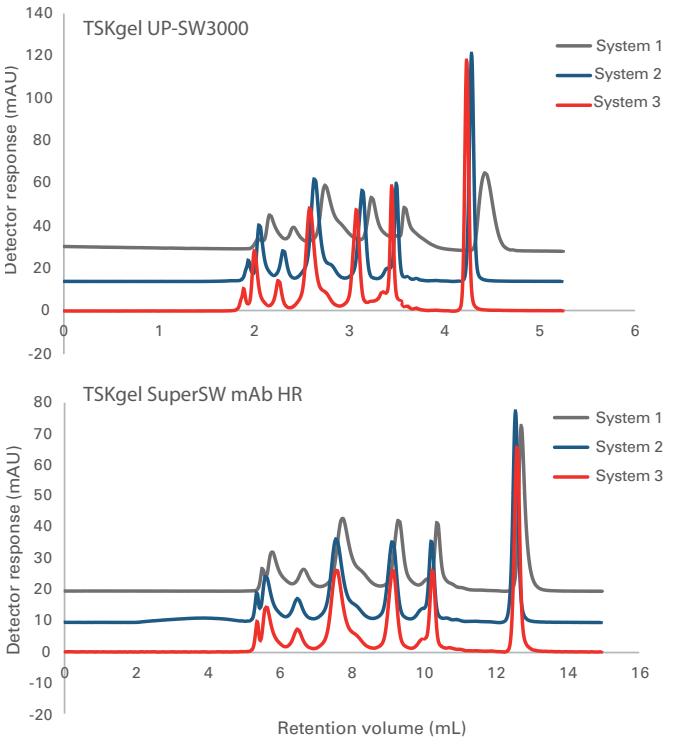

Figure 1

MAb INJECTIONS

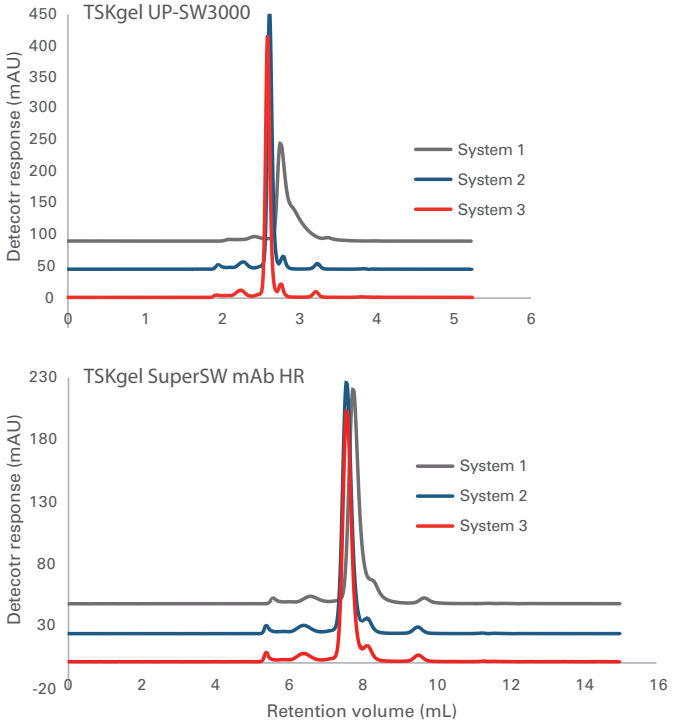

Figure 2

	UP-SW 3000 *	SuperSW mAb HR	UP-SW 3000	SuperSW mAb HR	UP-SW 3000	SuperSW mAb HR
(U)HPLC	System 1 (232 µL)		System 2 (15 µL)		System 3 (7 µL)	
N (pAba)	4.152	23.616	44.853	34.861	51.023	36.050
N (BI mAb)	3.220	5.604	13.752	6.410	16.327	6.731

** Due to pressure limit of fitting, flow was reduced to 0.15 mL/min)*

Table 2