MAbPac SCX 3 and 5 µm Particle Phases for Monoclonal Antibody (MAb) Variant Analysis

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Abstract

Monoclonal antibodies (MAbs) undergo several post-translational modifications including oxidation, deamidation, and truncation. Manufacturing of MAbs and subsequent stability testing procedures involve routine analysis and monitoring of the impurities resulting from these modifications.

Earlier, we introduced Thermo Scientific MAbPac strong cation-exchange phase (MAbPac SCX) based on 10 µm particles for the characterization of heterogeneity of MAbs. It is a complementary addition to the existing Thermo Scientific ProPac WCX-10 column that provides orthogonal selectivity for MAb charge variant analysis. Both ProPac™ WCX (Carboxylic acid functionality) and MAbPac™ SCX (Sulfonic acid functionality) stationary phases are based on nonporous, highly cross-linked styrenic polymeric media. They differ in proprietary hydrophilic coatings as well as functional groups and grafting processes.

Now, we are introducing the new MAbPac SCX columns with 5 µm and 3 µm particle phases for MAb variant analysis. The main objective for this development was to produce high-resolution columns comparable to the MAbPac SCX-10, 10 µm columns (4 × 250 mm), but with a much faster analysis time. The length advantage of a 250 mm column for producing high resolution is compensated by a smaller particle size in a 4 × 50 mm column. Since the column is shorter, analysis time can be reduced and throughput can be increased. Using these small particle columns, several fast applications including acidic, basic variant analysis, and lysine truncation variant characterization of a MAb were performed. Both salt and pH gradients were used in these experiments. In addition, a longer length version (4 × 250 mm) of the 5 µm phase columns are introduced for very high resolution purposes that takes advantage of both length and smaller particle size for superior resolution as compared to the 10 µm phase columns. Ruggedness of both the 3 and 5 µm phases is also demonstrated.

Introduction

Monoclonal antibodies generally exhibit charge heterogeneity from oxidation, asparagine deamidation, aspartic isomerization, lysine truncation, glycan modifications, and others. Therefore manufacturing and subsequent quality assurance and stability testing procedures of MAbs involve routine analyses and monitoring of the impurities resulting from these situations.

ProPac weak cation-exchange (WCX) columns are routinely used to characterize MAb heterogeneity as they are well suited for high-resolution analytical separations of both acidic and basic monoclonal antibody variants.

Earlier we introduced the MAbPac SCX-10, 10 µm, a strong cation exchange column for high resolution MAb charge variant characterization. Using this column, several applications, including characterization of lysine truncation variants, enzymatic digestions, and Fab and Fc analysis are performed.

This work describes the development of 5 µm and 3 µm small-particle, high-resolution MAbPac SCX columns for faster MAb analysis. These columns are compared with 4 × 250 mm ProPac WCX and MAbPac SCX 10 µm columns for different applications. Ruggedness of the 3 µm, as well as 5 µm columns, is presented.

Materials

Chromatographic Components

MAb separations were performed on an inert Thermo Scientific Dionex UltiMate 3000 Titanium system that included an inert gradient pump, VWD absorbance detector, biocompatible autosampler, and thermostatted column compartment.

Chromatography was controlled using the Thermo Scientific Dionex Chromeleon Chromatography Data System.

MAb sample was a gift from a local biotech company. Carboxypeptidase, cytochrome C, MES, and all other analytical grade chemicals were obtained from Sigma.

Columns

ProPac WCX-10, 10 µm, 4 × 250 mm (P/N 054993)
MAbPac SCX-10, 10 µm, 4 × 250 mm (P/N 074625)
MAbPac SCX-10, 3 µm, 4 × 50 mm (P/N 077907)
MAbPac SCX-10, 5 µm, 4 × 250 mm (P/N 078655)
MAbPac SCX-10, 5 µm, 4 × 50 mm (P/N 078656)

Separation Media and Mechanism

Ion Exchangers

ProPac WCX-10, 10 µm nonporous substrate

1. A proprietary surface modification process created a highly hydrophilic coating. No hydrophobic interactions are present.
2. A random polymerization approach was used for functional grafts.
3. Functional group: carboxylic acid.

MAbPac SCX-10, 10 µm, 5 µm, and 3 µm nonporous substrate

1. An alternative proprietary one-step surface modification process yielded a uniform hydrophilic coating. No hydrophobic interactions are present.
2. An ATRP-based grafting approach was used to control functional group chain length and the density of functional groups.
3. Functional group: sulfonic acid.

FIGURE 1. Separation media and mechanism of cation-exchange column.

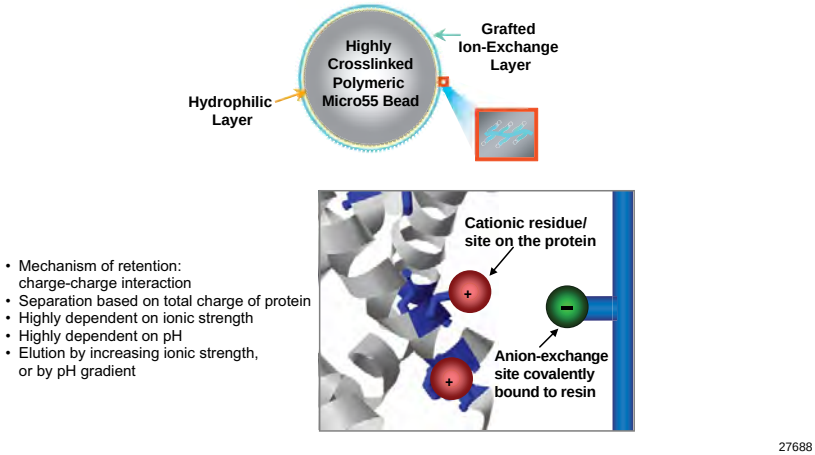


FIGURE 2. Selectivity differences: MAbPac SCX-10 vs ProPac WCX-10 columns.

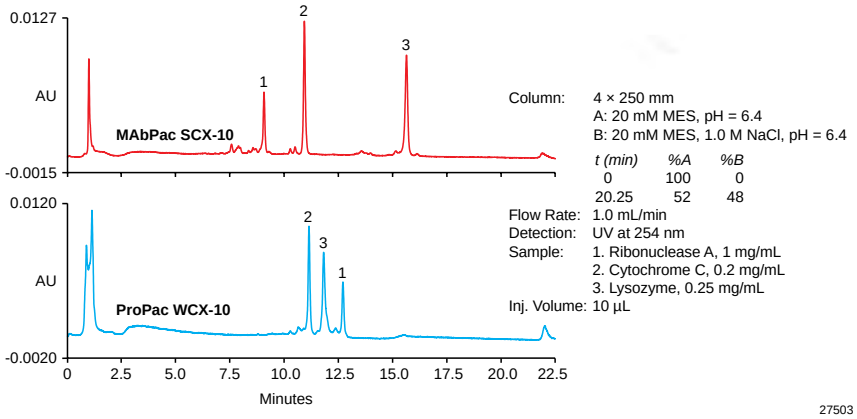
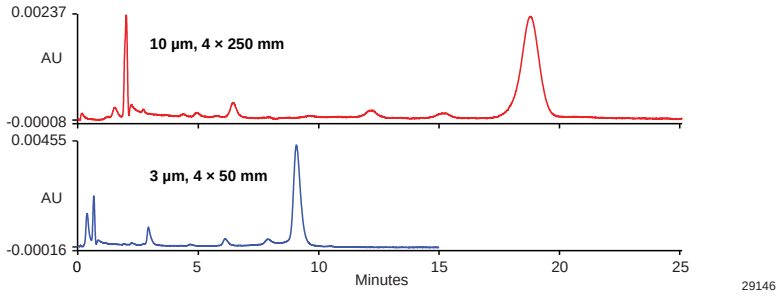


Figure 2 shows the separation of a protein mixture, demonstrating the selectivity differences beween ProPac WCX and MAbPac SCX columns. Cytochrome C elutes first on the ProPac WCX, followed by lysozyme and ribonuclease A. With the SCX column the elution order is reversed and ribonuclease A elutes first, followed by lysozyme and cytochrome C. Such selectivity differences could be very beneficial in characterizing the closely related protein variants.

FIGURE 3. Comparison of MAbPac SCX-10 10 µm, 4 × 250 mm column and 3 µm, 4 × 50 mm column: isocratic separation of Cytochrome C.



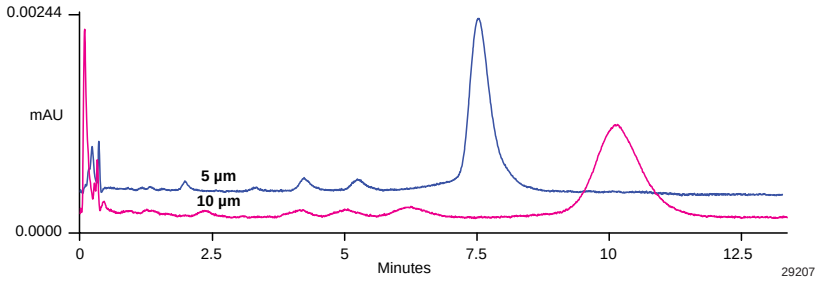
Efficiency for cytochrome C peak shows better values for MAbPac SCX-10, 3 µm, 4 × 50 mm column than the MAbPac SCX-10, 10 µm, 4 × 250 mm column (Figure 3). A comparison of retention time, asymmetry, back pressure, and efficiency are presented in Table 1.

Table 1. Comparison of MAbPac SCX-10 10 µm, 4 × 250 mm and 3 µm, 4 × 50 mm columns

Column Type	Resin Size, µm	Iso, $RT_{Cyt\ C}$	Asym	N	BP, psi at 0.5 mL/min
MAbPac SCX-10, 10 µm (4 × 250)	10.00	18.79	0.9	3035	950
MAbPac SCX-10, 3 µm (4 × 50)	3.00	9.07	1.6	4761	2800

Mobile phase: 20 mM MES, 160 mM NaCl, pH 6.0
Iso, $RT_{Cyt\ C}$: Retention time of Cyt C in isocratic test; Asym: Asymmetry of Cyt C;
N: Efficiency of Cyt C; BP: Column backpressure

FIGURE 4. Comparison of MAbPac SCX-10 10 µm, 4 × 50 mm column and 5 µm, 4 × 50 mm column: isocratic separation of Cytochrome C.



Efficiency for cytochrome C peak shows better values for MAbPac SCX-10, 5 µm, 4 × 50 mm column than the MAbPac SCX-10, 10 µm, 4 × 50 mm column (Figure 4). A comparison of retention time, asymmetry, back pressure, and efficiency are presented in Table 2.

Table 2. Comparison of MAbPac SCX-10, 10 µm, 4 × 50 mm and 5 µm, 4 × 50 mm columns

Column #	Resin Size, µm	Iso, $RT_{Cyt\ C}$ min	Asym	N	BP, psi
1	5	7.52	1.7	1748	1100
2	10	10.16	1.2	655	450

Column dimension: 4 × 50 mm
Mobile phase: 20mM MES, 130 mM NaCl, pH 6.5
Flow rate: 1 mL/min

FIGURE 5. Comparison of monoclonal antibody separation using the ProPac WCX-10, 10 µm, MAbPac SCX-10, 10 µm, and MAbPac SCX-10, 3 µm columns.

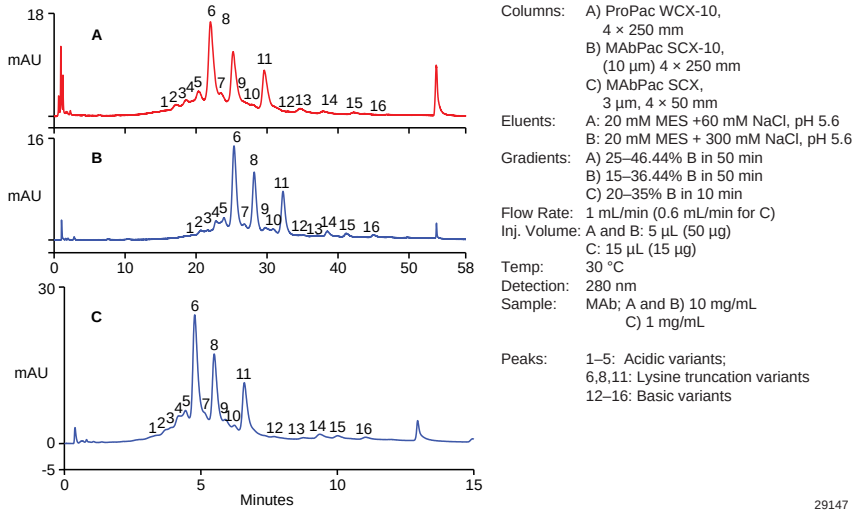


Figure 5 shows fast MAb analysis using a short (4 × 50 mm), 3 µm column. The MAb separation using the MAbPac SCX-10, 3 µm, 4 × 50 column is comparable to the MAbPac SCX-10, 10 µm, 4 × 250 mm column as well as the ProPac WCX-10, 10 µm column. But the time of analysis time is reduced from 58 minutes to 15 minutes.

FIGURE 6. pH gradient elution: MAb separation using MAbPac SCX-10, 3 µm column.

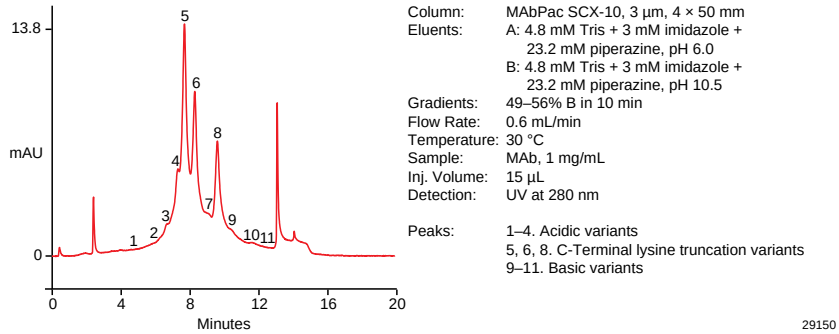
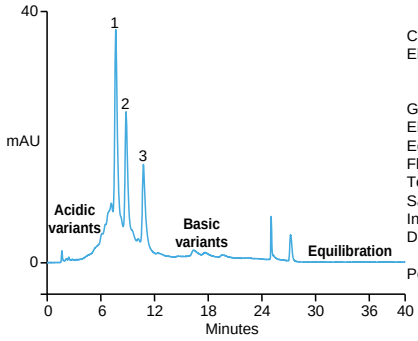


Figure 6 shows fast MAb separation on a 3 µm, 4 × 50 mm column using pH gradients. A similar pattern, as shown with the salt gradients in Figure 5, of acidic and basic variants including lysine truncation variants separation is achieved.

FIGURE 7. Salt Gradient Elution: MAb Separation using MAbPac SCX-10, 5 µm, 4 × 250 mm columns.



Column: MAbPac SCX-10, 5 µm, 4 × 250 mm
Eluents: A) 20 mM MES + 60 mM NaCl, pH 5.6
B) 20 mM MES + 300 mM NaCl, pH 5.6
C) 20 mM MES + 1 mM NaCl, pH 5.6 (for wash)
Gradients: 25–40% B in 20 min
Eluent C Wash: 2 min before equilibration
Equilibration: 15 min
Flow Rate: 0.7 mL/min
Temperature: 30 °C
Sample: MAb, 5 mg/mL
Inj. Volume: 10 µL
Detection: UV at 280 nm

Peaks: 1, 2, 3: C-terminal lysine truncation variants

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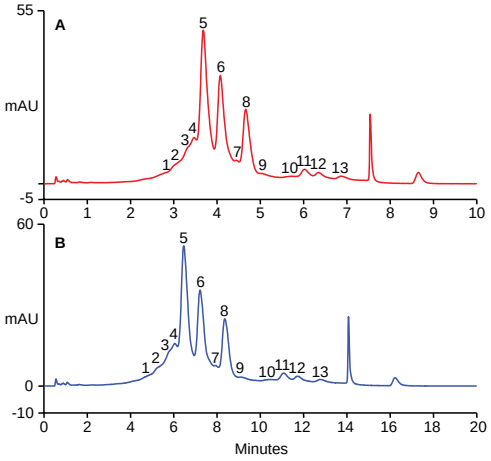
FIGURE 8. Salt gradient elution: MAb separation using MAbPac SCX-10, 5 µm columns.



Columns: MAbPac SCX-10, 5 µm, 4 × 50 mm
Eluents: A: 20 mM MES + 60 mM NaCl, pH 5.6
B: 20 mM MES + 300 mM NaCl, pH 5.6
Gradients: A) 18–33% B in 5 min
B) 18–33% B in 10 min
Flow Rate: 1 mL/min
Temperature: 30 °C
Sample: MAb, 5 mg/mL
Inj. Volume: 15 µL
Detection: UV at 280 nm

Peaks: 1–4. Acidic variants
5, 6, 8. C-Terminal lysine variants
9-13. Other basic variants

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Figure 8 shows fast MAb separation on a 5 µm, 4 × 50 mm column using MES based salt gradients. Panel A shows a 5 minute gradient and Panel B shows a 10 minute gradient. Higher resolution is achieved with a longer shallow gradient.

FIGURE 9. Faster Characterization of C-terminal lysine truncation variants.

Column: MAbPac SCX-10, 3 µm, 4 × 50 mm
Eluents: A: 20 mM MES (pH 5.6) + 60 mM NaCl
B: 20 mM MES (pH 5.6) + 300 mM NaCl
Gradient: 22–37% B in 5 min
Flow Rate: 0.5 mL/min
Temp: 30 °C
Inj. Volume: 6 µL
Detection: 280 nm
Sample: A) MAb, 2.5 mg/mL
B) MAb + 50 µg carboxypeptidase

Column: MAbPac SCX-10, 10 µm, 4 × 250 mm
Eluents: A: 20 mM MES (pH 5.6) + 60 mM NaCl
B: 20 mM MES (pH 5.6) + 300 mM NaCl
Gradient: 15–36.44% B in 50 min
Flow Rate: 1 mL/min
Temp: 30 °C
Inj. Volume: 5 µL
Detection: 280 nm
Sample: A) MAb, 9.0 mg/mL
B) MAb + 50 µg carboxypeptidase

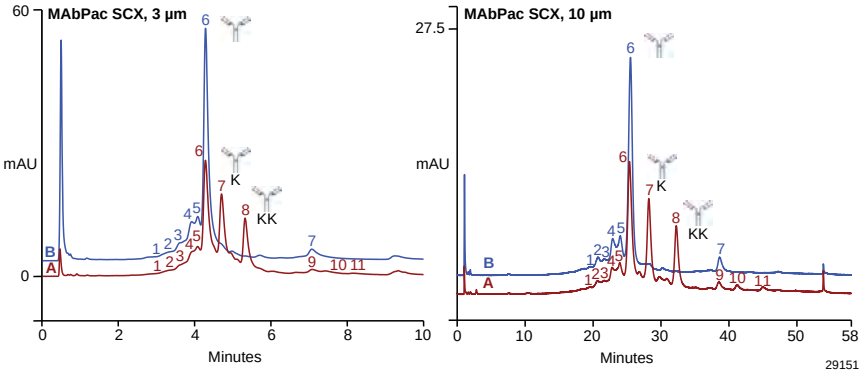

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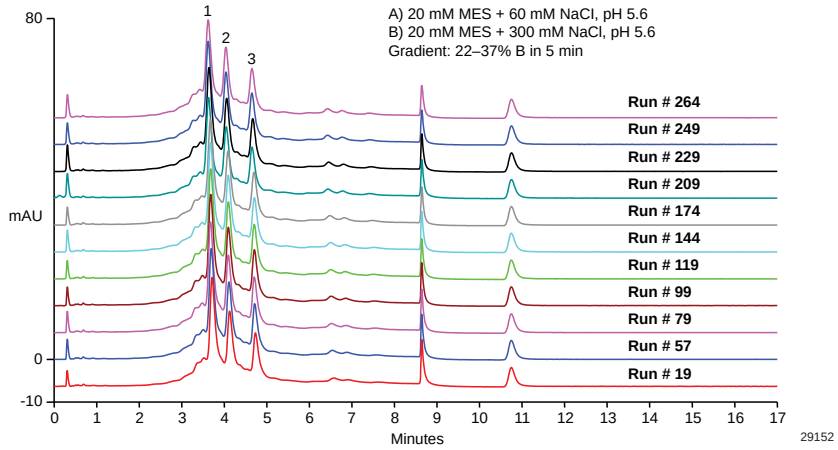
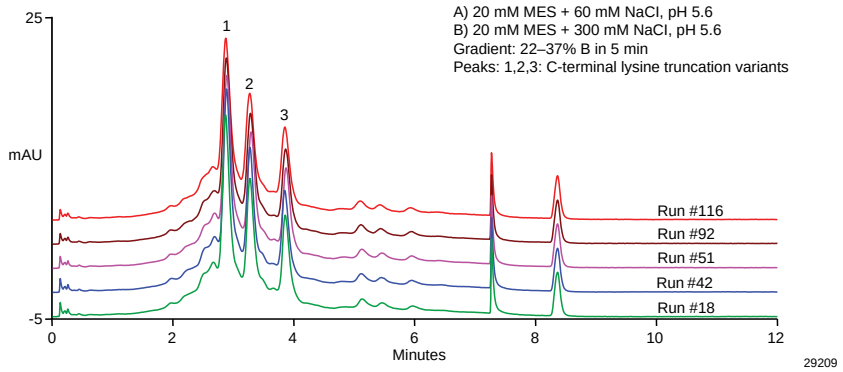
Figure 9 compares lysine (Lys) truncation variants characterization using a 3 µm, 4 × 50 mm column and 10 µm, 4 × 250 mm column using salt gradients. Bottom traces: No carboxypeptidase. Peaks 1– 5: Acidic variants; Peaks 6, 7, 8: C-terminal Lys truncation variants of main peak; Peaks 9, 10, 11: C-terminal Lys truncation variants of a minor variant peak. Top traces: After 50 µg carboxypeptidase incubation for 3 h at 37 °C. Lys truncation variant peaks 7, 8 loose their terminal lysine and become peak 6 which has no lysine after CBP treatment. Similarly, a minor variant lysine truncation peaks 10, 11 loose their terminal lysine and become peak 7 after CBP treatment.

FIGURE 10. Ruggedness testing of MAbPac SCX-10, 3 µm, 4 × 50 mm; Flow Rate: 0.8 mL/min.



MAbPac SCX-10, 3 µm column ruggedness (Figure 10) was tested for more than 264 runs at 0.8 mL/min. MAbPac SCX-10 5 µm column ruggedness (See below: Figure 11) was tested for more than 120 runs at 2 mL/min. Monoclonal antibody sample was injected intermittently every 3 to 5 runs. Selected chromatograms were overlayed. Both 3 and 5 µm phases exhibited excellent ruggedness.

FIGURE 11. Ruggedness testing of MAbPac SCX-10, 5 µm, 4 × 50 mm; Flow Rate: 2 mL/min.



Conclusion

- The new MAbPac family of columns are specifically developed for the separation and analysis of monoclonal antibodies.
- MAbPac SCX-10 is a complementary addition to existing ProPac WCX columns providing high resolution and orthogonal selectivity for various proteins and MAb charge variant characterization.
- The column is built with a 10, 5 or 3 µm nonporous, highly cross-linked styrene-type polymeric media. A controllable and robust ATRP surface grafting technique was applied that provided a uniform functional layer for ion-exchange process and sulfonic acid functionality.
- We have just introduced MAbPac SCX-10, 5 and 3 µm small particle phases for MAb variant analysis. The main objective for this development was to produce high-resolution columns that are comparable to the MAbPac SCX-10 columns (4 × 250 mm), but with a much faster analysis time. The longer length advantage from a 10 µm particle column for producing high efficiency, high resolution chromatography separation is compensated by a short, smaller particle size columns. Analysis time can be reduced and throughput is increased with the shorter column. Monoclonal antibody lysine truncation variant analysis time was reduced to 1/5th of the time when a small particle 3 µm, 4 × 50 mm high resolution column was used as compared to a 10 µm, 4 × 250 mm column.
- A longer 4 × 250 mm, 5 µm particle size column produced higher resolution when compared to the 10 µm similar length column.
- Both 5 and 3 µm columns exhibited excellent ruggedness.

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