

Optimal Method Development for Separation of Monoclonal Antibodies Using ProPac Weak Cation-Exchange (ProPac WCX) Columns: Effect of Iron on Column Performance

Srinivasa Rao and Christopher Pohl, Dionex Corporation, Sunnyvale, CA, USA

ABSTRACT

Charge heterogeneity is generally exhibited by monoclonal antibodies (MAbs) which can result from various reasons including oxidation, aspartic isomerization, asparagine deamidation, lysine truncations, and glycan modifications. Manufacturing and subsequent stability testing procedures of MAbs involve routine analysis and monitoring of the impurities resulting from these situations. ProPac® weak cation-exchange (WCX) columns are widely used to characterize MAb heterogeneity.

ProPac WCX columns are packed with non-porous particles which are well suited for high resolution analytical separations of both acidic and basic monoclonal antibody variants. Using ProPac WCX columns, we separated MAbs using MES (pH 5.6), ACES (pH 6.7), and Tris (pH 7.3) and compared the influence of these conditions. Further, we studied the influence of varying pH, gradient conditions, and temperature and discussed optimal conditions for separation of monoclonal antibodies.

We attempted and achieved fast separations of MAb within 2.5 minutes using a steep gradient on a ProPac WCX-10 4 × 50 mm column. Even under these conditions, lysine variants could be separated.

We explored the problems associated with metal poisoning of ProPac WCX columns when stainless steel HPLC systems were used. Metal components in such HPLC systems corrode and form a metal complex when salts and low pH eluents are in contact with them. Such metal complexes from the corrosion will leach on to the column and inhibit its performance. We have tested this phenomenon with a laboratory experiment employing Fe³⁺ conjugates on the separation of MAb using ProPac WCX columns on inert HPLC systems. The loss of resolution and capacity were completely reversible after washing the column with oxalate solution suggesting that it was due to the metal binding on cation-exchange sites. Earlier, we have shown that SST HPLC systems have a seasoning effect. These observations provide evidence that inert systems are ideal for MAb separations by cation-exchange chromatography.

INTRODUCTION

Monoclonal antibody (MAb) micro-heterogeneity can be attributed to glycosylation, oxidation, mutation, phosphorylation, amino terminal modifications (e.g., to pyroglutamate), incomplete processing of the C-terminus, and asparagine (Asn) deamidation. These variations in protein composition occur in many types of proteins and can impact the activity and stability of biotherapeutics. Monitoring stability of therapeutic proteins and peptides is regarded as essential for demonstrating safety and efficacy of these drugs, and is required by the FDA and other regulatory agencies.

ProPac WCX columns are packed with non-porous 10 µm particles which are well suited for high resolution analytical separations of both acidic and basic monoclonal antibody variants. ProPac packings are pellicular polymeric supports with hydrophilic coatings and grafted surface chemistry, which exhibits minimal hydrophobic character. In addition, these particles exhibit a wide range of pH stability with high selectivity and minimal band spreading.

In this study we present optimal method development conditions for MAb separations using ProPac WCX. We tested the effects of pH, temperature, and NaCl gradients on MAb separation. Using ProPac WCX columns, we separated MAbs using MES (pH 5.6), ACES (pH 6.7), and Tris (pH 7.3) eluents and compared the influence of these conditions. Also, we demonstrated fast MAb separations using ProPac WCX 4 × 50 columns using different gradients. Earlier, we had developed an optimized MES/heat conditioning step to recover occasionally underperforming ProPac WCX columns. We noticed that this conditioning step did not help to recover the metal poisoned columns.

We explored the problems associated with the metal poisoning of ProPac WCX columns caused by rusted stainless steel components. We tested this phenomenon by injecting Fe³⁺ conjugates directly on to the ProPac WCX columns, followed by the separation of MAbs on inert HPLC systems. The loss of resolution and capacity observed were completely reversible after washing the column with oxalate solution, suggesting that poor performance was due to the metal binding on cation-exchange sites. Earlier, we had shown that SST HPLC systems have a seasoning effect. These observations provide evidence that inert HPLC systems are ideal for MAb separations by cation-exchange chromatography.

RESULTS

In this study, we present data collected using the ProPac WCX column. Baseline separations were achieved for the majority of the IgG charge variants 1-3 (Figure 3). We investigated the effect of different temperatures (Figure 4) and NaCl gradients (Figure 5) on MAb separation. Also, we tested the effect of different buffering eluents including MES (pH 5.6), ACES (pH 6.7) and Tris (pH 7.3) with varying pH on a MAb separation (Figure 6). While all these eluents were effective in separating lysine variants and basic variants, acidic variants were best resolved using MES buffers, suggesting that the optimal choice of buffer and pH of the eluent play an important role in method development. We also noted that gravimetric preparation of eluents improved the retention time accuracy and reproducibility of the method.

We explored the problems associated with metal poisoning of ProPac WCX columns when stainless steel (SST) HPLC systems are used. Metal components in SST HPLC systems corrode and form a metal complex (rust) when high concentrations of salts containing low pH eluents are in contact with them. Metal complexes from the corrosion will leach on to the column and inhibit its performance. We tested this phenomenon by injecting Fe³⁺ conjugates onto ProPac WCX columns followed by the separation of MAb on inert HPLC systems. The loss of resolution and capacity observed were reversible after washing the column with oxalate solution, suggesting the poor performance was due to the metal binding on cation-exchange sites (Figures 7, 8, and 9). In addition, we showed that SST HPLC systems have a seasoning effect (Figures 10 and 11). These observations provide evidence that inert systems are ideal for MAb separations by cation-exchange chromatography. Figure 12 shows the fast chromatography separation using the ProPac WCX-10 4 × 50 mm column (under 2 minutes).

We observed that occasionally some MAbs did not resolve well on WCX columns. We developed a simple procedure to heat treat the WCX column in the presence of 20 mM MES (at pH 5.6 or 6.5) at 50 °C for 48 h (Figure 13) or 70 °C for 7 h (Figure 14). MAb separations on the MES/heat-conditioned columns exhibited higher efficiency, better peak shapes, and improved overall performance when compared to separations on unconditioned control columns. We hypothesize that a MES/heat step brings favorable changes in the conformation of the polymer grafts that may be responsible for this improved performance. Also, we noticed that this conditioning step will not help to recover the metal poisoned columns.

Materials

Chromatographic Components

All PEEK™/inert system:

ICS-3000 DP gradient pump, VWD absorbance detector, UltiMate® autosampler, and TCC-100 thermostatted column compartment were from Dionex Corporation

SST system:

P680 gradient pump, AD20 absorbance detector, ASI-100 autosampler, and TCC-100 thermostatted column compartment were from Dionex Corporation

Chromatography was controlled by Chromeleon® Chromatography Management Software (Dionex Corporation)

Chemicals

MES, HEPES, ACES, oxalic acid dihydrate, ferric sulfate hydrate, and all other analytical grade chemicals were obtained from Sigma. Ferric oxide model rust particles (38 nm) were prepared in the laboratory. MAb was a gift from a local biotech company.

Columns

ProPac WCX-10 analytical 4 × 250 mm (P/N 054993) and ProPac WCX-10G guard 4 × 50 mm (P/N 054994) columns were from Dionex Corporation.

Methods

Metal Removal Procedure*

Step 1: Wash the ProPac column with 200 mM oxalic acid dihydrate at 0.2 mL/min overnight (room temp).
You may skip connecting to the detector.

Step 2: Wash the column with 20 mM NaOH at 0.5 mL/min for 30 min (room temp).

Step 3: Equilibrate your column with your routine buffer before proceeding to analysis.

*Should be performed using inert HPLC pumps only.

MES/Heat Treatment Procedure

Treat the ProPac WCX column with 20 mM MES, pH 5.6 or pH 6.5, for 7 h at 70 °C at a flow rate of 0.2 mL/min.

Alternatively, the column may be treated for 48 h at 50 °C.

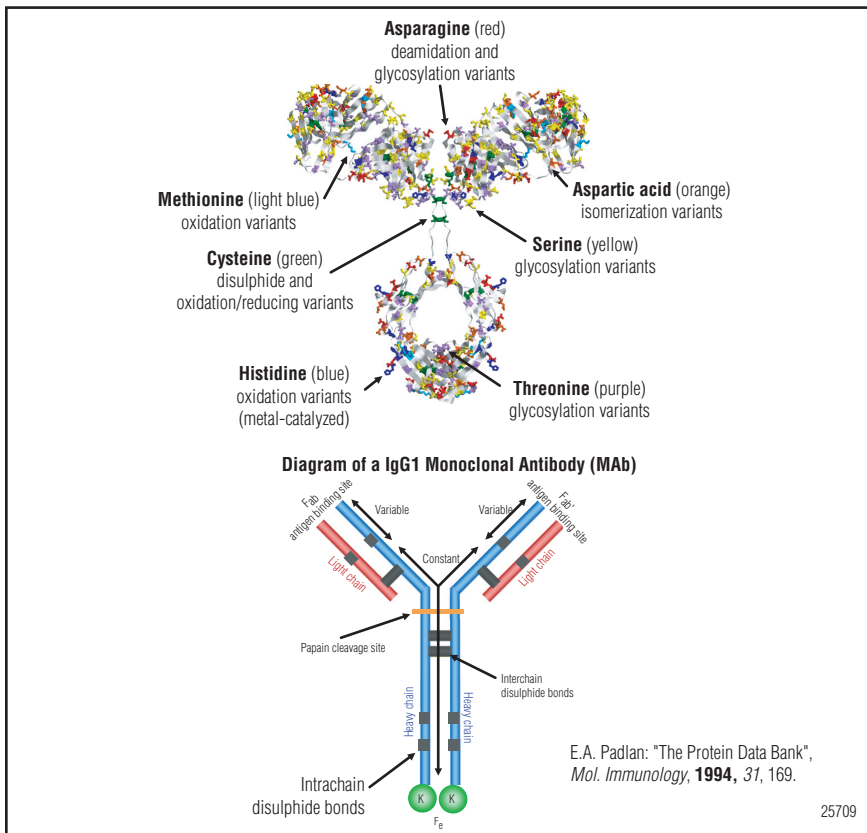


Figure 1. Crystal structure of a human IgG1 MAb.

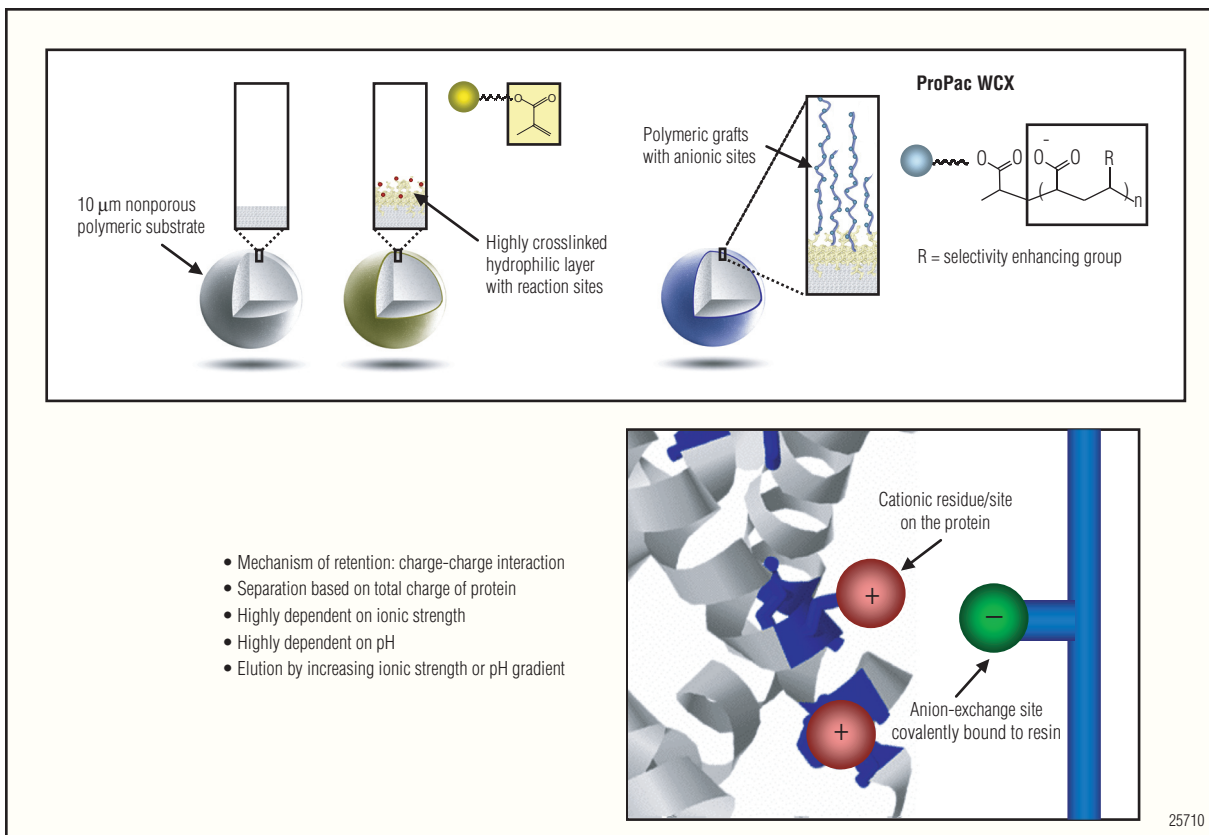


Figure 2. Diagram and description of ProPac WCX-10 stationary phase.

MAB-Method Development

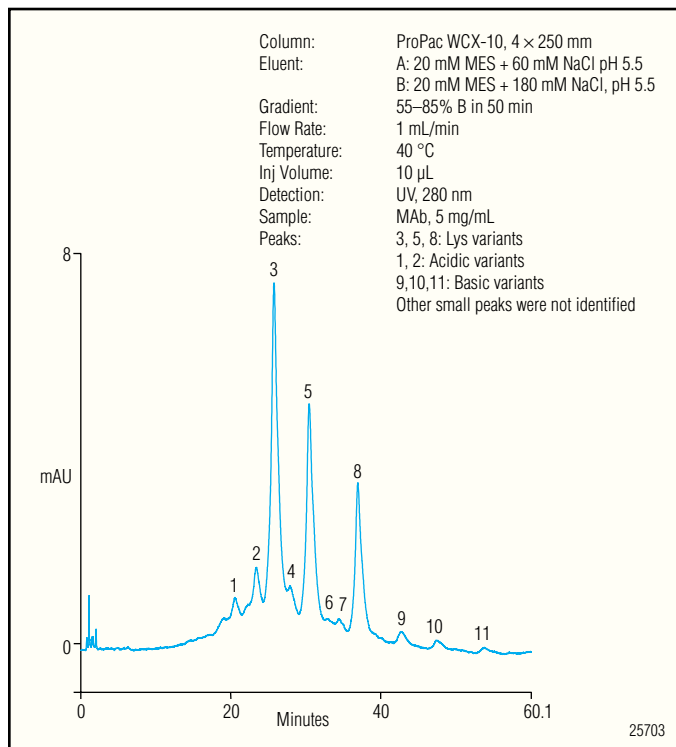


Figure 3. Separation of MAb on a ProPac WCX column using MES eluents.

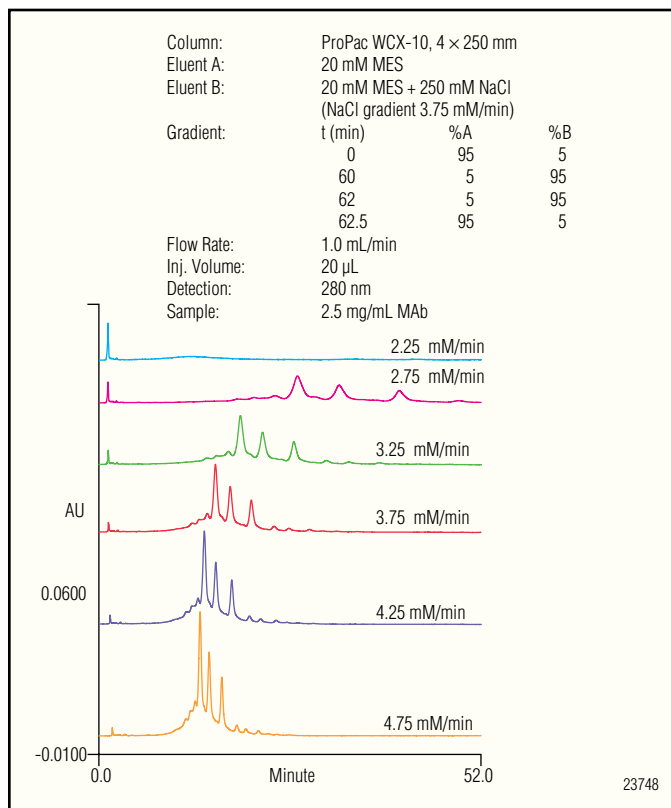


Figure 5. Effect of salt gradients (4.75 mM/min to 2.25 mM/min) on MAb separation at pH 5.5 is shown.

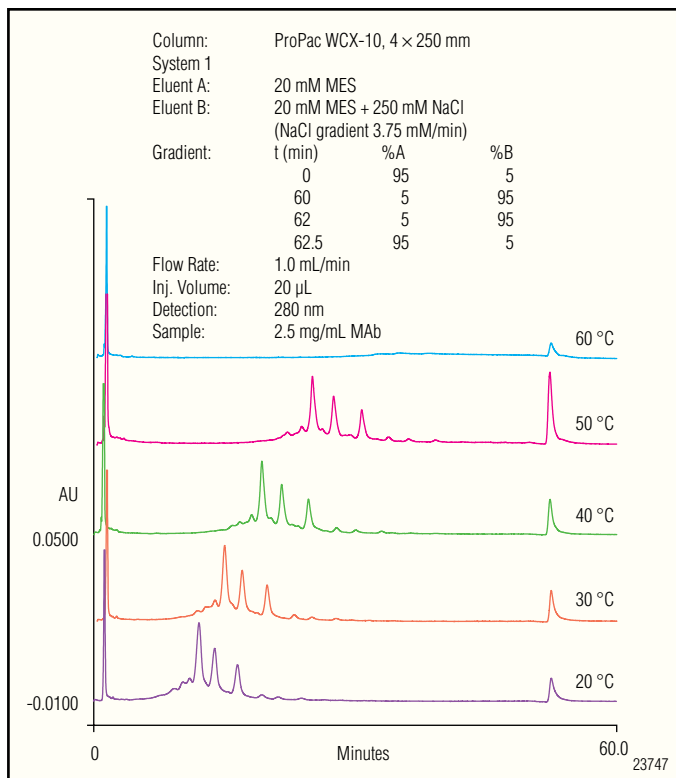


Figure 4. Effect of temperature (20 °C to 60 °C) on MAb separation at pH 5.5 is shown.

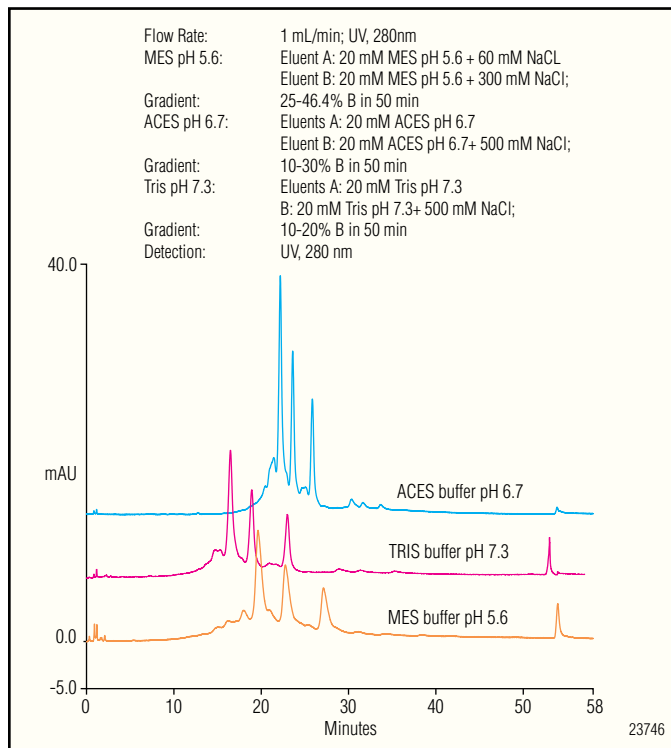


Figure 6. Effect of various buffers with different pH on MAb separation is shown. Three different buffers were used.

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Effect of Iron



Figure 7. A picture of inlet frit of ProPac WCX column after Fe^{3+} loading experiment (left). An unused frit is shown for comparison (right).

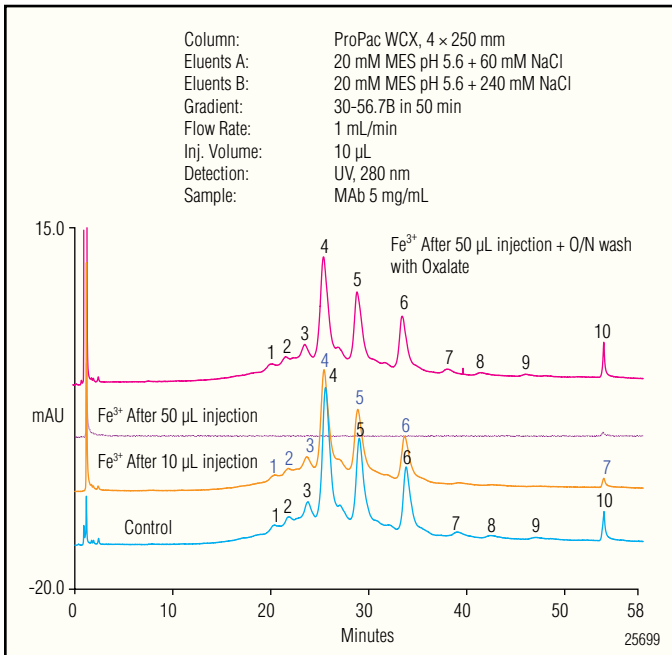


Figure 8. MAb separation on ProPac WCX-10: Effect of Fe^{3+} sulfate and reversal with oxalic acid hydrate treatment.

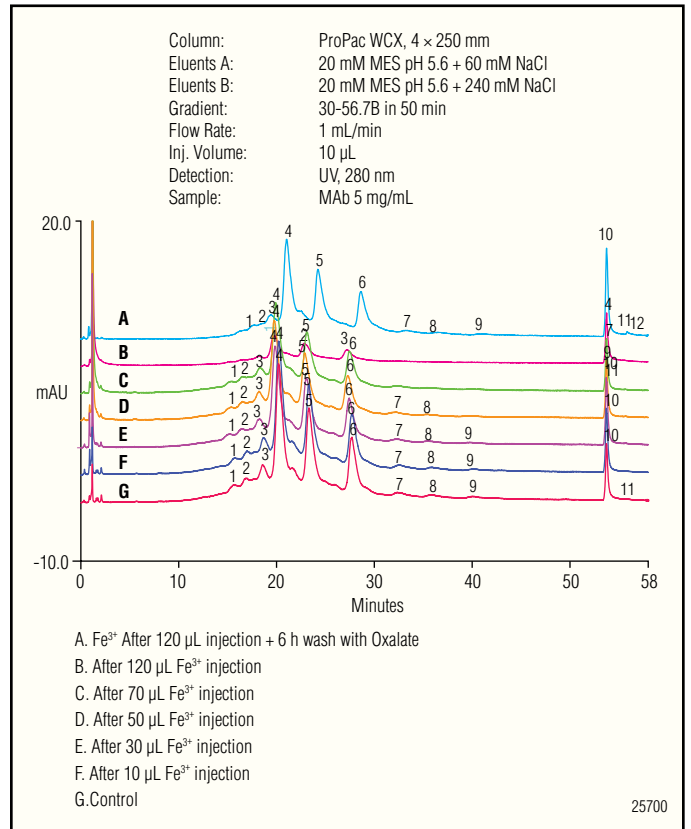


Figure 9. MAb separation on ProPac WCX-10: Effect of feric oxide model rust particles and reversal with oxalic acid hydrate treatment.

PEEK vs SST HPLC Systems

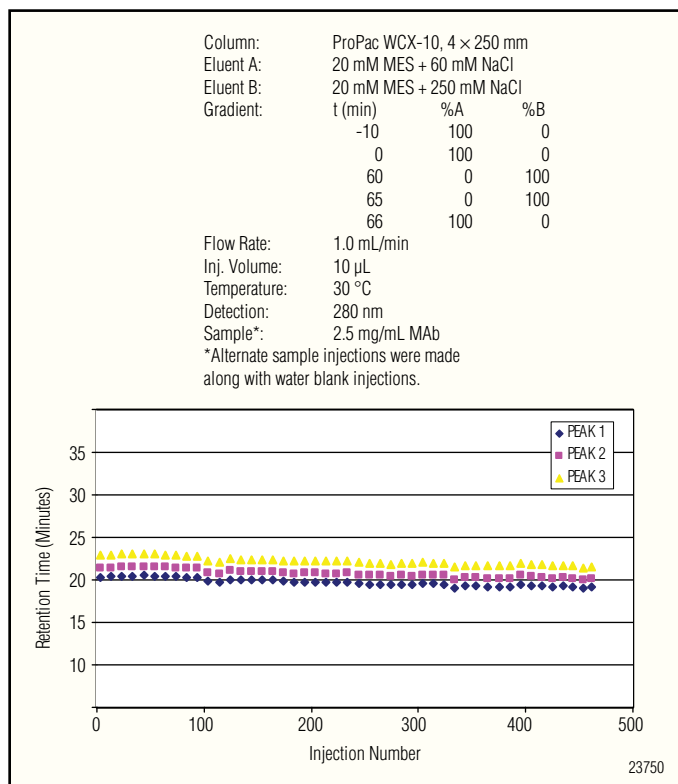


Figure 10. MAb was separated on a ProPac WCX using an all-PEEK HPLC system. A total of 460 cycles were analyzed. The data produced at various cycles (one data point for every tenth cycle) is shown. Peaks 1, 2, and 3 represent C-terminal lysine variants.

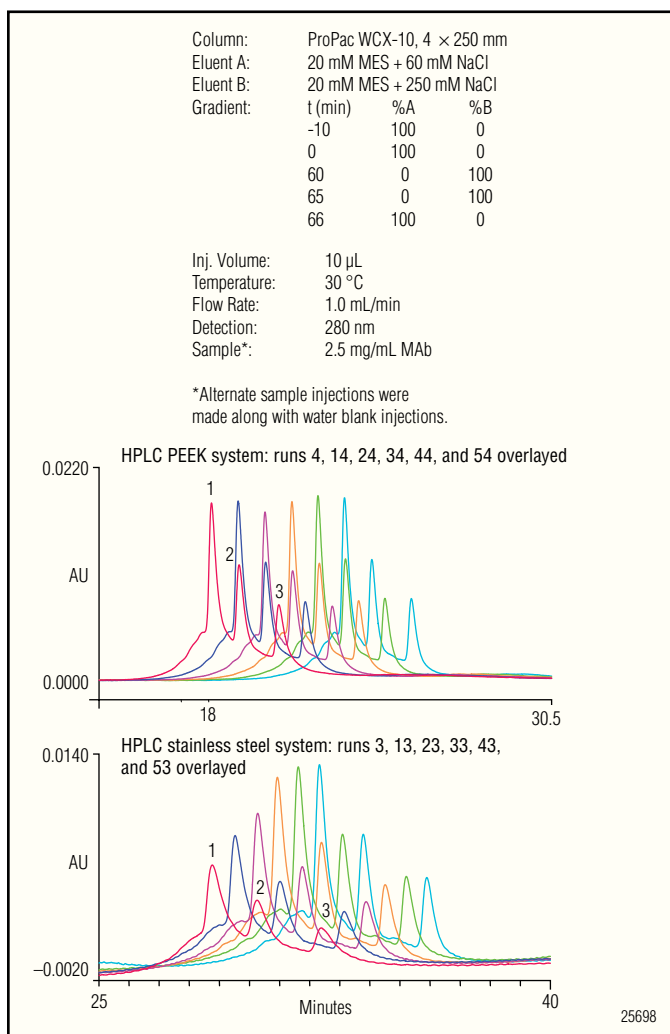


Figure 11. Separation of MAb: Comparison of results (first 55 cycles) obtained on an all PEEK HPLC system vs an all SST HPLC system. The data produced at various cycles (one data point for every tenth cycle) is shown. A clear sea-soning effect was observed with SST HPLC system compared to the all PEEK system. It took 43 cycles for SST to stabilize the peak areas, whereas PEEK systems produced consistent peak areas from the beginning (cycle 4).

Fast MAb Separations

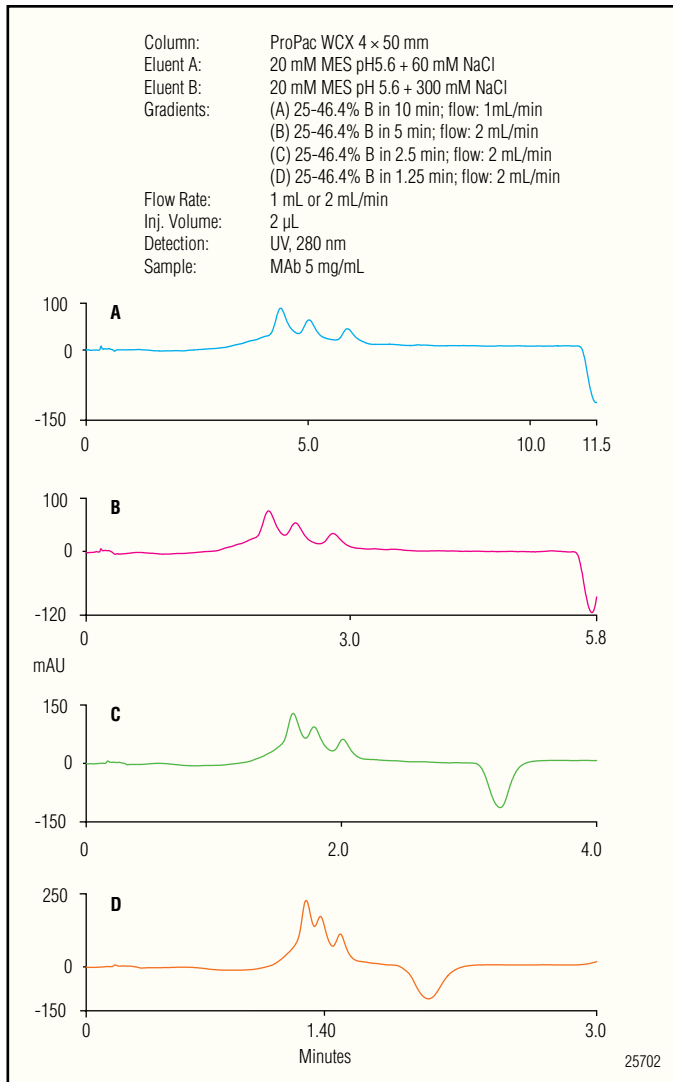


Figure 12. Fast MAb analysis on ProPac WCX 4 × 50 mm using different gradients at various flow rates.

MES/Heat Treatment

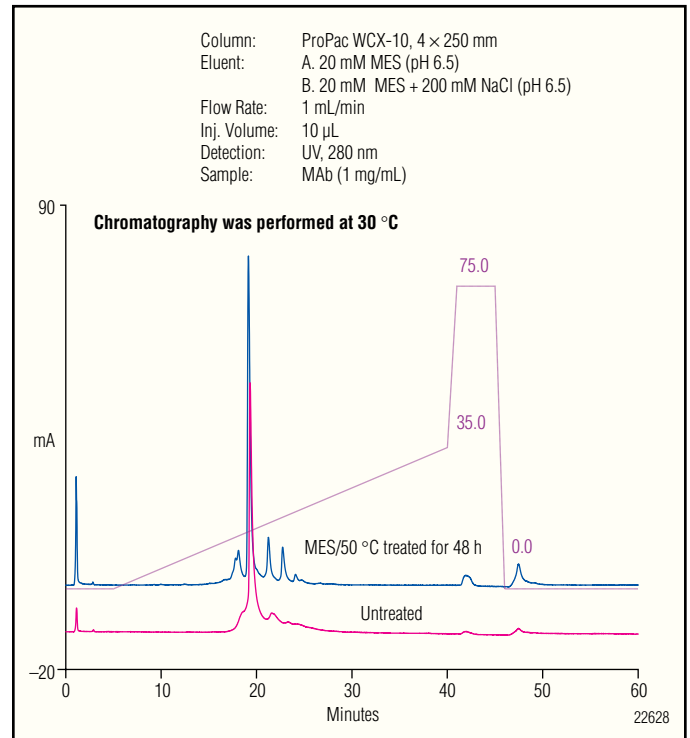


Figure 13. The ProPac WCX-10 column was treated with 20 mM MES pH 6.5 at 50 °C at 48 h. The treated column was used to separate MAb and compared with an untreated column. The chromatography was performed at 30 °C.

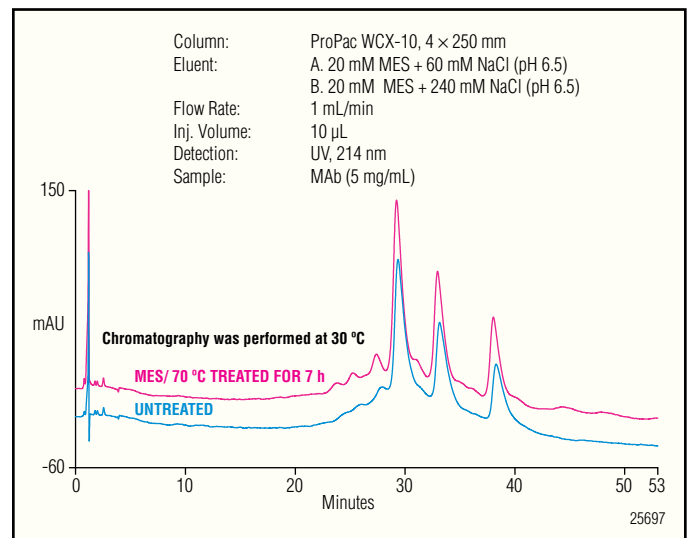


Figure 14. The ProPac WCX column was treated with 20 mM MES pH 5.6 for 7hrs at 70 °C. The treated column was compared with an untreated control column and the chromatography was performed at 30 °C.

CONCLUSION

The ProPac WCX-10 is the column of choice for the separation of acidic and basic charge variants of monoclonal antibodies (MAb).

The choice of buffering eluents and pH play an important role in method development of MAb separation. We tested the separation of MAb using MES (pH 5.6), ACES (pH 6.7) and Tris (pH 7.3) eluents. We also investigated the effect of different temperature and NaCl gradients on MAb separation. All these parameters appear to influence MAb separation and therefore play an important role in method development. Gravimetric eluent preparation methods improve reproducibility of MAb separation.

We noticed that metal complexes from the corroded SST HPLC systems will leach onto a ProPac WCX column and inhibit its performance. We tested this phenomenon by injecting Fe³⁺ conjugates onto ProPac WCX columns, followed by the separation of MAb on inert HPLC systems. The loss of resolution and capacity observed were completely reversible after prolonged washing of the column with oxalate solution, suggesting that the poor performance was due to the metal binding to cation-exchange sites. This metal removal process is very tedious and depends on the severity of the metal poisoning. In addition, we have shown that SST HPLC systems have a seasoning effect. All these observations provide evidence and suggest that inert HPLC systems are ideal for MAb separations by cation-exchange chromatography.

We have attempted and achieved fast separations of MAb within 2.5 minutes using a steep gradient on a ProPac WCX-10, 4 × 50 mm column. Even under these conditions, lysine variants could be separated.

MES/heat conditioning of the ProPac WCX column improves the overall performance of the occasional under-performing WCX columns. When MAbs are separated on these heat conditioned columns, higher efficiency, better peak shapes, and improved overall performance are observed as compared to unconditioned control columns. Once the column is heat treated, there is no need to repeat this step for subsequent runs. We assume that this change is permanent. However, how long this effect will last is unknown. We hypothesize that a change in the conformation of the polymer grafts may be responsible for this improved performance. Also, we observed that this conditioning step will not help to recover the metal poisoned columns.

REFERENCES

1. Dionex Application Notes 125, 127, and 128.
2. Harris, R. J., *et al* (2001) *J. Chromatogr B*, 752: 233-245.
3. Moorhouse K. G., *et al* (1997) *J. Pharm. and Biomed Anal.* 16: 593-603.

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Dionex Corporation

1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

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U.S. (847) 295-7500
Canada (905) 844-9650

South America

Brazil (55) 11 3731 5140

Europe

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