Application Note 21008

High-Throughput, High-Resolution Monoclonal Antibody Analysis with Small Particle Size HPLC Columns

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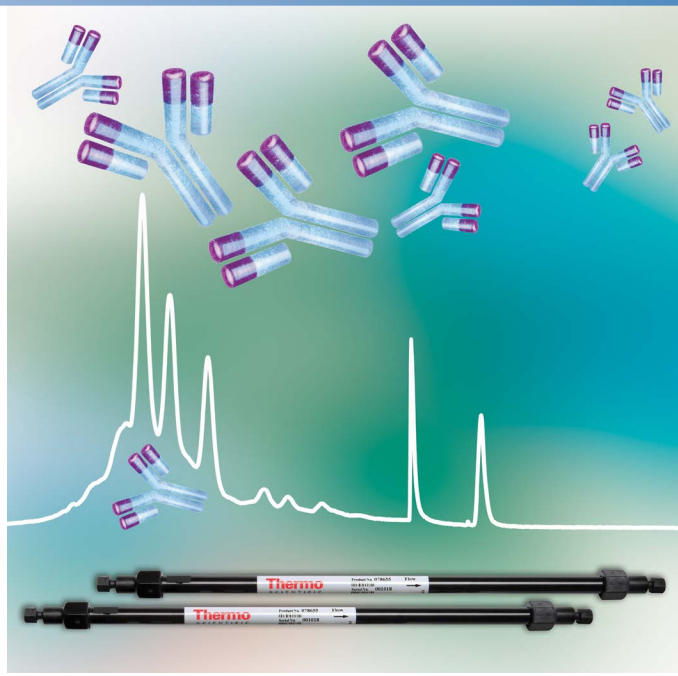
Key Words
MAbPac SCX-10, ProPac WCX-10, high-throughput analysis, lysine truncation, mAb separation, mAb analysis, mAb heterogeneity

Abstract
High-throughput applications of the Thermo Scientific™ MAbPac™ SCX-10 column are described, showing acidic and basic variant analysis, including mAb lysine truncation variant characterization. The ruggedness of the 3 µm small particle phase column is also demonstrated.

Introduction
Monoclonal antibodies (mAbs) can undergo a number of different chemical modifications including oxidation, deamidation, and C-terminal lysine truncation during the manufacturing process [1–6]. Manufacturing and subsequent quality control and stability assessment of mAbs can be very challenging tasks. The increasing focus on the development of mAbs in the pharmaceutical industry is driving a growing demand for improved high-resolution stationary phases for characterization of mAbs. Higher throughput is required for quality control due to an increasing sample load in many labs.

The MAbPac SCX-10 column is a strong acid cation-exchanger that is complementary to the widely used Thermo Scientific™ ProPac™ WCX-10 column. It provides high resolution and alternative selectivity for various proteins and mAbs. Both ProPac WCX-10 and MAbPac SCX-10 stationary phases are based on a nonporous highly cross-linked vinylaromatic type of polymeric media with a proprietary hydrophilic coating. The ProPac WCX-10 column is available in only a 10 µm particle size; however, the MAbPac SCX-10 is available in 10, 5, and 3 µm particle sizes. MAbPac SCX-10 3 µm and 5 µm small particle columns are available in different formats to suit a variety of application needs. The 150 mm and 250 mm length columns are designed for high-resolution separations, and shorter 50 mm columns are used when high throughput is desired.



This application note shows the selectivity differences between ProPac WCX-10 and MAbPac SCX-10 phases for protein separation. Applications using smaller particle columns for high-throughput, high-resolution mAb separations are also described. The ruggedness of small particle phase columns is presented.

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Experimental Details

Sample Handling Equipment
MAb separations were performed on an inert Thermo Scientific™ Dionex™ UltiMate™ 3000 Titanium system that was equipped with the following:
DGP-3600 BM Biocompatible dual gradient pump
SRD-3600 Solvent rack with six degasser channels
WPS-3000TBFC Analytical thermostatted autosampler
VWD-3400 UV Detector equipped with micro flow cell
TCC-3000SD Thermostatted column compartment

Columns	Part Number
ProPac WCX-10, 10 µm, 4 × 250 mm	054993
MAbPac SCX-10, 10 µm, 4 × 250 mm	074625
MAbPac SCX-10, 5 µm, 4 × 50 mm	078656
MAbPac SCX-10, 5 µm, 4 × 150 mm	085198
MAbPac SCX-10, 5 µm, 4 × 250 mm	078655
MAbPac SCX-10, 3 µm, 4 × 50 mm	077907

Sample Preparation	Part Number
The mAb sample was a gift from a local biotech company. Cytochrome C (equine), ribonuclease A, lysozyme proteins, carboxypeptidae B (CPB), MES, NaCl and other chemicals were obtained from Sigma-Aldrich®.	
Samples were prepared and injected from Thermo Scientific 2 mL Target DP polypropylene vials, 300 µL, with 9 mm AVCS screw thread cap with pre-slitted silicone/PTFE septum	C4000-11 C5000-55B
Carboxypeptidase B Preparation: A 5 mg sample was dissolved in 1 mL deionized water and sub-aliquots were stored frozen (-20 °C) until use.	

Mobile Phase for Protein Analysis	
Eluent A:	20 mM MES (pH 6.4)
Preparation:	20 mM MES (pH 6.4) was prepared by adding 3.9 g of MES to 900 mL of Milli-Q® water. Adjust the pH with NaOH to 6.4 and volume to 1 liter and filter before use.
Eluent B:	20 mM MES (pH 6.4) + 1 M NaCl Preparation: 20 mM MES (pH 6.4) was prepared by adding 3.9 g of MES to 900 mL of Milli-Q water. Adjust the pH with NaOH to 6.4 and add 58.44 g of NaCl, adjust volume to 1 liter and filter before use.

Mobile Phase for mAb Analysis	
Eluent A:	20 mM MES (pH 5.6) + 60 mM NaCl
Preparation:	20 mM MES (pH 5.6) is prepared by adding 3.9 g of MES to 900 mL of Milli-Q water. Adjust the pH with NaOH to 5.6 and add 3.506 g of NaCl, adjust volume to 1 liter and filter (0.22 µm) before use.
Eluent B:	20 mM MES (pH 5.6) + 300 mM NaCl
Preparation:	20 mM MES (pH 5.6) is prepared by adding 3.9 g of MES to 900 mL of Milli-Q water. Adjust the pH with NaOH to 5.6 and add 17.53 g of NaCl, adjust volume to 1 liter and filter before use.
Eluent C:	20 mM MES (pH 5.6) + 1 M NaCl
Preparation:	20 mM MES (pH 5.6) is prepared by adding 3.9 g of MES (Sigma-Aldrich) to 900 mL of Milli-Q water. Adjust the pH with NaOH to 5.6 and add 58.44 g of NaCl, adjust volume to 1 liter and filter (0.22 µm) before use.

Data Processing	
Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

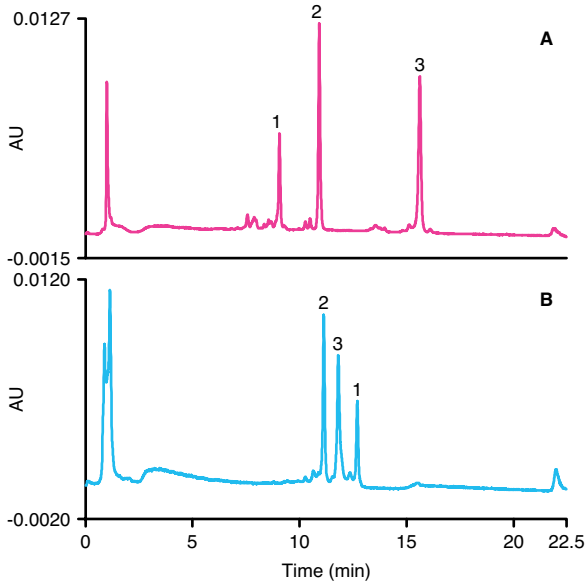
ProPac WCX-10 and MAbPac SCX-10 Phases for mAb Analysis

There are differences between the cation exchange phases of the ProPac WCX-10 and MAbPac SCX-10 columns (Table 1). The ProPac WCX-10 phase is a weak acid cation exchanger with a carboxylic function group, and the MAbPac SCX-10 phase is a strong acid cation exchanger with a sulfonic acid functionality. They exhibit selectivity differences for these protein separations. Both phases are routinely used for high-resolution mAb separations.

Physical Characteristic	ProPac WCX-10	MAbPac SCX-10
Substrate	Nonporous, highly cross-linked vinyl aromatic media	Nonporous, highly cross-linked vinyl aromatic media
Coating	Hydrophilic layer	Different hydrophilic layer
Particle size	10 µm	10 µm, 5 µm, 3 µm
Grafting	Conventional radical polymerization	ATRP
Functional group	Carboxylic acid	Sulfonic acid

Table 1: Physical characteristics of ProPac WCX-10 and MAbPac SCX-10 phases

When a three protein mixture was separated on these columns, under similar conditions, ribonuclease A eluted first on the MAbPac SCX-10 column, followed by cytochrome C and lysozyme. Conversely, cytochrome C eluted first on the ProPac WCX-10 column, followed by lysozyme and ribonuclease A (Figure 1), demonstrating selectivity differences between the two column chemistries.



Column:	A. **MAbPac SCX-10** B. **ProPac WCX-10**
Dimension:	4 × 250 mm
Eluent A:	20 mM MES, pH = 6.4
Eluent B:	20 mM MES, 1.0 M NaCl, pH = 6.4
Gradient:	Time(min) %A %B -15.0 100 0 0.0 100 0 20.25 52 48 21.0 0 100 24.0 0 100 25.0 100 0
Flow Rate:	1.0 mL/min
Inj. Volume:	10 µL
Detection:	UV at 254 nm
Sample:	1. Ribonuclease A 1 mg/mL 2. Cytochrome C 0.2 mg/mL 3. Lysozyme 0.25 mg/mL

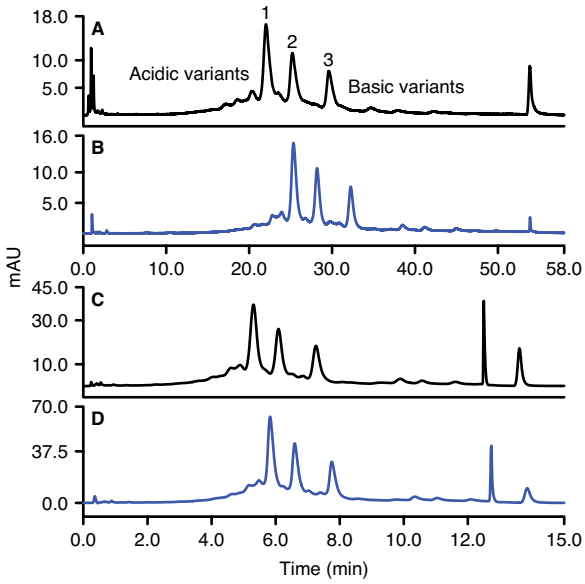
Figure 1: Selectivity differences of MAbPac SCX-10 and ProPac WCX-10 columns

Monoclonal Antibody Analysis

Both the ProPac WCX-10 and MAbPac SCX-10 columns are routinely used for separation of monoclonal antibodies and their variants. Both salt gradients and pH gradients can be used for the separation of mAbs. A detailed method is described and applications using pH gradients has been previously described [7].

High Throughput and High Resolution by Small Particle Columns

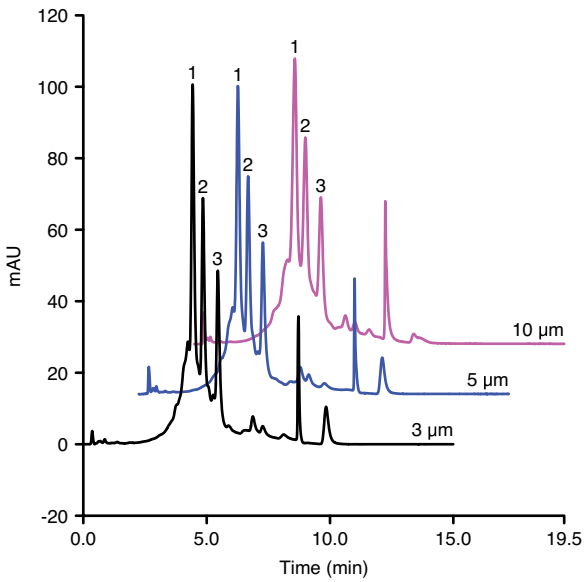
A comparison of mAb separations using the ProPac WCX-10 column and the alternative selectivity MAbPac SCX-10 column in a variety of particle sizes is shown in Figure 2.



Columns:	A: **ProPac WCX-10**, 10 µm, 4 × 250 mm B: **MAbPac SCX-10**,10 µm, 4 × 250 mm C: **MAbPac SCX-10**, 5 µm, 4 × 50 mm D: **MAbPac SCX-10**, 3 µm, 4 × 50 mm
Eluent A:	20 mM MES + 60 mM NaCl pH 5.6
Eluent B:	20 mM MES + 300 mM NaCl, pH 5.6
Gradients:	A: 25–46.44% B in 50min B: 15–36.44% B in 50min C: 20–35% B in 10min D: 22–37% B in 10min
Flow Rate:	1mL/min (0.7mL/min for D)
Temp.:	30 °C
Sample:	mAb; 50 µg (10 µL) for A&B and 15 µg (15 µL) for C&D
Detection:	280 nm
Peaks:	Lysine variants, 1, 2, 3; acidic variants and basic variants are shown

Figure 2: Comparison of mAb analysis with ProPac WCX-10 and MAbPac SCX-10 cation exchange columns

The 10 µm, 4 × 250 mm columns require a gradient of 50 minutes for well-resolved separation of variants. By comparison, 3 and 5 µm, 4 × 50 mm columns require much shorter gradients of 10 minutes to produce comparable chromatography. These smaller particle size columns compensate for the longer length required by the 10 µm particle column.



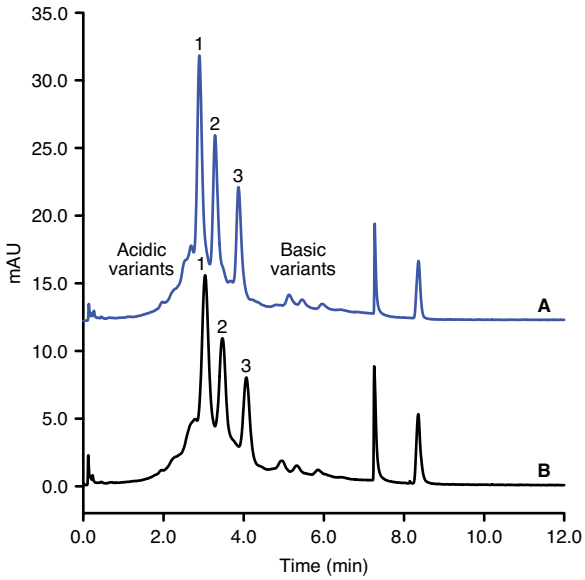
Column:	4 × 50 mm
Eluent A:	20 mM MES + 60 mM NaCl, pH 5.6
Eluent B:	20 mM MES + 300 mM NaCl, pH 5.6
Gradient:	22–37%B in 5 min (3 & 5 µm) 20–35%B in 5 min (10 µm)
Flow Rate:	0.7 mL/ min
Inj. Volume:	5 µL
Sample:	mAb, 5 mg/mL

Resolution values are shown in the inset for 3 lysine truncation variants

Particle size	Peak 1	Peak 2	Peak 3
3 µm	2.191	3.054	6.331
5 µm	1.884	2.727	6.239
10 µm	1.532	2.336	3.655

Figure 3: Comparison of mAb separation on different particle size MAbPac SCX-10 columns

Figure 3 shows the improved resolution of an mAb separation on a small particle column when compared to a larger particle column. Column dimensions are kept constant in this example. Using the same flow rate (0.7 mL/min) and gradient slope, resolution values for three lysine truncation variants of an mAb are assessed on 10 µm, 5 µm and 3 µm particle size columns. Results show that when compared to a 10 µm column for lysine truncation, the resolution of peaks 1, 2, and 3 is improved by 23%, 16.7%, and 71%, respectively, for 5 µm columns and 43%, 30.7% and 73%, respectively, for 3 µm columns. Even at a flow rate of 2 mL/ min, a 5 µm particle 4 x 50 mm column produces higher resolution of 22%, 32%, and 80% for lysine truncation peaks 1, 2 and 3, respectively, when compared to a 10 µm particle column (Figure 4).



Columns:	A: **MAbPac® SCX-10**, 5 µm, 4 × 50 mm B: **MAbPac® SCX-10**, 10 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 C) 20 mM MES + 1M NaCl, pH 5.6
Gradients:	20–35% B in 5 min
Eluent:	C wash: 1 min Eluent C wash before equilibration
Equilb. Time:	4 min
Flow Rate:	2 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Sample:	mAb, 5 mg/mL
Detection:	UV at 280 nm
Peaks:	1, 2, 3 : C-terminal lysine truncation variants. Resolution is shown in inset.

Particle size	Peak 1	Peak 2	Peak 3
5 µm	1.85	2.78	5.53
10 µm	1.52	2.10	3.08

Figure 4: Comparison of mAb analysis on MAbPac SCX-10 10 µm, and 5 µm columns at high flow rate of 2 mL/min

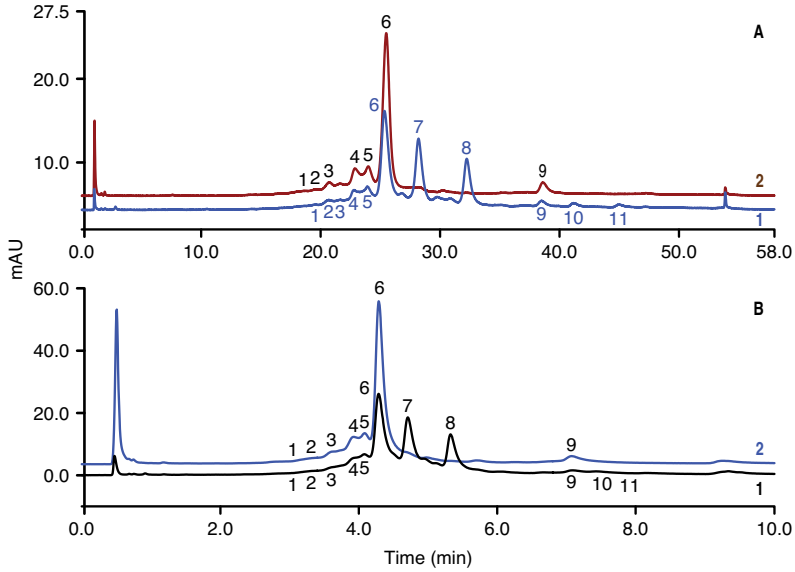
High-Throughput Analysis Using a Small Particle Column; Monitoring Processing of C-terminal Lysine Residues of mAbs

Charge heterogeneity can result if the processing of C-terminus of a protein is incomplete [5]. The resulting charge heterogeneity of the variant forms can be identified by cation exchange chromatography.

In this example, the MAbPac SCX-10 column was used to separate variants of a mAb containing lysine residue variation at the C-terminal of the heavy chains. A comparative analysis was performed on a 10 µm MAbPac SCX-10, 4 × 250 mm column (Figure 5A) and a 3 µm MAbPac SCX-10, 4 × 50 mm column (Figure 5B) using different flow rates and gradient conditions. For the 10 µm, 250 mm column, a 50 minute gradient time was used, which is considerably shortened to a 5 minute gradient time in the case of the 3 µm column. By doing this, overall run time was decreased by more than five-fold and resulted in increased throughput with the small particle column. In both cases, three variant forms differing by the presence of lysine at the C-terminal of the heavy chains with either no lysine, or 1 lysine, or 2 lysine residues are separated as individual peaks (Figure 5A and B; sample 1).

To verify that the different retention times of the three peaks were due to the different heavy chain C terminal lysine content, the mAb was treated with carboxypeptidase B. This is an exopeptidase that specifically cleaves C terminal lysine residues (Figure 5A and B; sample 2). This treatment of the mAb sample resulted in the absence of peaks 7 and 8 (containing 1 and 2 terminal lysine residues, respectively, on their heavy chains). The decreased peak areas in peaks 7 and 8 are accompanied by a corresponding increase in area of peak 6 where no lysine is present. Another minor variant with lysine truncations, peaks 9-11, collapsed to peak 9 after carboxypeptidase B treatment (Figure 5A and B).

High-throughput monitoring of C-terminus lysine truncations on a small particle 3 µm column is shown in Figure 5B. Total analysis time is reduced more than five-fold for the 3 µm column when compared to the 10 µm, 4 × 250 mm column.



Eluents:	A: 20 mM MES (pH 5.6) + 60 mM NaCl B: 20 mM MES (pH 5.6) + 300 mM NaCl
Temp:	30 °C
Detection:	280 nm
Sample:	1. mAb 2. mAb + Carboxypeptidase B (CPB)

A.

Column:	MAbPac SCX-10, 10 µm, 4 × 250 mm
Sample:	1. mAb, 9 mg/mL 2. 0.9 mg of mAb was treated with 50 µg of CPB. Final mAb concentration was 8.2 mg/mL
Gradient:	15–36.44%B in 50 min
Flow Rate:	1 mL/min
Inj. Volume:	5 µL

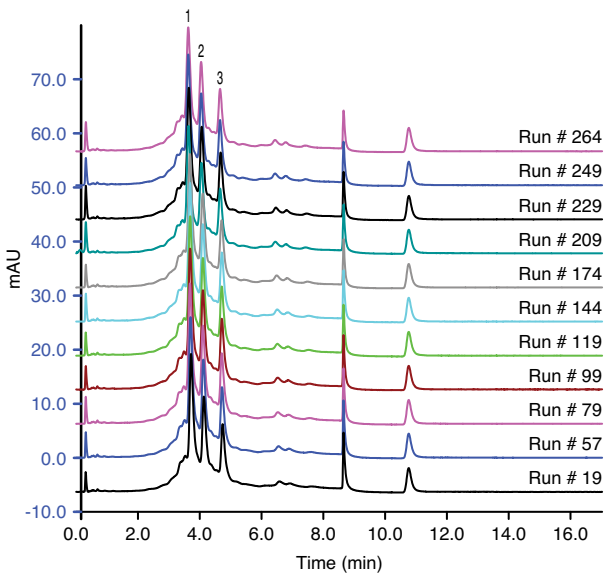
B.

Column:	**MAbPac SCX-10**, 3 µm, 4 × 50 mm
Sample:	1. mAb, 2.5 mg/mL 2. 0.25 mg of mAb was treated with 50 µg of CPB. Final mAb concentration was 2.3 mg/mL.
Gradient:	22–37 % B in 5 min
Flow Rate:	0.5 mL/min
Inj. Volume:	6 µL
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

Figure 5: Characterization of mAb C-terminal lysine truncation variants using MAbPac SCX-10 10 µm (Panel A) or 3 µm (Panel B) columns. 3 µm, 4 × 50 mm column separation is more than five times faster than MAbPac SCX-10 10 µm, 4 × 250 mm column. (note that time scales are different)

Ruggedness of MAbPac SCX-10 Small Particle Columns

Figure 6 demonstrates that more than 260 runs were performed without significant loss of resolution. Peak widths at half height values were determined for lysine truncation variants (Table 2). Fast, 5 minute gradients were used in this assessment. The ruggedness of the column improved if columns were washed with a high salt concentration buffer after each run.



Columns:	**MAbPac SCX-10**, 3 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 C) 20 mM MES + 1M NaCl, pH 5.6 (for wash)
Gradients:	22–35% B in 5 min
Eluent C Wash:	1 min Eluent C wash before equilibration
Equilib. Time:	4 min
Flow Rate:	0.8 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV at 280 nm
Sample:	mAb, 5 mg/mL (Injected intermittently at every 3 to 5 runs;
Peaks:	1, 2, 3 : C-terminal lysine truncation variants. PWHH values are shown in Table 2

Figure 6: Ruggedness of MAbPac SCX-10 3 µm, 4 × 50 columns

Time (min)	Peak 1	Peak 2	Peak 2
19	0.122	0.12	0.122
57	0.121	0.117	0.118
79	0.122	0.116	0.119
99	0.121	0.115	0.118
119	0.122	0.116	0.12
144	0.123	0.118	0.121
174	0.129	0.124	0.121
209	0.124	0.12	0.122
229	0.123	0.117	0.118
249	0.121	0.116	0.119
264	0.126	0.118	0.123
Average	0.123	0.118	0.121
RSD (%)	2.01	2.11	2.11

Table 2: Ruggedness of MAbPac SCX-10 3 µm columns

Conclusion

This application note demonstrates high-throughput, high-resolution mAb separation applications using MAbPac SCX-10 small particle columns.

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