Evaluation and application of salt- and pH-based ion-exchange chromatography gradients for analysis of therapeutic monoclonal antibodies

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Keywords

NIBRT, biopharmaceutical, bioproduction, QA/QC, biotherapeutic, IgG, monoclonal antibody (mAb), critical quality attribute, intact protein analysis, charge variant analysis, cetuximab, trastuzumab, bevacizumab, infliximab, innovator, ion exchange chromatography, MAbPac SCX-10, pH gradient buffer, CX-1 pH buffer, Vanquish Flex UHPLC

Application benefits

- Simplified ion exchange analysis (IEX) of charge variants using a pH gradient

- Easy and fast method development for pH gradient based IEX compared to salt gradients

- Reduced risk of method variability of pH gradients compared to salt gradients for IEX

Goal

To demonstrate a simple, easily prepared pH buffer system that is capable of charge variant determination for the majority of therapeutic monoclonal antibody species. To show the ease of optimization and improved reproducibility of the CX-1 pH gradient buffer system when compared to salt-based gradient systems used for charge variant analysis.

Introduction

Monoclonal antibodies (mAbs) are well-established pharmacological therapeutics with high specificity to target antigens, long serum half-life in humans, and capabilities for the treatment of a wide range of ailments, such as inflammatory diseases and cancer. Recombinant mAbs are highly heterogeneous biomolecules that may undergo a wide range of enzymatic and chemical modifications during bioprocessing and storage, which have

the potential to alter the safety and efficacy profile of mAbs by impacting protein binding, reducing drug activity, and giving rise to adverse toxicological and immunological responses in vivo.[1] Hence, it is essential to detect, characterize, and quantify mAb variants and modifications (i.e. their critical quality attributes (CQAs)), to ensure the safety, quality, and efficacy of therapeutic biomolecules and thereby enable regulatory approval of mAbs for clinical use. Charge-related microheterogeneity of mAbs is frequently observed following bioprocessing due to both enzymatic processes and non-enzymatic degradation of therapeutic proteins.[2]

Determination of a mAb charge variant profile provides information regarding commonly encountered product CQAs.[3] Bioprocess-related protein modifications may impart a positive or negative charge on mAbs, resulting in the generation of modified mAb variants with different isoelectric point (pI) values. Chemical modifications, such as deamidation, may give rise to a negative charge on mAbs, thereby decreasing their pI value and resulting in the formation of acidic mAb variants.[4,5] Similarly, C-terminal cleavage of lysine residues results in the loss of positively charged amino acids on the mAb also creating acidic protein variants.[6] Other modifications that may affect the charge of mAbs include cyclisation of N-terminal glutamine to form pyroglutamate and peptide bond cleavage.[7]

Ion-exchange chromatography (IEX), using either a salt or pH separation gradient, is the gold standard for determination of the charge variant profile of therapeutic mAbs.[8] Traditionally, salt-based gradients, wherein the pH is maintained at a constant value while the ionic strength is increased over the duration of the gradient, were most frequently used for IEX. However, optimization of a salt gradient for charge variant analysis of individual mAbs often requires significant effort. Salt gradient buffer optimization may include the choice of salt reagent, salt concentration, and the pH value of the buffers. Use of pH gradients for IEX separation has been demonstrated as an excellent alternative to salt-based gradients for IEX of mAbs. pH-gradient-based ion-exchange chromatographic methods are reported to have greater resolving power and peak capacities when compared to ionic strength elution ion-exchange chromatography.[9]

A protein's pI value corresponds to the pH value at which the protein has no net charge. In pH gradient SCX, the charge on the protein is reduced as the pH increases;

elution occurs when the protein has no electrostatic interaction with the SCX stationary phase. Unlike a salt gradient, optimization of a pH gradient for IEX does not require alteration of the mobile phase composition and hence facilitates more rapid method optimization. Furthermore, reproducible batch-to-batch preparation of salt-based buffers may be challenging when using certain buffer reagents. In this application note, the reproducibility of different salt buffers prepared across multiple preparation dates by different analysts is evaluated for both salt- and pH-based gradient buffers. In addition, two approaches to the generation of a pH gradient were investigated. One approach consisted of quaternary pump mixing of an acid, a base, a salt solution, and water to produce a pH separation gradient. The second approach was generation of a pH gradient using a Thermo Scientific™ cation-exchange pH gradient buffer platform consisting of a low pH buffer at pH 5.6 (Thermo Scientific™ CX-1 pH Gradient Buffer A) and a high pH buffer at pH 10.2 (Thermo Scientific™ CX-1 pH Gradient Buffer B). 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 may be generated. Since the majority of mAbs have pI values in the range of pH 6 to 10, pH-gradient-based separation methods based on the Thermo Scientific pH buffer platform and Thermo Scientific™ MAbPac™ SCX-10 columns may serve as a generic platform for mAb charge variant analysis. Furthermore, the described platform enables rapid, simple method optimization by merely using a shallower pH gradient over a narrower pH range following initial separations in the typical mAb pI range of pH 6 to 10.

Currently, six of the top 10 selling biopharmaceutical products are therapeutic mAbs, all of which will have come off patent by 2019.[10] As a consequence, many biosimilars corresponding to these innovator mAbs are currently being marketed or are under development. Thus, the availability of a simple, readily optimized, widely applicable platform for charge variant analysis is of great benefit to producers of innovator therapeutic mAbs and biosimilars. The charge variant profile of the commercial mAbs bevacizumab, cetuximab, infliximab, and trastuzumab were also determined in this application note using a MAbPac SCX-10 column on a Thermo Scientific™ Vanquish™ Flex UHPLC with CX-1 pH gradient buffers. Minimal method optimization in <1.5 hours facilitated excellent separation of mAb variants. This approach could be easily applied to multiple mAbs and mAb biosimilar products.

Experimental

Recommended consumables

- CX-1 pH Gradient Buffer A, pH 5.6 (P/N 085346)

- CX-1 pH Gradient Buffer B, pH 10.2 (P/N 085348)

- Deionized (DI) water, 18.2 MΩ·cm resistivity

- MabPac SCX-10, 4 × 250 mm, 10 μm column (P/N 074625)

- Thermo Scientific™ Virtuoso™ Vial, clear 2 mL kit with septa and cap (P/N 60180-VT402)

- Thermo Scientific™ Virtuoso™ Identification System (P/N 60180-VT100)

- Carboxypeptidase B (150 units/mg) (Roche Diagnostics™) (P/N 10103233001)

- Sodium phosphate monobasic (Acros Organics™) (P/N 10226410)

- Sodium phosphate dibasic (Acros Organics) (P/N 10036020)

- Sodium chloride (Fisher Chemicals™) (P/N 10428420)

- MES monohydrate (Acros Organics) (P/N 10517241)

Sample preparation

All untreated mAb samples were injected directly in formulation buffer.

To identify peaks corresponding to mAb variants containing C-terminal lysine, samples were digested with carboxypeptidease B as follows: 200 μg of mAb and 15 units of carboxypeptidase B were placed in a microcentrifuge tube, prepared to 60 μL with DI water, and mixed by pipette action. Samples were incubated for 2 hours at 37 °C.

Buffer preparation

Phosphate buffers

Eluent A:	20 mM sodium phosphate, pH 6.2
Eluent B:	20 mM sodium phosphate, pH 6.2, 300 mM NaCl

MES buffers

Eluent A:	20 mM MES, pH 5.6, 60 mM NaCl
Eluent B:	20 mM MES, pH 5.6, 300 mM NaCl

pH gradient buffers: Four solution blending gradient

Eluent A:	100 mM MES monohydrate
Eluent B:	100 mM sodium phosphate dibasic
Eluent C:	1 M sodium chloride
Eluent D:	Deionized water

pH gradient buffers: Two solution linear gradient

Eluent A:	10-fold dilution of CX-1 buffer A (pH 5.6) with DI water
Eluent B:	10-fold dilution of CX-1 buffer B (pH 10.2) with DI water. Mobile phase B was stored in an amber borosilicate glass bottle.

Separation conditions

Instrumentation

Vanquish Flex Quaternary UHPLC system equipped with:
- Quaternary Pump F (P/N VF-P20-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A) with a 25 μL Sample Loop
- Diode Array Detector HT (P/N VH-D10-A) with a Thermo Scientific™ LightPipe™ 10 mm Standard Flow Cell (P/N 6083.0100)

Instrument settings

Flow rate:	1.0 mL/min
Column temperature:	30 °C
Injection details:	50 μg of mAb (2 μL of 25 mg/mL bevacizumab, 10 μL of 5 mg/mL cetuximab, 5 μL of 10 mg/mL infliximab, 2.4 μL of 21 mg/mL trastuzumab)
DAD detector settings:	280 nm
Gradient:	Tables 1–3

Table 1. Gradient conditions for mAb charge variant separations by IEX using either a salt gradient (phosphate- and MES-based buffers) or pH gradient (CX-1 buffer kit)

Time (minutes)	Optimized Gradient Conditions (% Eluent B)		
	Phosphate Buffers	MES Buffers	pH Gradient Buffers (Two Solution Gradient)
0.0	10	15	10
30.0	50	45	50
30.1	10	15	10
40.0	10	15	10

Table 2. Gradient conditions for mAb charge variant separations using a pH gradient of pH 6.5 to 7.2, generated using quaternary pump mixing of four eluents: 100 mM MES monohydrate (A), 100 mM sodium phosphate dibasic (B), 1 M sodium chloride (C), and water (D)

Time (minutes)	% Eluent A	% Eluent B	% Eluent C	% Eluent D	pH	Salt Concentration (mmol/L)
0.00	13.3	6.7	4	76	6.5	40
15.00	7.7	12.3	4	76	7.2	40
15.01	7.7	12.3	50	30	7.2	500
20.01	7.7	12.3	50	30	7.2	500
20.02	13.3	6.7	4	76	6.5	40
35.02	13.3	6.7	4	76	6.5	40

Table 3. pH gradient conditions used for mAb charge variant separations by IEX using a Thermo Scientific CX-1 pH gradient buffer kit

Time (minutes)	% Eluent B				
	Initial Gradient Conditions	Optimized Gradient Conditions			
		Bevacizumab	Cetuximab	Infliximab	Trastuzumab
-10.0	0	20	10	15	20
0.0	0	20	10	15	20
30.0	100	50	40	45	60
30.1	0	20	10	15	20
40.0	0	20	10	15	20

Data processing

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.4 was used for data acquisition and analysis.

Results and discussion

Evaluation of buffer preparation reproducibility for both salt and pH-based IEX eluents

Preparation of IEX buffers from salts followed by manual adjustment of pH has been reported to result in variations in chromatography from preparation to preparation of eluents. To evaluate the buffer preparation reproducibility for salt- and pH-based IEX eluents, phosphate salt buffers, MES salt buffers, and CX-1 pH gradient buffers were prepared by two different analysts on three different days. These buffers were then applied for charge variant analysis using a Vanquish Flex Quaternary UHPLC system. For each buffer system, triplicate injections of infliximab were performed. Excellent reproducibility was observed for replicate mAb injections analyzed using the same buffer preparation, as shown in the example in Figure 1, reflecting the excellent instrument precision achievable with a the Vanquish Flex UHPLC.

Charge variant analysis performed using phosphate buffers resulted in slight retention time shifts for different buffers prepared by different analysts on different days despite using the same procedure, reagent, and equipment (Figure 2). However, the charge variant profile was unchanged following analysis with different phosphate buffer preparations. MES-based salt buffers were found to result in increased reproducibility when compared to phosphate buffers with very minor variations in retention time observed (Figure 3). Charge variant profiles generated using pH gradient buffers were found to be the most reproducible of the eluents evaluated when comparing different buffer preparations (Figure 4). Furthermore, the pH gradient yielded the best separation of peaks, resulting in the resolution of 12 mAb variants compared to 10 mAb species separated using salt gradients, with minimal buffer optimization (<1.5 hours).
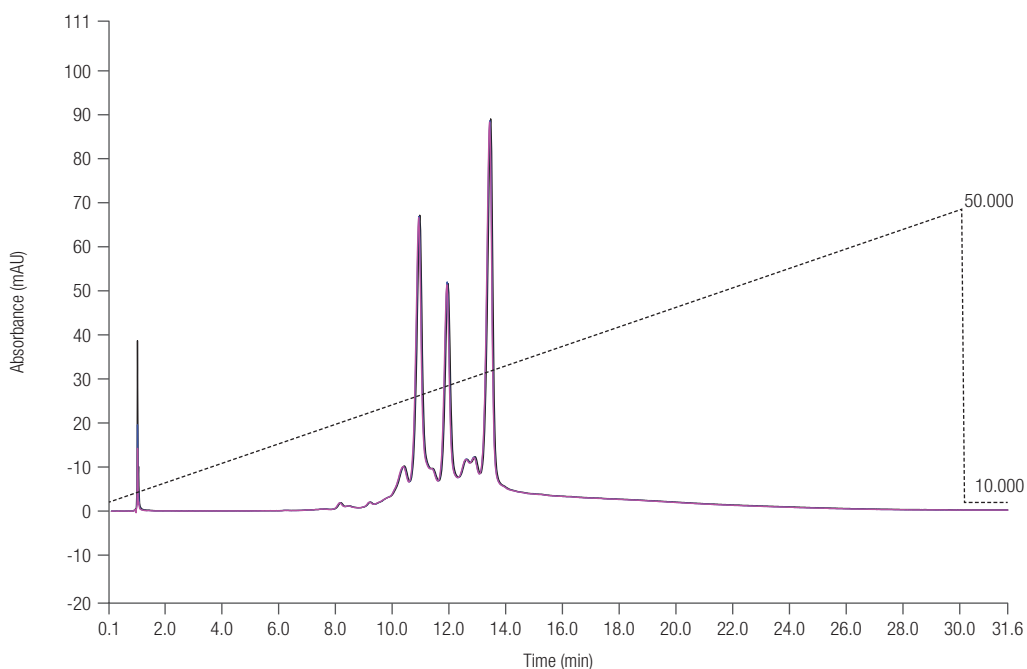

Figure 1. Overlay of chromatograms generated from triplicate injections of infliximab with a salt gradient using the same phosphate buffer preparation
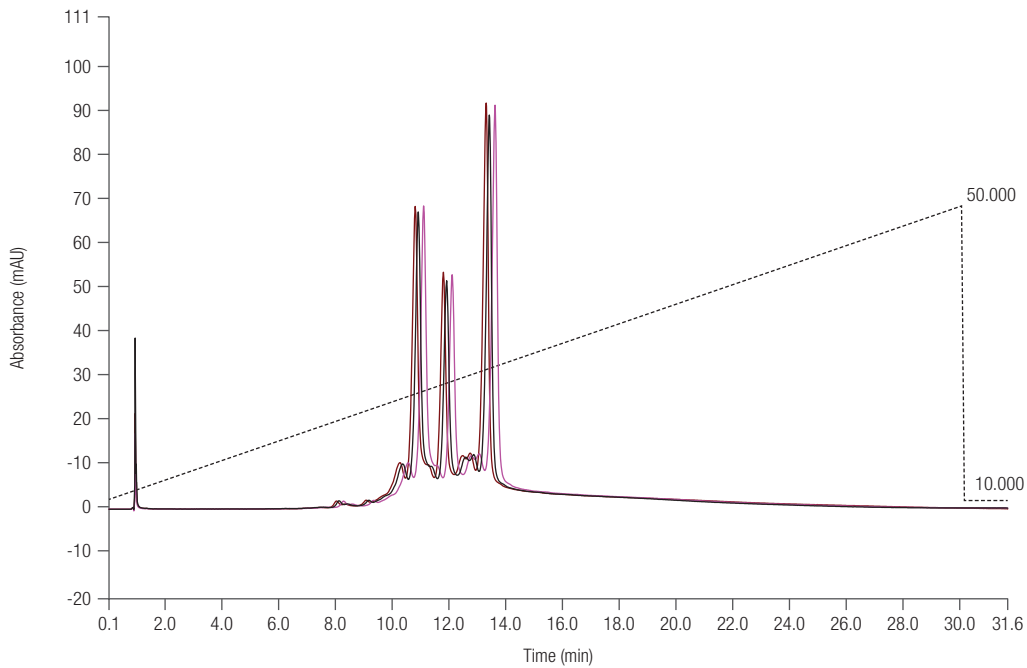
Figure 2. Overlay of chromatograms generated from IEX analysis of infliximab with a salt gradient using different phosphate buffer eluents, prepared by two different analysts on three different days
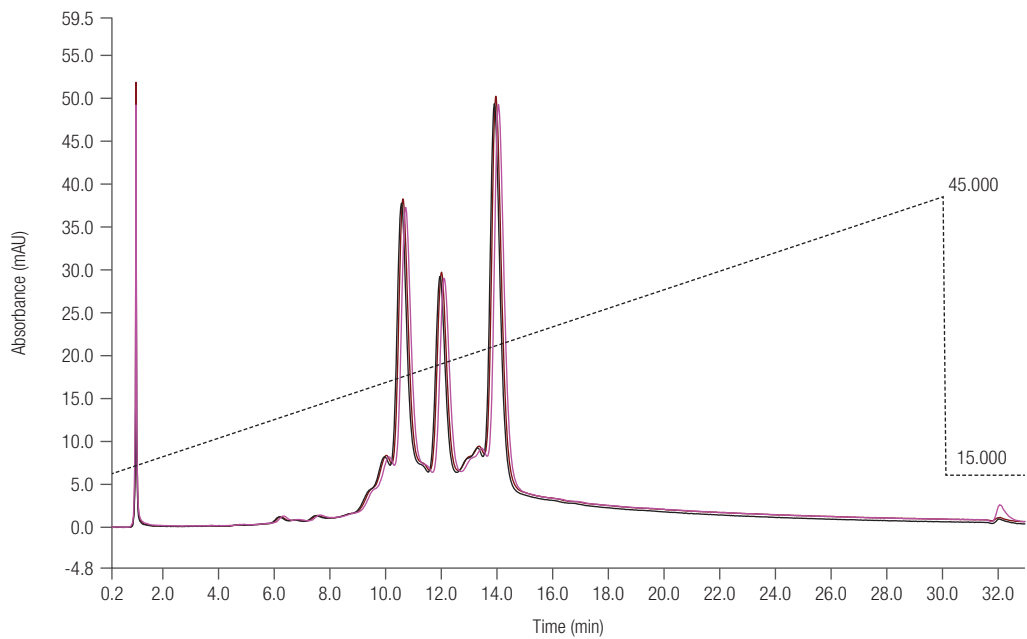

Figure 3. Overlay of chromatograms generated from IEX analysis of infliximab with a salt gradient using different MES buffer eluents, prepared by two different analysts on three different days
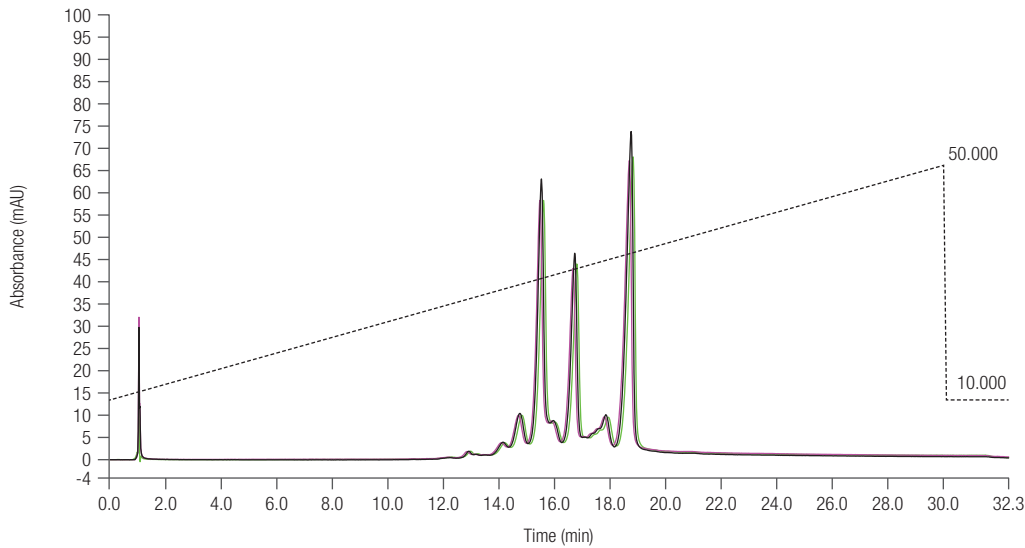
Figure 4. Overlay of chromatograms generated from IEX analysis of infliximab using three different pH buffer eluents, prepared by two different analysts on three different days. pH gradient buffers were prepared using a Thermo Scientific CX-1 buffer kit (pH range 6.1 to 7.9).

Comparison of CX-1 buffer kits with other pH gradient generation strategy.

The Vanquish Flex UHPLC is equipped with a biocompatible, low-pressure mixing quaternary pump. This quaternary pump enables the generation of a pH gradient using a four-solution blending system comprising a weak acid and cognate base for pH buffer adjustment, and sodium chloride and water to alter the ionic strength of the buffer. Similar to the CX-1 pH gradient buffer kit, a pH gradient generated using a four-solution mixing gradient resulted in excellent separation

of infliximab charge variants (Figure 5). However, this pH gradient required considerable optimization to achieve an optimized separation gradient, increased buffer preparation times, and is only possible with a high-performing, inert quaternary pump capable of efficient, reproducibly mixing of eluents. The use of MES/phosphate buffer is restricted to pH 5.5 to 8 as it does not perform with decent buffering capacity outside these pH values. mAb samples with IP values outside this region would require method optimization with a different buffer system.
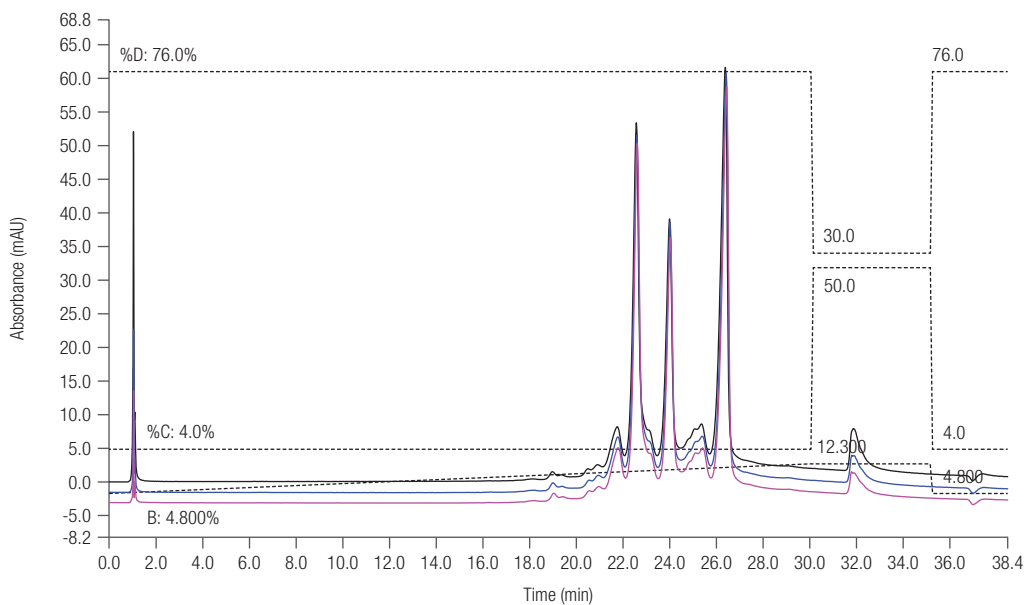

Figure 5. Overlay of chromatograms generated from IEX analysis of infliximab using a pH gradient from pH 6.5 to 7.2, generated from quaternary pump mixing of an acid, a base, a salt solution, and water

Application of CX-1 pH buffer kits for IEX analysis of therapeutic monoclonal antibodies.

Charge variant analysis of four therapeutic mAbs was performed with a pH gradient mode, produced using CX-1 pH gradient buffers on a MAbPac SCX-10 column (4.0 x 250 mm, 10 µm) and a Vanquish Flex UHPLC. Use of a linear gradient from 100% of CX-1 Gradient Buffer A to 100% of CX-1 Gradient Buffer B results in the generation of a pH gradient from pH 5.6 to pH 10.2. As the majority of therapeutic mAbs have pI values that fall within this pH range, CX-1 pH gradient buffers may be applied for the charge variant analyses of most therapeutic mAbs. Bevacizumab (Figure 6-A), cetuximab (Figure 7-A), infliximab (Figure 8-A), and trastuzumab (Figure 9-A) were analyzed using the described platform using the full pH gradient method from 100% of CX-1 buffer A to 100% of CX-1 buffer B, showing good

separation of mAb variants for each therapeutic protein. Following initial survey runs, the method was optimized for each therapeutic mAb by decreasing the pH range and hence gradient slope of the method (Figures 6-B to 9-B). Ease of optimization was facilitated by the use of a linear pH gradient method in combination with a MAbPac SCX-10 column. For each therapeutic mAb analyzed the pH gradients, as outlined in Table 3, were fully optimized in <1.5 hours. Simple optimization of the method enabled the separation of mAb variants, which were initially undetected for all mAbs determined. This straightforward, easily optimized, widely applicable approach to charge variant analysis of therapeutic mAbs has the potential to greatly expedite the characterisation of mAb charge variants during drug development and routine quality control analyses.
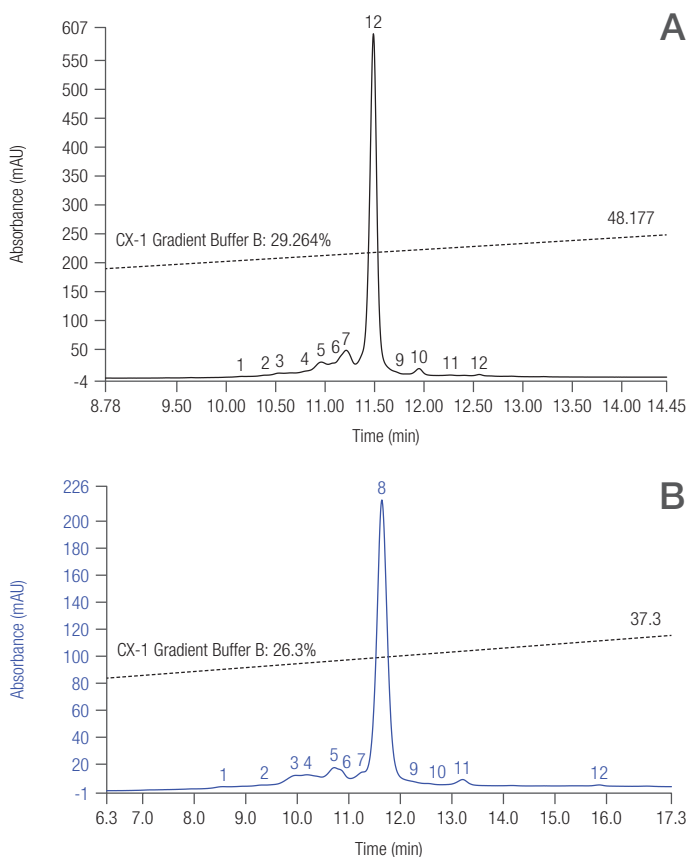

Figure 6. Chromatographic separation of bevacizumab charge variants using pH gradient (A) 0–100% over 30 min and (B) 20–50% B over 30 min, on a MAbPac SCX-10, 10 µm, 4 × 250 mm column
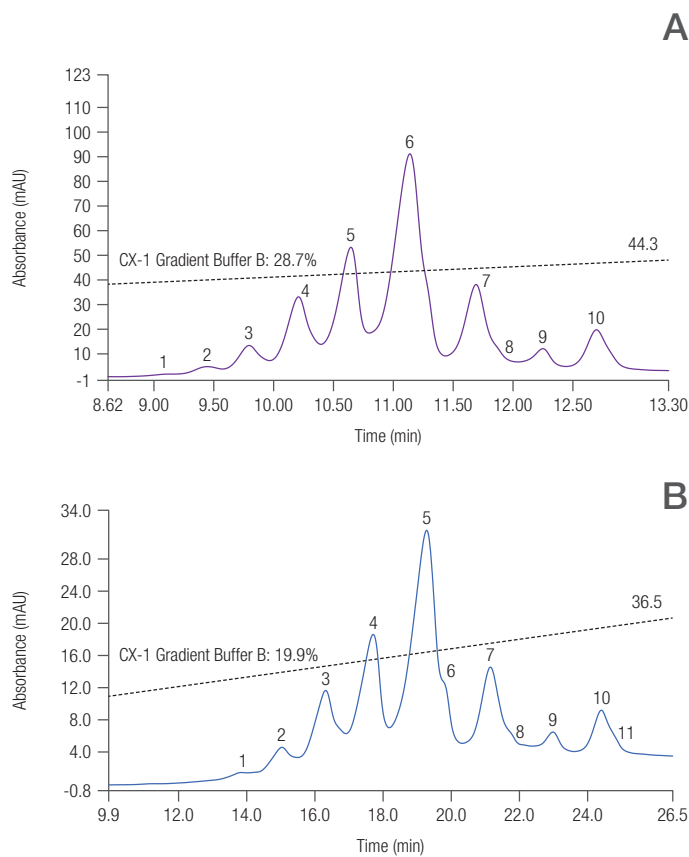

Figure 7. Chromatographic separation of cetuximab charge variants using pH gradient (A) 0–100% over 30 min and (B) 10–40% B over 30 min, on a MAbPac SCX-10, 10 µm, 4 × 250 mm column
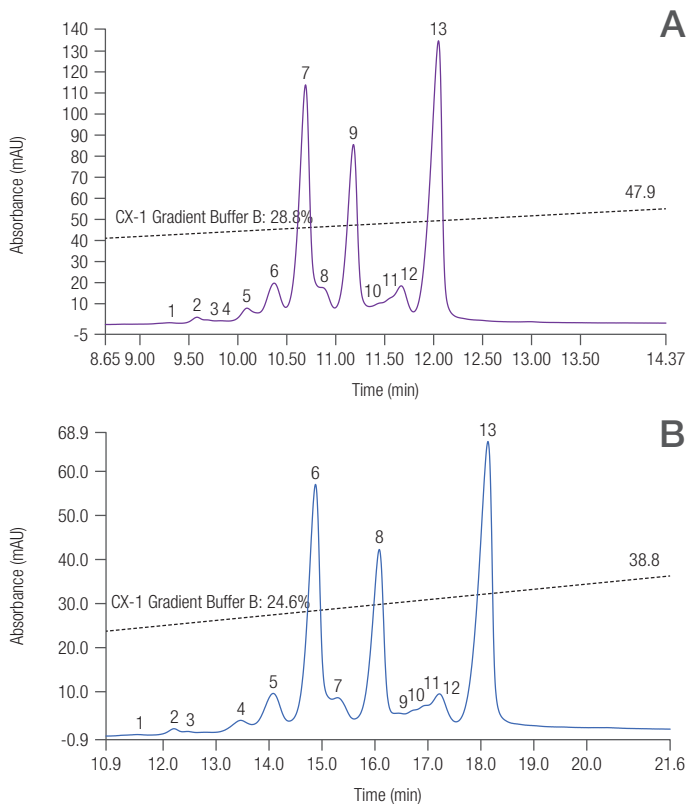
Figure 8. Chromatographic separation of infliximab charge variants using pH gradient (A) 0–100% over 30 min and (B) 10–40% B over 30 min, on a MAbPac SCX-10, 10 µm, 4 × 250 mm column

Figure 9. Chromatographic separation of trastuzumab charge variants using pH gradient (A) 0–100% over 30 min and (B) 10–40% B over 30 min, on a MAbPac SCX-10, 10 µm, 4 × 250 mm column

Both enzymatic and non-enzymatic modifications result in charge heterogeneity in therapeutic mAbs, e.g. glycosylation, deamidation, and lysine truncation. Two common mAb variants include moieties incorporating either one or two C-terminal lysine residues, which are reported to produce distinctive peaks eluting after the main mAb peak upon SCX analysis.[11] To determine which chromatographic peaks detected correspond to mAb variants containing zero, one, or two C-terminal lysine residues, each of the mAb samples under determination were processed with carboxypeptidase B, an endopeptidase that cleaves C-terminal lysines with high specificity. Treatment of the therapeutic mAbs with carboxypeptidase B resulted in the removal of peaks in the basic region of the chromatograms acquired for charge variant analysis for the majority of the mAbs. A loss of peaks 7 and 8 was observed for bevacizumab
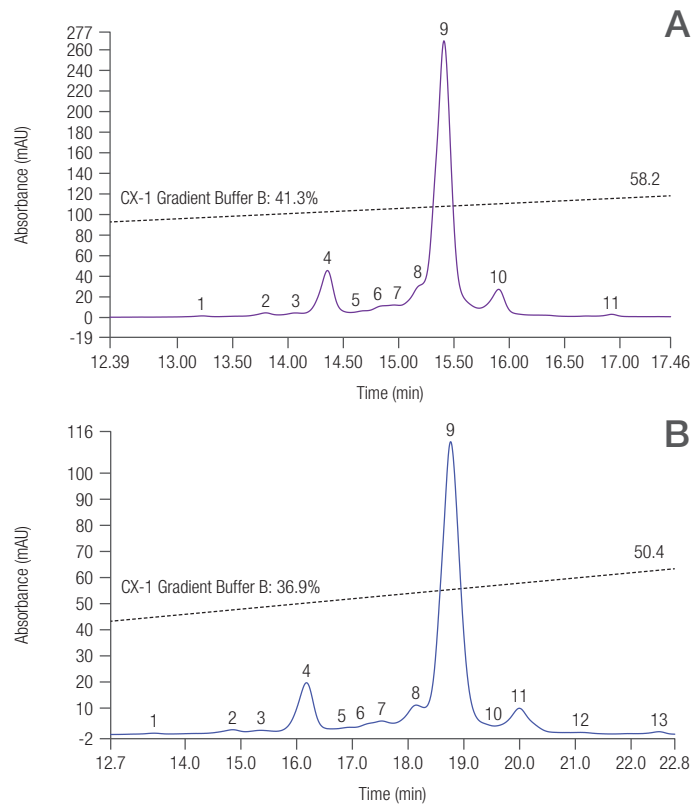
(Figure 10), while multiple peaks were eliminated from the chromatogram showing the charge variant profile of cetuximab, namely peaks 6 to 10 (Figure 11). Digestion of infliximab with carboxypeptidase B resulted in the loss of peaks 7 to 13 from the chromatogram and a notable increase in peak 6 which is the non-truncated form, as shown in Figure 12. Notably, digestion of trastuzumab drug product with carboxypeptidase B did not appear to result in the removal of peaks from the chromatograms suggesting the absence of C-terminal lysine variants in the drug substance (Figure 13).
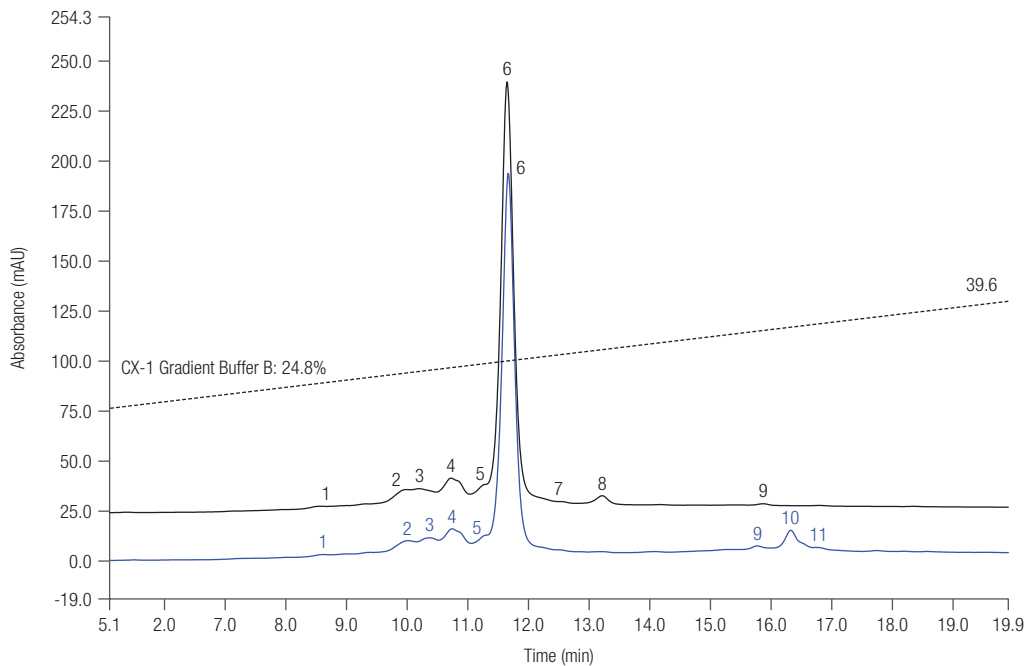
Figure 10. Characterization of bevacizumab drug product C-terminal lysine truncation variants using MAbPac SCX-10 column (4.0 × 250 mm, 10 μm) and pH gradient mode
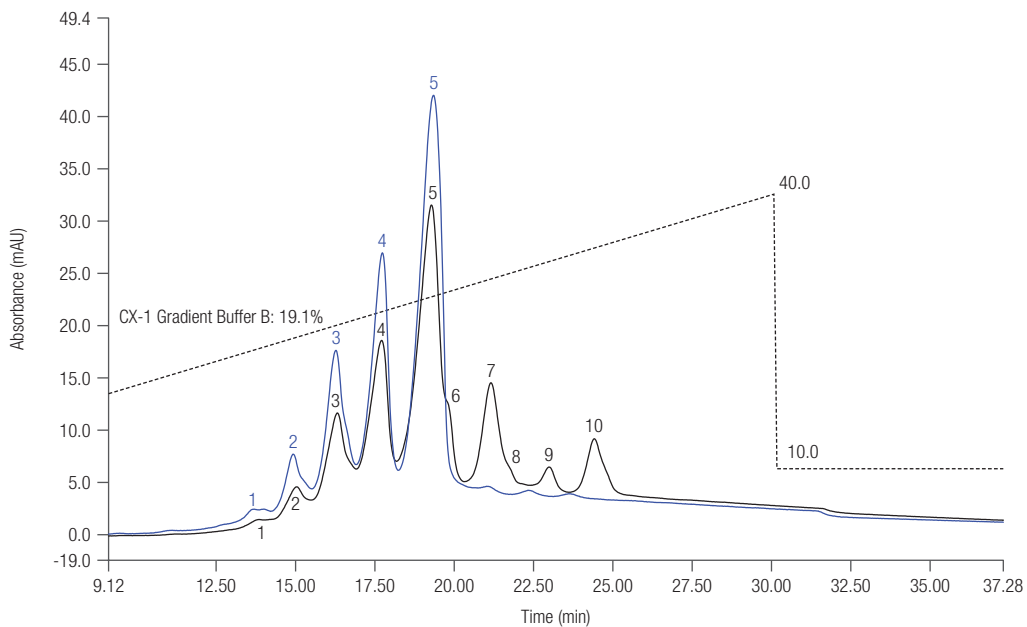

Figure 11. Characterization of cetuximab drug product C-terminal lysine truncation variants using MAbPac SCX-10 column (4.0 × 250 mm, 10 μm) and pH gradient mode
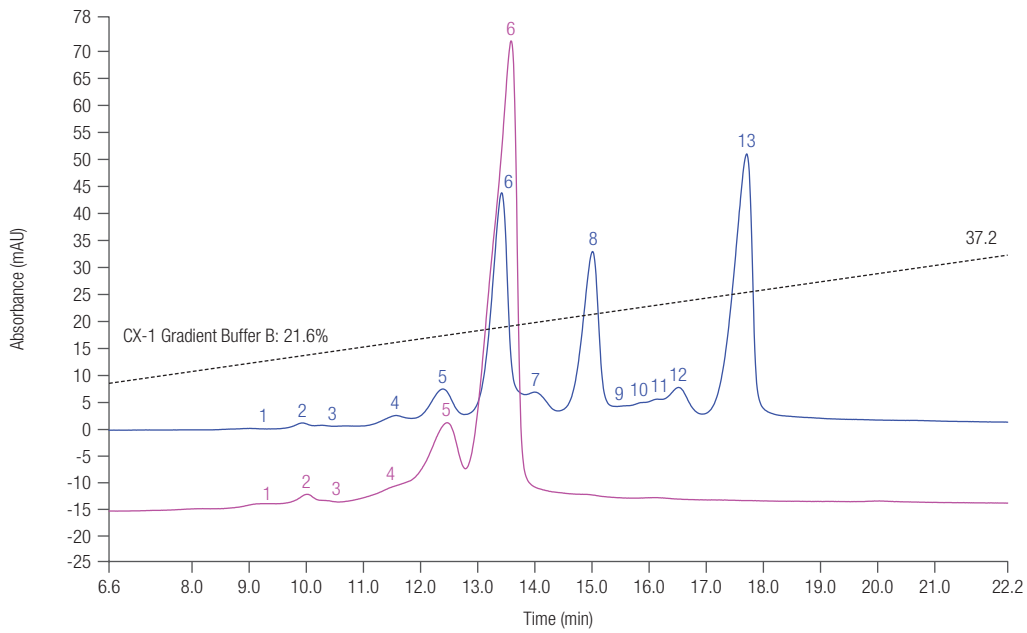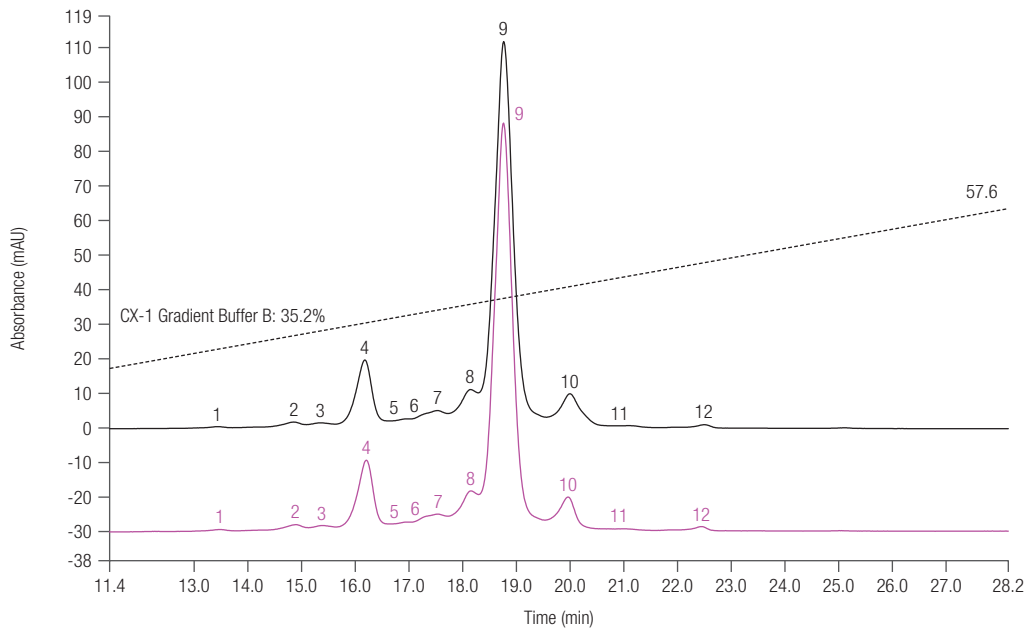
Figure 12. Characterization of infliximab drug product C-terminal lysine truncation variants using MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH gradient mode

Figure 13. Characterization of trastuzumab drug product C-terminal lysine truncation variants using MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH gradient mode

Conclusions

- Thermo Scientific CX-1 pH gradient buffers in combination with Thermo Scientific MAbPac SCX-10 analytical columns enable rapid method development for charge variants analysis of most mAb biotherapeutics.

- Use of a pH gradient for mAb charge variant analysis by IEX enables increased reproducibility of chromatography across different buffer preparations.

- This straightforward, easily optimized, widely applicable approach to charge variant analysis of therapeutic mAbs generated using Thermo Scientific CX-1 pH gradient buffers has the potential to greatly expedite the characterization of mAb charge variants during drug development and routine quality control analyses.

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