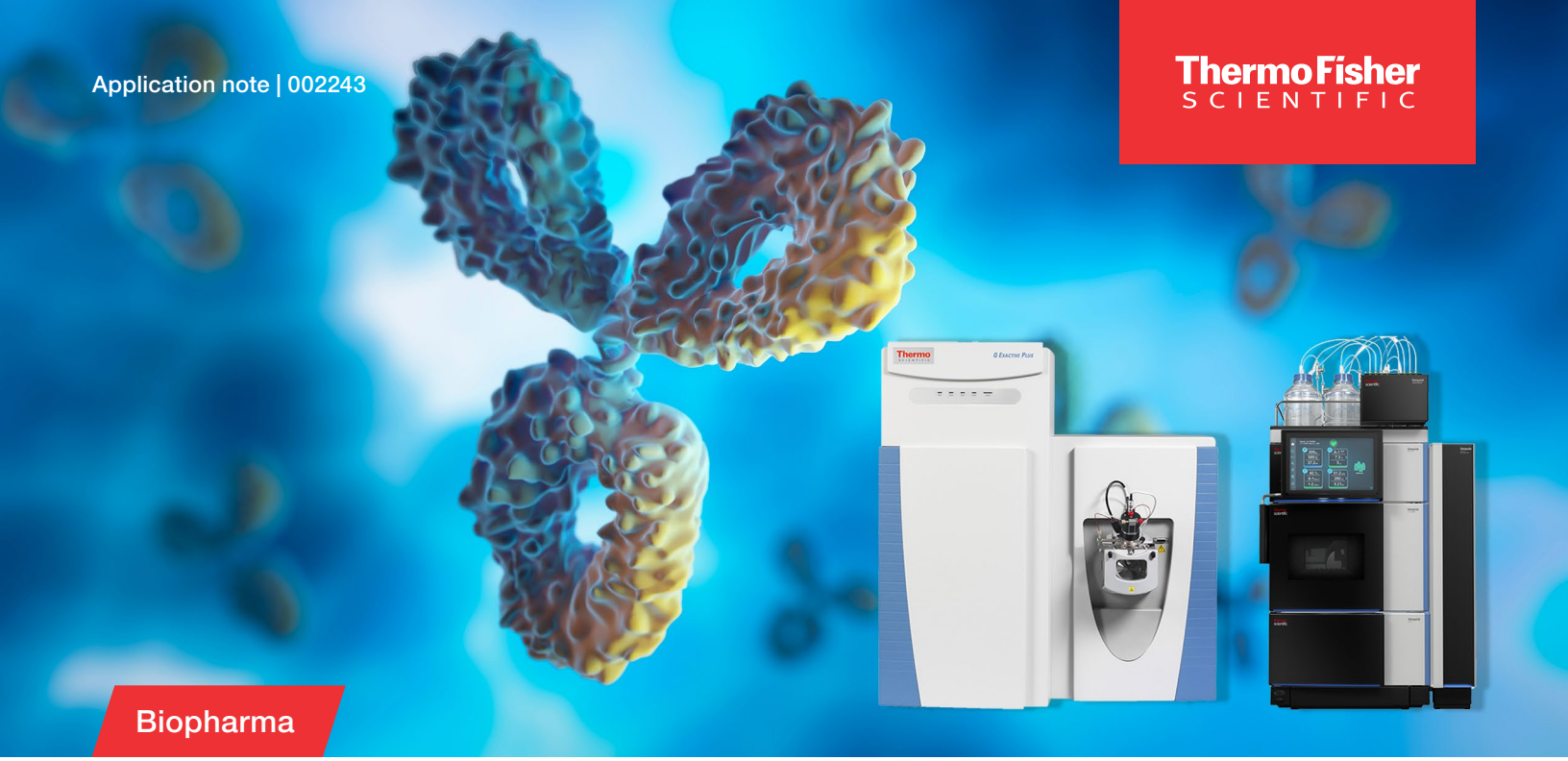
Application note | 002243



Biopharma

Coupling ion exchange chromatography with mass spectrometry to characterize charge heterogeneity of mAbs under near-native conditions using a ProPac 3R SCX column

Authors

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Keywords

Protein charge variants, durvalumab, NISTmAb, rituximab, trastuzumab, MS-compatible pH gradient, heat stress, charge variant analysis, ProPac 3R SCX column

Application benefits

- High-resolution chromatographic separation of protein charge variants
- Easy, straightforward method development with MS compatible pH gradient buffer
- Suitable for different kinds of therapeutic monoclonal antibodies (mAbs), including durvalumab, NISTmAb, rituximab, and trastuzumab

Goal

Method development and high-resolution mass spectrometry analysis of monoclonal antibodies using MS-compatible pH gradient buffer with the new generation Thermo Scientific™ ProPac™ 3R SCX columns.

Introduction

Proteins have been used as a major class of therapeutics for the treatment of various diseases including cancer, cardiovascular disease, and autoimmune disorders. The market for proteins as therapeutics is expected to continue growing for the foreseeable future. Proteins typically have an isoelectric point (pI) ranging from 4.0 to 12.0, based

on their amino acid composition, glycosylation profile, and other post-translational modifications. Ion exchange chromatography (IEX) is a standard technique for analyzing proteins and their associated variants based on their accessible surface charge.

Charge heterogeneity is considered a critical quality attribute in therapeutic monoclonal antibodies (mAbs) and needs to be thoroughly characterized and monitored throughout the drug development stages. IEX is one of the main techniques used to recognize the overall charge heterogeneity of mAbs. Mass spectrometry (MS)-based tools have played a critical role in charge variants characterization. Recent advances in both instrumentation and methodology made it possible for online coupling of multiple charge-based separation techniques with direct MS detection.

Protein strong cation exchange (SCX) technology is widely used in biopharmaceutical development and qualification of protein-based therapeutics and their associated variants. The Thermo Scientific™ ProPac™ 3R SCX 3 µm HPLC column is designed to achieve high-efficiency protein separations. The packing material is based on a 3 µm, nonporous, divinylbenzene monodisperse polymer particle, which results in shorter diffusion distances, yielding better reproducible mass transfer and narrower peaks. Due to increased capacity associated with smaller particles, shorter columns can also be used for reduced run times with improved separation relative to larger particle media that require longer column lengths to achieve the same separation.

pH gradient is a widely used method since the elution profile can be predicted based on the pI value of the protein. The pH gradient buffer can be easily prepared; however, the commercially available pH gradient buffer is not MS-compatible. In this application note, we developed and optimized a MS-compatible pH gradient buffer-based cation exchange chromatography coupled with native MS (CEX-MS) methods and explored its utility in charge heterogeneity characterization of durvalumab. The new generation IEX column, the ProPac 3R SCX column (2 × 50 mm, 3 µm), was used. Heat stress testing was executed by putting the durvalumab at 50 °C for 1 day, 2 days, 3 days, 6 days, and 7 days.

Experimental

Instrumentation

- Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:
  - Vanquish System Base (P/N V-S01-A-01)
  - Vanquish Binary Pump F (P/N VF-P10-A)
  - Vanquish Split Sampler FT (P/N VF-A10-A)
  - Vanquish Column Compartment H (P/N VH-C10-A)
  - Vanquish Variable Wavelength Detector F (P/N VF-D40-A)
- Thermo Scientific™ Orion Star™ A211 pH Meter (P/N XW3-10683)
- ProPac 3R SCX 2 × 50 mm, 3 µm column (P/N 43103-052068)
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (P/N 0726030)
- Thermo Scientific™ National™ Target DP™ Vial, PP, 300 µL, 100/PK (P/N C4000-11)
- Thermo Scientific™ AVCS™ Blue Cap Vial, 100/PK (P/N C5000-54B)

Software

- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Version 7.2.10
- Thermo Scientific™ BioPharma Finder™ 5.1 Software

Reagents and consumables

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Ammonium bicarbonate, Sigma-Aldrich™ (P/N 09830-500G)
- Acetic acid, LC/MS grade, Fisher Scientific™ (P/N A113-50)
- Ammonium hydroxide solution, 1 M, Sigma-Aldrich (P/N 09859-1L)
- Commercial durvalumab injection solution
- Thermo Scientific™ CX-1 pH gradient buffer A (P/N 083273)
- Thermo Scientific™ CX-1 pH gradient buffer B (P/N 085348)

Sample preparation

Durvalumab: Commercially available durvalumab was diluted to 1 mg/mL using H_2O.

Heat stress treatment durvalumab: durvalumab samples were stressed at 50 °C for 1 day, 2 days, 3 days, 6 days, and 7 days.

Mobile phase buffer preparation

For the UHPLC-only pH gradient method, these solutions were used as mobile phase:

- 10-fold dilution of CX-1 buffer A (pH 5.6) with DI water
- 10-fold dilution of CX-1 buffer B (pH 10.2) with DI water

For the MS-compatible pH gradient buffer method optimization, these solutions were used as mobile phase:

- 25 mM ammonium bicarbonate and 30 mM acetic acid in water at pH 5.3
- 10 mM ammonium hydroxide in water at pH 10.2

The pH was adjusted with acetic acid or ammonium hydroxide, since the method is pH sensitive and ammonium hydroxide is volatile, the mobile phase is used within 48 h of preparation.

Liquid chromatography

The commercial pH buffer kit chromatographic condition and MS-compatible pH gradient buffer chromatographic conditions are described in Tables 1 and 2. For MS-compatible pH gradient buffer chromatographic conditions, three gradients were compared and optimized.

Table 1. Commercial pH buffer kit chromatographic conditions

Experimental conditions			
Column:	ProPac 3R SCX column, 2 × 50 mm, 3 µm (P/N 43103-052068)		
Gradient 1	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	0	100	0
	3.5	100	0
	30	0	100
	32	0	100
	35	100	0
	40	100	0
Flow rate	0.3 mL/min		
Column temperature	30 °C		
Sample compartment temperature	4 °C		
Injection volume	10 µL		
UV detection	280 nm, 2 Hz, 2 response time		

Table 2. MS-compatible pH gradient buffer chromatographic conditions

Experimental conditions			
Column	ProPac 3R SCX column, 2 × 50 mm, 3 µm (P/N 43103-052068)		
Mobile phase	A: 25 mM ammonium bicarbonate and 30 mM acetic acid in water at pH 5.3 B: 10 mM ammonium hydroxide in water at pH 10.2		
Gradient 1	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	0	85	15
	3	85	15
	28	25	75
	30	25	75
	35	85	15
	40	85	15
Gradient 2	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	0	70	30
	3	70	30
	28	20	80
	30	20	80
	35	70	30
	40	70	30
Gradient 3	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	0	80	20
	3	80	20
	28	15	85
	30	15	85
	35	70	30
	40	70	30
Flow rate	0.3 mL/min		
Column temperature	30 °C		
Sample compartment temperature	4 °C		
Injection volume	10 µL		
UV detection	280 nm, 2 Hz, 2 response time		

Mass spectrometry

All experiments presented in this application note were performed on the Q Exactive Plus mass spectrometer fully controlled by Chromeleon CDS. The MS parameters are summarized in Table 3.

Table 3. MS parameters for durvalumab analysis

Full MS – HMR mode, trapping gas pressure setting = 1.0	
Sheath gas	30 Arb
Aux gas	10 Arb
Spray voltage	3.6 kV
Capillary temp.	275 °C
Aux gas heater temp.	200 °C
In-source CID	100 eV
S-lens RF level	200
Microscans	10
Resolution	35,000 @ *m/z* 200
Scan range	2,500-8,000 Da

Results and discussion

Commercial pH buffer kit method

In a pH gradient, the charge on the protein is reduced as the pH increases. As such, buffer pH has a significant effect on the separation of the proteins and associated variants. For cation exchange chromatography, the mobile phase pH should be lower than the isoelectric point (pI) of the protein and variants of interest to promote binding to the solid phase. If the mobile phase pH is higher than the pI, the protein or variants may not be retained nor separated. Using a gradient from 100% of CX-1 pH gradient buffer A to 100% of CX-1 pH gradient buffer B, a linear pH gradient from pH 5.6 to pH 10.2 could be generated. Since the majority of mAbs have pI values in the range of pH 6 to 10, pH-gradient-based separation methods using the Thermo Scientific pH buffer platform and ProPac 3R SCX columns can serve as a general platform for mAb charge variants analysis. Figure 1 displays the liquid chromatography for durvalumab, NIST mAb, rituximab, and trastuzumab using pH gradient separation.

MS-compatible pH gradient buffer method optimization

Although the commercial pH buffer kit method is widely used, it is not compatible with online MS coupling. The need for MS compatible methods for charge variants analysis is increasing with the growing demand for therapeutic monoclonal antibodies (mAbs) analysis using LC-MS. Figure 2 displays how we optimized the MS-compatible pH gradient buffer method for durvalumab analysis. Using a gradient from 15% of mobile phase B to 75% mobile phase B achieved the best separation and peak shape for both acidic and basic peaks. For stress testing and MS analysis, the gradient from 15%B to 75%B was applied.

The MS-compatible pH gradient buffer method can also be used for different therapeutic monoclonal antibodies (mAbs). Since the pI value of rituximab (pI = 9.0–9.5) and denosumab (pI = 8.5–9.0) are high, a higher pH mobile phase B (pH 10.9) was used to avoid slow elution as well as loss of resolution because of reduced charge interaction. Figure 3 displays the liquid chromatography for rituximab and denosumab. The ProPac 3R SCX column can be used for different kinds of mAb under the MS-compatible pH gradient buffer conditions.

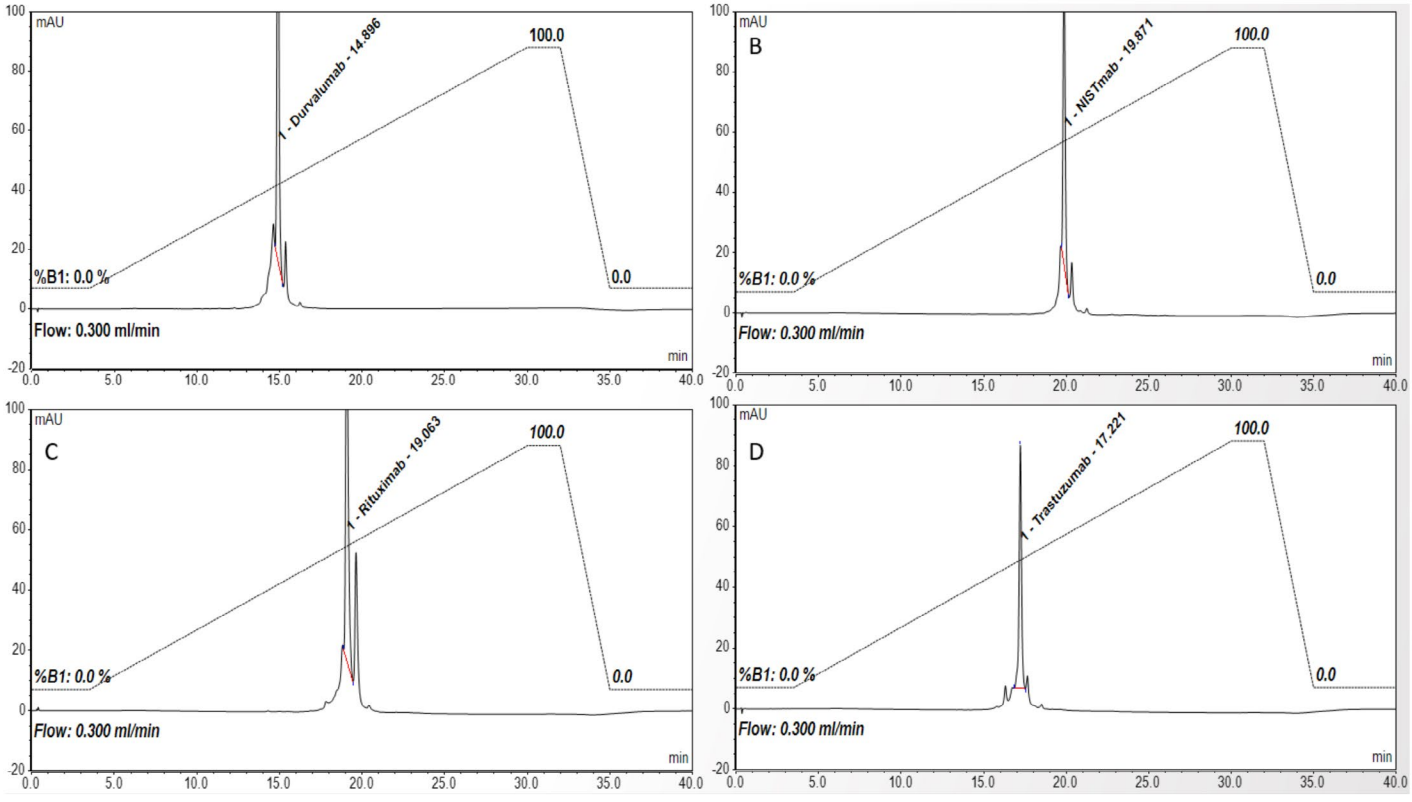

Figure 1. Chromatograms for (A) durvalumab, (B) NISTmAb, (C) rituximab, and (D) trastuzumab using the general pH gradient method

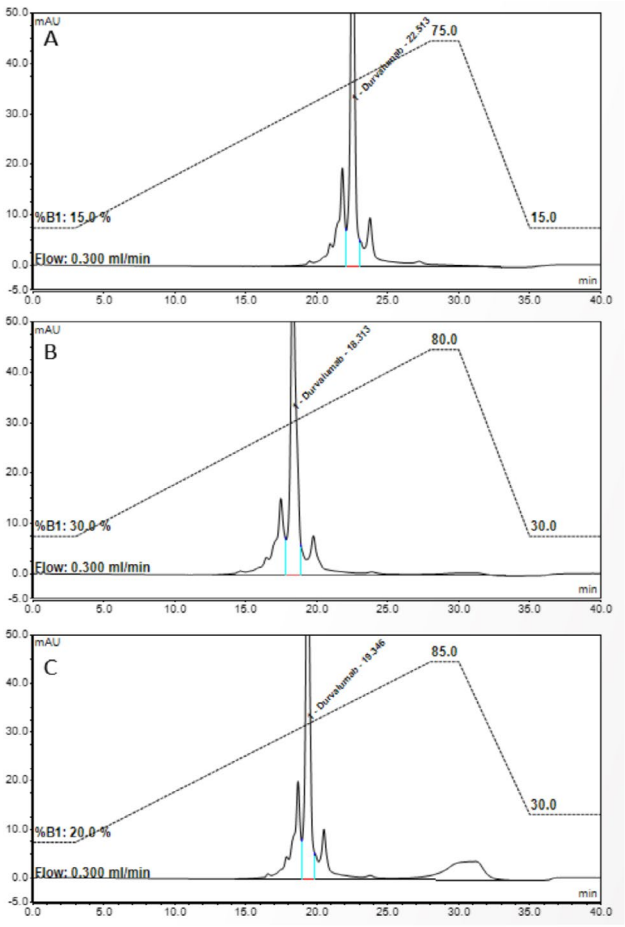

Figure 2. Liquid chromatography for durvalumab by using different MS-compatible pH gradient buffer methods with gradient conditions elution from:

(A) 15% of mobile phase B to 75% mobile phase B

(B) 30% of mobile phase B to 80% mobile phase B

(C) 20% of mobile phase B to 85% mobile phase B

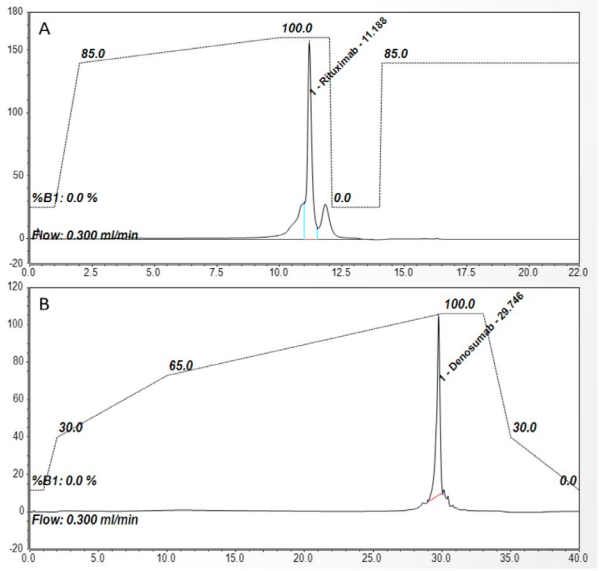

Figure 3. Liquid chromatography for rituximab and denosumab using different MS compatible pH gradient methods

Lot-to-lot comparison

Using the MS-compatible pH gradient 1 method shown in Table 2, we compared the performance of two different lots of the ProPac 3R SCX column and the reproducibility. There are three injections for durvalumab analysis by both lots of the column for 3 days. Figure 4 displays the chromatography for the analysis at different days, and Figure 5 displays the overlay chromatography for durvalumab analysis by applying two lots of columns. Table 4 displays the %RSD for durvalumab analysis by using the Lot 1 SCX column and the Lot 2 SCX column at different days as well as the %RSD for the two different lots of the SCX column. The highest %RSD for the two different lots is 4.75% and the lowest %RSD is 0.17%. Excellent reproducibility is observed.

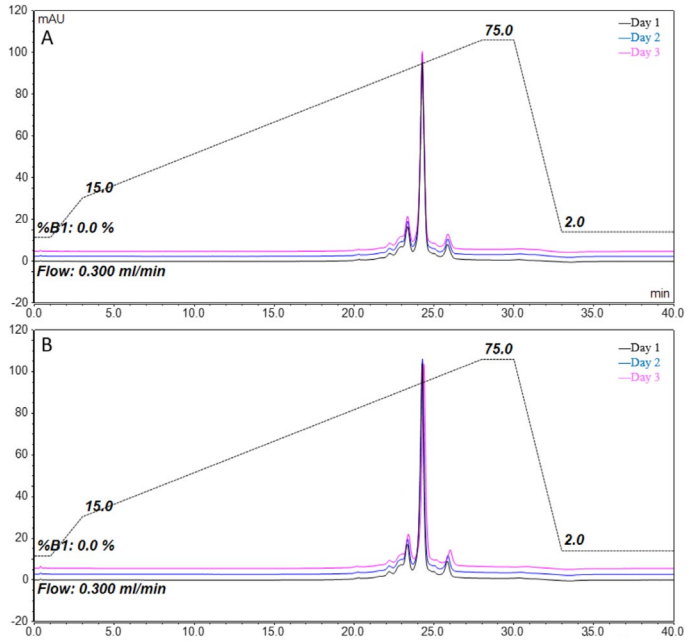

Figure 4. Liquid chromatography for durvalumab analysis at three different days with (A) Lot 1 SCX column and (B) Lot 2 SCX column

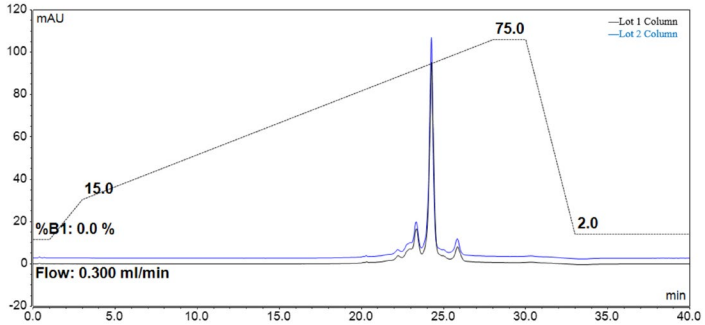

Figure 5. Overlay liquid chromatography for durvalumab analysis by applying two lots of the SCX column

Table 4. The %RSD for durvalumab analysis by two different lots of the SCX column

Peak	%RSD		
	Lot 1	Lot 2	Between Lot 1 and Lot 2
Acid peak 1	3.50	2.65	3.36
Acid peak 2	1.24	1.69	3.30
Acid peak 3	2.74	1.81	3.69
Acid peak 4	2.20	0.43	1.76
Acid peak 5	0.18	0.31	0.23
Main peak	0.14	0.90	0.17
Basic peak 1	2.18	0.40	3.05
Basic peak 2	3.02	2.74	4.75
Basic peak 3	0.49	0.53	0.47

Heat stressed samples

To demonstrate the utility of the ProPac 3R SCX column, we provide a durvalumab sample that has been stressed at 50 °C for 1 day, 2 days, 3 days, 6 days, and 7 days to induce heat stress. Figure 6 compares these unstressed and stressed durvalumab samples. Figure 7 and Table 5 display the change of peak area% of each peak. The peak area of acid peak 3 and acid peak 5 have increased 100% after 7 days thermal stress treatment compared to the unstressed sample. Heat stressing of samples typically results in an increase in the number of acidic variants such as deamidation, the relative abundance of acidic peaks also increased. For basic peaks, the basic peak 2 area decreased. The peak area of basic peak 1 increased because of oxidation% growth, which was induced by heat stress. The temperature treatment of the protein results in the presence of more acidic and less basic variants with the exception of basic peak 1.

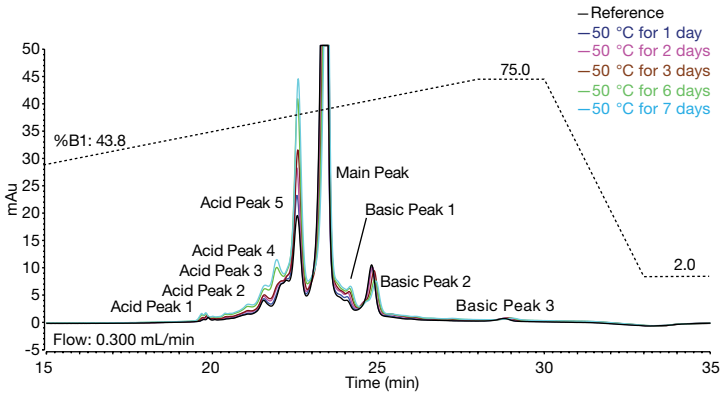

Figure 6. The overlay liquid chromatography for unstressed durvalumab and stressed durvalumab samples under 50 °C for different days

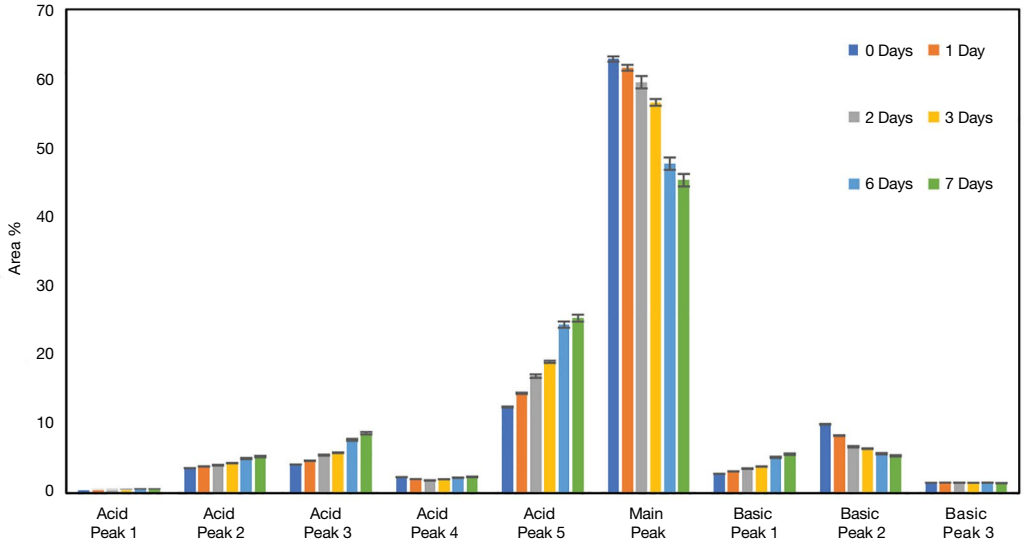

Figure 7. The peak area for unstressed durvalumab and stressed durvalumab samples under 50 °C for different days

Table 5. The area% for each acidic and basic peak of stressed durvalumab samples

	Acid peak 1	Acid peak 2	Acid peak 3	Acid peak 4	Acid peak 5	Main peak	Basic peak 1	Basic peak 2	Basic peak 3
0 day	0.37%	3.61%	4.15%	2.32%	12.46%	62.82%	2.79%	9.97%	1.50%
1 day	0.41%	3.86%	4.69%	2.04%	14.46%	61.54%	3.15%	8.83%	1.52%
2 days	0.42%	4.05%	5.51%	1.85%	16.94%	59.46%	3.56%	6.71%	1.51%
3 days	0.46%	4.34%	5.85%	2.01%	19.00%	56.54%	3.85%	6.44%	1.50%
6 days	0.57%	5.02%	7.71%	2.23%	24.39%	46.67%	5.18%	5.71%	1.51%
7 days	0.56%	5.30%	8.67%	2.36%	25.32%	45.28%	5.64%	5.40%	1.46%

MS analysis for durvalumab

The MS-compatible pH gradient buffer method has been optimized in HPLC and then applied in MS. Figure 8 displays the MS spectrum and deconvolution result of non-stressed durvalumab. Figure 8A displays the MS spectrum and 8B shows the deconvolution result. The top abundance components from each peak in the non-stressed sample are shown in Table 6, based on high-resolution MS data deconvolution results. Acidic peak 1 is partial mAb, with mass range between 100 and 110 kDa. The dominant modification from acidic peak 2 to acidic peak 5 is deamidation, and A2S1G0F/A2S1G1F starts to be eluted in acidic peak 5. In basic peak 1, K truncation at one heavy chain C-terminal and GK truncation at the other heavy chain C-terminal can be observed, with three oxidations. In basic peak 2, K truncation and GK truncation at heavy chain C-terminal, and K truncation at one heavy chain C-terminal only can be detected. In basic peak 3, two types of variants appear, both heavy chains keep lysine at C-terminal, or both chains lose GK.

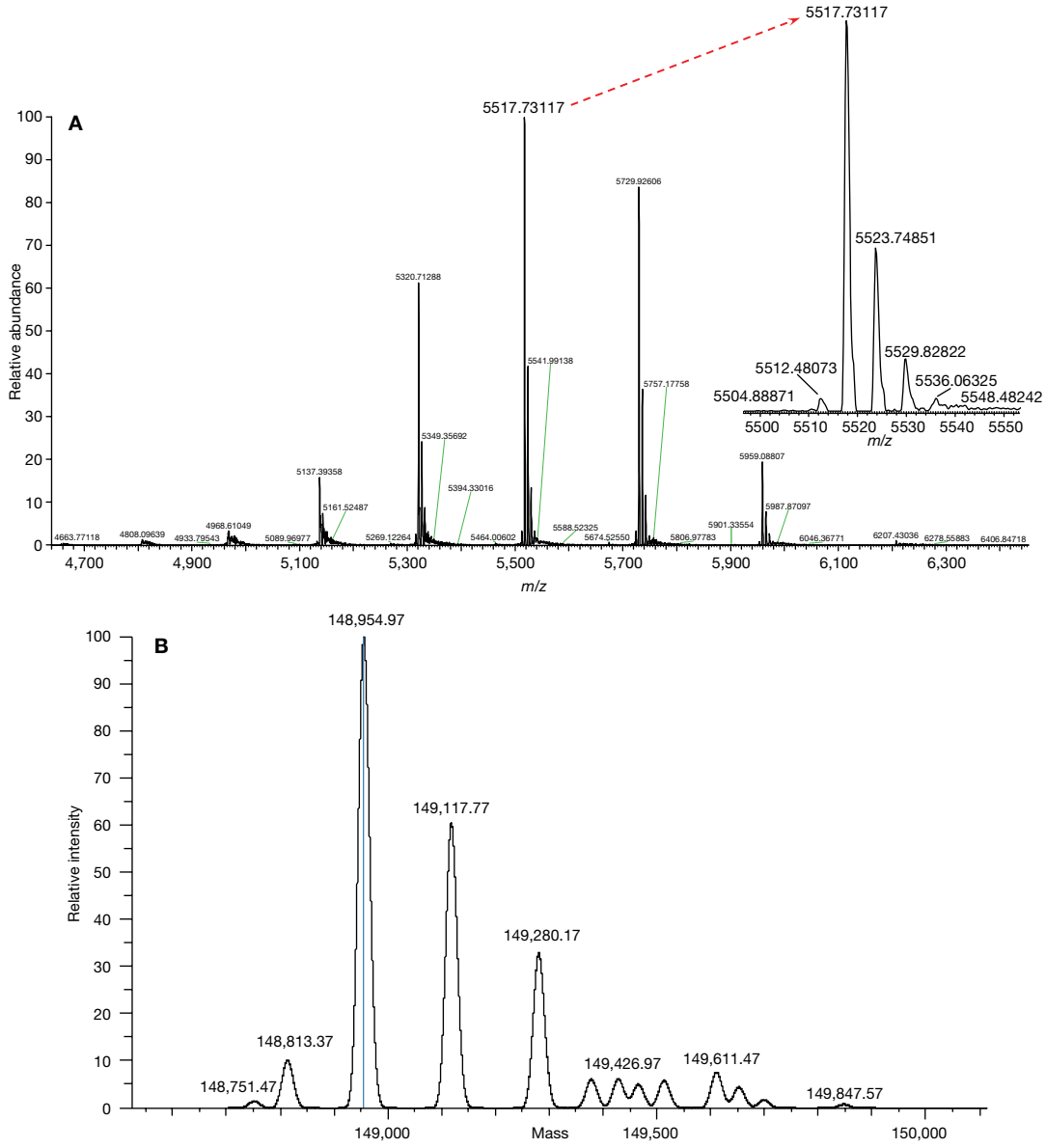

Figure 8. MS spectrum (A) and deconvolution results (B) of non-stressed durvalumab

Table 6. Top abundance components from each peak in non-stressed sample

Acid peak 1 modification	Acid peak 2 modification	Acid peak 3 modification	Acid peak 4 modification	Acid peak 5 modification
Fragments, 100~110 kDa	2×deamidation 2×K loss	2×deamidation 2×K loss	3×deamidation 2×K loss	1×deamidation A2S1G0F/A2S1G1F 2×K loss
Main peak modification	**Basic peak 1 modification**	**Basic peak 2 modification**	**Basic peak 3 modification**	
2×K loss	1×K loss, 1×GK loss, 3×oxidation	1×K loss, 1×GK loss 1×K loss	2×GK loss 0×K loss	

The heat stressed durvalumab samples have also been analyzed by the mass spectrometer and the results are shown in Figure 9. The mass accuracy of glycoform 2×A2G0F, 2× K loss from the main peak in all stressed samples is shown in Table 7; matched mass error between measured and theoretical mass are all less than 6 ppm. In Figure 9, there are some new peaks that appear, especially after 6 and 7 days of heat stressing. One new acidic peak was observed between acidic peak 5 and the main peak, resulting from deamidation and oxidation. Three new basic peaks appeared because of oxidation. The top abundance components from the new peaks in stressed samples are shown in Table 8.

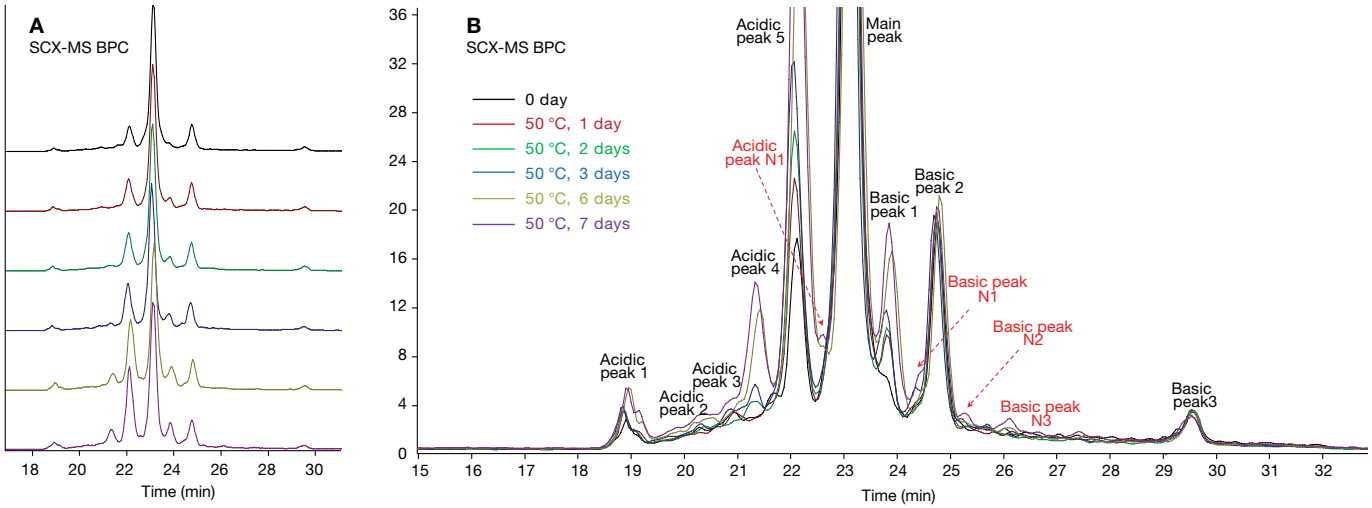

Figure 9. SCX – MS profile of thermally stressed durvalumab. (A) Base peak chromatogram (BPC) of durvalumab at 50 °C for 0/1/2/3/6/7days. (B) Zooming-in spectra, overlaid

Table 7. Mass accuracy of glycoform 2×A2G0F, 2× K loss from main peak in all stressed samples

Condition	Average mass (Da)	Theoretical mass (Da)	Matched mass error (ppm)
0 day	148954.91	148955.38	-2.75
50 °C, 1 day	148984.56	148955.38	-5.45
50 °C, 2 days	148954.70	148955.38	-4.54
50 °C, 3 days	148954.78	148955.38	-4.04
50 °C, 6 days	148955.30	148955.38	-0.56
50 °C, 7 days	148955.23	148955.38	-0.98

Table 8. Top abundance components from new peaks in stressed samples

Acidic peak N1 modifications	Basic peak N1 modifications	Basic peak N2 modifications	Basic peak N3 modifications
1×deamidation 2×K loss; A2G0F/A2G1F+A1G1F, 2×K loss, 2×oxidation	1×K loss, 1×GK loss, 3×oxidation	1×K loss, 1×GK loss, 4×oxidation	2×GK loss, 8×oxidation

Conclusion

- ProPac 3R SCX columns provide excellent separation of durvalumab and associated charge variants using a MS-compatible pH gradient to give high resolution, robust performance, and excellent reproducibility.
- The temperature treatment of the protein results in the presence of more acidic and less basic variants. High-resolution MS data provides modification details.
- Consistent performance for durvalumab charge variants analysis was demonstrated for different lots of the ProPac 3R SCX column.
- This online SCX-MS workflow can be used for charge variant analysis in the biopharma industry.

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