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Development of pH Gradient Mobile Phase Concentrates for Robust, High Resolution mAb Charge Variant Analysis

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Abstract

This application note describes the design, optimization, and performance of BioResolve CX pH concentrates and their corresponding mobile phase system. A new pH gradient mobile phase system, based on BioResolve CX pH Concentrates, has been developed to provide universally applicable cation-exchange separations of mAbs having a wide range of isoelectric point (pI) values. The composition and concentrations of buffer salts, as well as the pH range, were optimized using theory and empirical observations to achieve robust and high-resolution separation of mAbs. The performance of this method has been confirmed to be reproducible from batch-to-batch and to afford column lifetimes up to and beyond 500 injections. Moreover, it has been found that it is particularly advantageous to employ these mobile phase concentrates with BioResolve SCX mAb Columns. Method implementation and optimization has been simplified while both throughput and resolution have been improved. The BioResolve CX pH Concentrates, together with BioResolve SCX mAb Columns, provide an effective and efficient approach to mAb charge variant analysis.

Benefits

- Platform method for mAbs with pI values from 6 to 10
- Optimized for robust and high resolution separations with BioResolve SCX mAb Columns

- Easy implementation
- QC batch tested with a monoclonal antibody charge variant profile

Introduction

Protein therapeutics, such as monoclonal antibodies (mAbs), have rapidly grown to be stalwarts of the pharmaceutical industry and have been successfully applied to the treatment of many different diseases. Their post-translational modifications, an intrinsic outcome from biologically-based manufacturing, need to be carefully characterized, since minor structural changes can have significant impacts on drug stability, potency, and efficacy. One such type of variation that must be understood is charge heterogeneity. For this, ion-exchange chromatography has been widely applied.[1] Time-consuming method optimization is generally required for each individual product, if an analyst is to rely on the use of salt gradients. On the other hand, there is the potential for well-developed pH gradient techniques that can be made applicable to many different samples.

Zhang[2] and Lin[3] have demonstrated that salt-mediated pH gradient methods show promise to provide reproducible separations with cation-exchange chromatography. Both of their efforts produced noteworthy resolution for mAbs with low to medium isoelectric point (pI) values. Nevertheless, the resolution observed for mAbs with higher pI values suggested there might be room for the experimental approach to be further optimized, especially since 17 of the 23 FDA/EMA approved mAbs exhibit pIs between 8.0 and 9.4.[4] To that end, BioResolve CX pH Concentrates were developed to facilitate high resolution separations of mAbs over the pI range of 6–10. Together with BioResolve SCX mAb, 3 µm Columns, BioResolve CX pH Concentrates provide a robust, high resolution, and simple-to-implement pH gradient method for charge variant analysis of mAbs. In this application note, we describe the design, optimization, and performance of these concentrates and their corresponding mobile phase system.

Experimental

Sample preparation

Panitumumab, infliximab, trastuzumab, adalimumab, and NIST mAb (reference material 8671) were diluted

with 18.2 MΩ water to 5 mg/mL. The mAb Charge Variant Standard (p/n: 186009065) was reconstituted with 100 µL of 18.2 MΩ water.

LC conditions

Instrument:	ACQUITY UPLC H-Class Bio with Titanium (5 mm, 1500 nL) Flow Cell
Data management:	Empower 3
Column temp.:	30 °C
Detection (UV):	20 Hz, 280 nm
Sample manager wash:	18.2 MΩ water
Seal wash:	10% HPLC-grade methanol/90% 18.2 MΩ water v/v (interval set to 0.5 min)

Method conditions (Figures 1 and 2)

Columns:	Waters Prototype SCX Column, 2.1 x 50 mm BioResolve SCX mAb, 3 µm, 4.6 × 50 mm (p/n: 186009058) BioResolve SCX mAb, 3 µm, 4.6 × 100 mm (p/n: 186009060)
Mobile phase A:	1X prototype CX pH mobile phase A or 1X BioResolve CX pH Concentrate A (p/n: 186009063)
Mobile phase B:	1X prototype CX pH mobile phase B or 1X BioResolve CX pH Concentrate B (p/n: 186009064)

Flow rate:	1.00 mL/min or as specified (4.6 mm I.D.), 0.15 mL/min (2.1 mm I.D.)
Injection volume:	5 µL (4.6 mm I.D.), 1 µL (2.1 mm I.D.)
pH/Conductivity measurement:	GE Healthcare Monitor pH/C-900 pH trace sampling rate: 120 points/min

Method conditions (Figure 3, 4, and 6)

Columns:	BioResolve SCX mAb, 3 µm, 4.6 × 50 mm (p/n: 186009058)
	Competitor SCX, 3 µm, 4.0 × 50 mm
	Competitor SCX, 10 µm, 4.0 × 250 mm
Mobile phase A:	1X BioResolve CX pH concentrate A (p/n: 186009063)
Mobile phase B:	1X BioResolve CX pH concentrate B (p/n: 186009064)
Mobile phase C:	1X competitor CX pH concentrate A
Mobile phase D:	1X competitor CX pH concentrate B
Flow rate:	1.00 mL/min (4.6 mm I.D.) 0.76 mL/min (4.0 mm I.D.)
Injection volume:	10 µL (mAb Charge Variant Standard) 5 µL (infliximab, trastuzumab, adalimumab, and NIST mAb)

Gradients for Figure 3

Time	%A	%B	Curve
Initial	100	0	Initial
1.0	100	0	6
31.0	0	100	6
32.0	0	100	6
33.0	100	0	6
40.0	100	0	6

Time	%C	%D	Curve
1.0	100	0	6
27.5	0	100	6
28.5	0	100	6
29.5	100	0	6
40.0	100	0	6

Gradients for Figure 4

Time	%A	%B	Curve
Initial	100	0	Initial

Time	%A	%B	Curve
1.0	100	0	6
21.0	0	100	6
22.0	0	100	6
23.0	100	0	6
30.0	100	0	6

Time	%C	%D	Curve
Initial	100	0	Initial
5.0	100	0	6
55.0	0	100	6
60.0	0	100	6
65.0	100	0	6
100.0	100	0	6

Gradient for Figure 6

Time	%A	%B	Curve
Initial	100	0	Initial
1.0	100	0	6

Time	%A	%B	Curve
31.0	0	100	6
32.0	0	100	6
33.0	100	0	6
40.0	100	0	6

Method conditions (Figure 5)

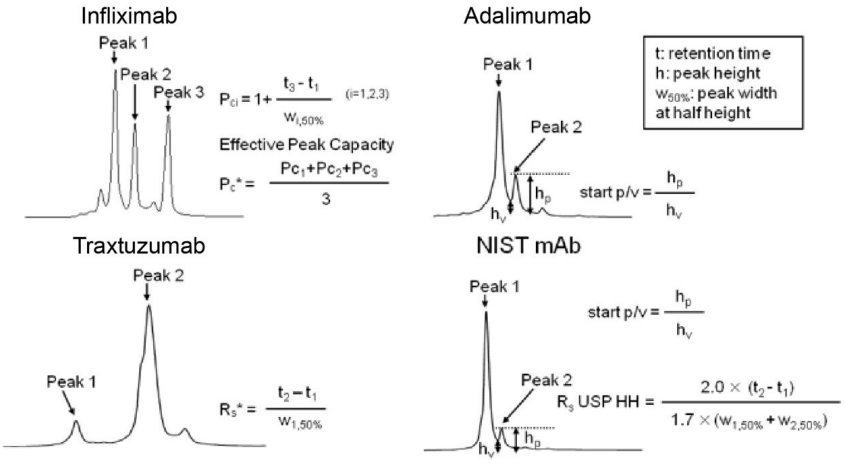
Columns:	BioResolve SCX mAb, 3 µm, 4.6 x 50 mm (p/n: 186009058)
Mobile phase A:	1X BioResolve CX pH Concentrate A (p/n: 186009063)
Mobile phase B:	1X BioResolve CX pH Concentrate B (p/n: 186009064)
Flow rate:	1.00 mL/min
Injection volume:	2.4 µL

Time	%A	%B	Curve
Initial	100	0	Initial
1.0	100	0	6
34.0	0	100	6
35.4	0	100	6

Time	%A	%B	Curve
36.9	100	0	6
43.2	100	0	6

Calculations and data analysis

Five different mAbs (panitumumab, infliximab, trastuzumab, adalimumab, and NIST mAb [RM 8671]) were selected for use in this study because of their diverse pI values, unique retention behavior, and distinct charge variant profile. The calculation of resolution metrics of four mAbs, an effective peak capacity (P_c*) of infliximab, a pseudo resolution value for the separation of an acidic variant (R_S*) of trastuzumab, peak-to-valley (p/v) or a USP half-height (HH) measurement (R_S) of the major basic variant of adalimumab and NIST mAb (RM 8671), are demonstrated below. Due to the presence of a partially resolved pre-peak/shoulder of the main peak of trastuzumab, which interferes peak width measurement, a pseudo resolution (R_S*) was applied to track the resolution of the acidic variant against the main peak. The retention and charge variant profile of panitumumab, a low pI mAb, were inspected for all the prototype CX pH mobile phases.



Mean absolute percent error (MAPE) was calculated as follows:

$$MAPE = \frac{100\%}{n} \sum_{t=0}^{n} \left| \frac{pH\ meas\ (t) - pH\ calc\ (t)}{pH\ calc\ (t)} \right|$$

Results and Discussion

Development of a Platform Method for mAb Charge Variant Analysis

A recent study with imaged capillary isoelectric focusing revealed that the pI values of 23 FDA and EMA approved mAbs ranged from 6.1 to 9.4 and that the majority of them have pI values above 8.0.[4] Accordingly, an ideal mobile phase system for pH gradient mAb charge variant analysis must offer tightly controlled pH and constant buffer capacity in the pH range of 6 to 10. Such a mobile phase system must also be composed of biologically compatible buffer salts with evenly distributed pK_a values over the pH range. These considerations led to a binary mobile phase system and corresponding BioResolve CX pH Concentrates that are comprised of four buffer salts, namely succinic acid (pK_a values of 4.1 and 5.5), BIS-TRIS propane (pK_a values of 6.8 and 9.1), triethanolamine (pK_a value of 7.8), and CAPSO (pK_a value of 9.6), each of which is a biological buffer salt commonly used in protein ion-exchange chromatography.[5] The development of these concentrates was based both on theoretical predictions and empirical optimization according to observations relating mobile phase composition to resolving power. Mobile phase optimization was performed on BioResolve SCX mAb Columns[6] and their late-stage prototypes to ensure compatibility with the newly developed cation-exchange stationary phase. In order to develop a platform method for mAb charge variant analysis, the retention and resolution of five mAbs with diverse pI values and unique retention behavior (panitumumab, infliximab, trastuzumab, adalimumab, and NIST mAb [RM 8671]) were monitored. Detailed calculations of resolution metrics can be found in the experimental section.

Shown in Figure 1 are mAb charge variant profiles obtained with a BioResolve SCX mAb Column and several different mobile phase compositions. Separations achieved with the finely tuned compositions and concentrations of the BioResolve CX pH Concentrates are portrayed in Figure 1A. Compared to alternatives, no other mobile phase system could produce separations that were as equally well balanced for retention and resolution of each of the tested mAbs (Figures 1B–E). For instance, when succinic acid was replaced with MES, which is a zwitterionic buffer salt with comparable pK_a, no benefit to resolution was observed for infliximab, trastuzumab, adalimumab, or NIST mAb, yet the retention of the low pI mAb, panitumumab, was markedly

impacted (Figure 1B). In another case, BIS-TRIS propane, a diprotic compound, was exchanged with two separate buffer salts of comparable pK_a values (N-(2-acetamido)-2-aminoethanesulfonic acid and 2-amino-2-methyl-1,3-propanediol). While similar separations were observed for panitumumab and infliximab, the resolution metrics for trastuzumab, adalimumab, and NIST mAb were negatively affected (Figure 1E). In some cases, the variation of mAb retention and resolution with different mobile phase compositions can be explained by their unique pH and conductivity traces, while it is possible that the physicochemical properties of buffer salts play an important role.

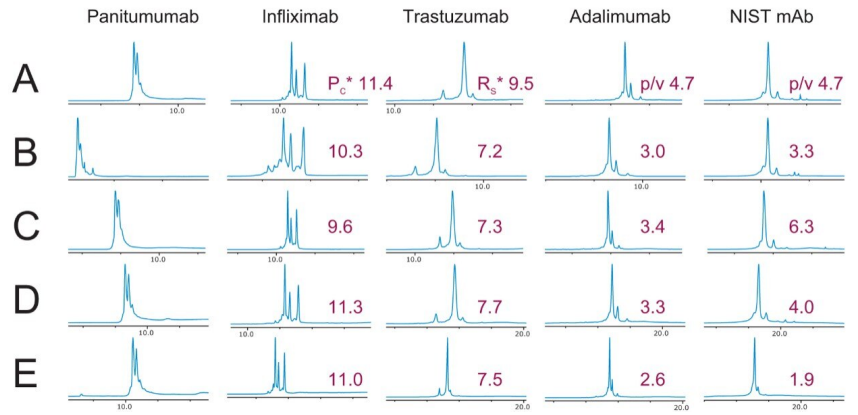


Figure 1. Optimization of pH gradient mAb charge variant separations using mobile phases composed of different buffer salts. The UV chromatograms of panitumumab, infliximab, trastuzumab, adalimumab, and NIST mAb (RM 8671) were obtained on IEX prototype columns using prototype cation exchange (CX) pH mobile phases at a gradient slope of 0.46 pH unit/min. The measured resolution metrics are noted on the chromatograms, including effective peak capacity (P_c) of infliximab, a pseudo resolution value for the separation of an acidic variant (R_s*) of trastuzumab, and peak-to-valley (p/v) numbers for the major basic variant of adalimumab and NIST mAb (RM 8671).*

After extensive exploration of mobile phase compositions, the pH range of mobile phases composed of succinic acid, BIS-TRIS propane, triethanolamine, and CAPSO was optimized by varying the pH of mobile phase A from 3.5 to 5.5 while keeping the pH of mobile phase B at 10.2. The peak capacities of infliximab and the p/v ratios of the basic variant of NIST mAb slightly improved as the pH of mobile phase A increased from 3.5 to 5.5, while the resolution of trastuzumab and adalimumab remain relatively constant (data not shown). On the other hand, the retention of panitumumab, a mAb with a pI of 6.7, was poor when the pH of mobile phase A was titrated to

5.5, and the peak profile was distorted in comparison to established results when the pH of mobile phase A was 5.2.

Given these and other similar observations, it was determined that a pH 5.0 to 10.2 buffer system based on succinic acid, BIS-TRIS propane, triethanolamine, and CAPSO affords one of the most capable mAb charge variant separations as performed with a state-of-the-art, cation-exchange stationary phase, such as that provided with the technology of the BioResolve SCX mAb Column. This mobile phase system has been commercialized in the form of two convenient 10x concentrates that can be used with a 10-fold water dilution to prepare a simple, binary mobile phase system.

pH Linearity and Kinetic Effects

In addition to desirable chromatographic effects, it was imperative for this mobile phase system to deliver linear pH traces so that it would be robust and reproducible upon implementation. To this end, the concentrations of components in the mobile phase system were optimized to afford linear pH dependence within the elution window of most mAb species. A theoretical prediction provided a starting point for each individual reagent concentration.[5] Experimentally, it was found that the pH trace obtained with this mobile phase system showed some deviation from an ideal linear trace. Concentrations were accordingly adjusted until pH linearity was optimized. In turn, an optimal ratio of succinic acid:BIS-TRIS propane:triethanolamine:CAPSO was successfully determined without sacrificing mAb resolution.

While making the above observations, it was observed that pH linearity is dependent on numerous factors, including kinetics. More specifically, it has come to be understood that the pH linearity is a function of gradient steepness. It has been widely accepted that peak resolution improves with increasing gradient times. As shown in Figure 2A, this trend can be demonstrated with a separation of infliximab using the BioResolve CX pH Concentrates with either a 4.6 × 50 mm or a 4.6 × 100 mm BioResolve SCX mAb Column. Similar to all forms of chromatography, ion exchange performed with pH gradient elution is affected by kinetic variables. Figures 2B and C present empirically derived charts that relate pH linearity to gradient steepness. Mean absolute percent error (MAPE), where smaller values correlate to better pH linearity, was used to quantify the linearity of pH traces obtained with BioResolve CX pH Concentrates on BioResolve SCX mAb Columns. It was observed that pH linearity improved with increasing gradient time. Similar trends of MAPE versus ΔpH/Δcolumn volume (CV) were observed with columns of differing dimensions, including 2.1 versus 4.6 mm I.D.s and 50 versus 100 mm lengths. To achieve high precision pH traces, it is recommended that a gradient steepness (ΔpH/ΔCV) of 0.5 or lower be employed. Hence, a potential method condition to consider for a 4.6 × 50 mm column entails a flow rate of 1.00 mL/min across a 30 min 0–100% B gradient, as shown in Figure 2D.

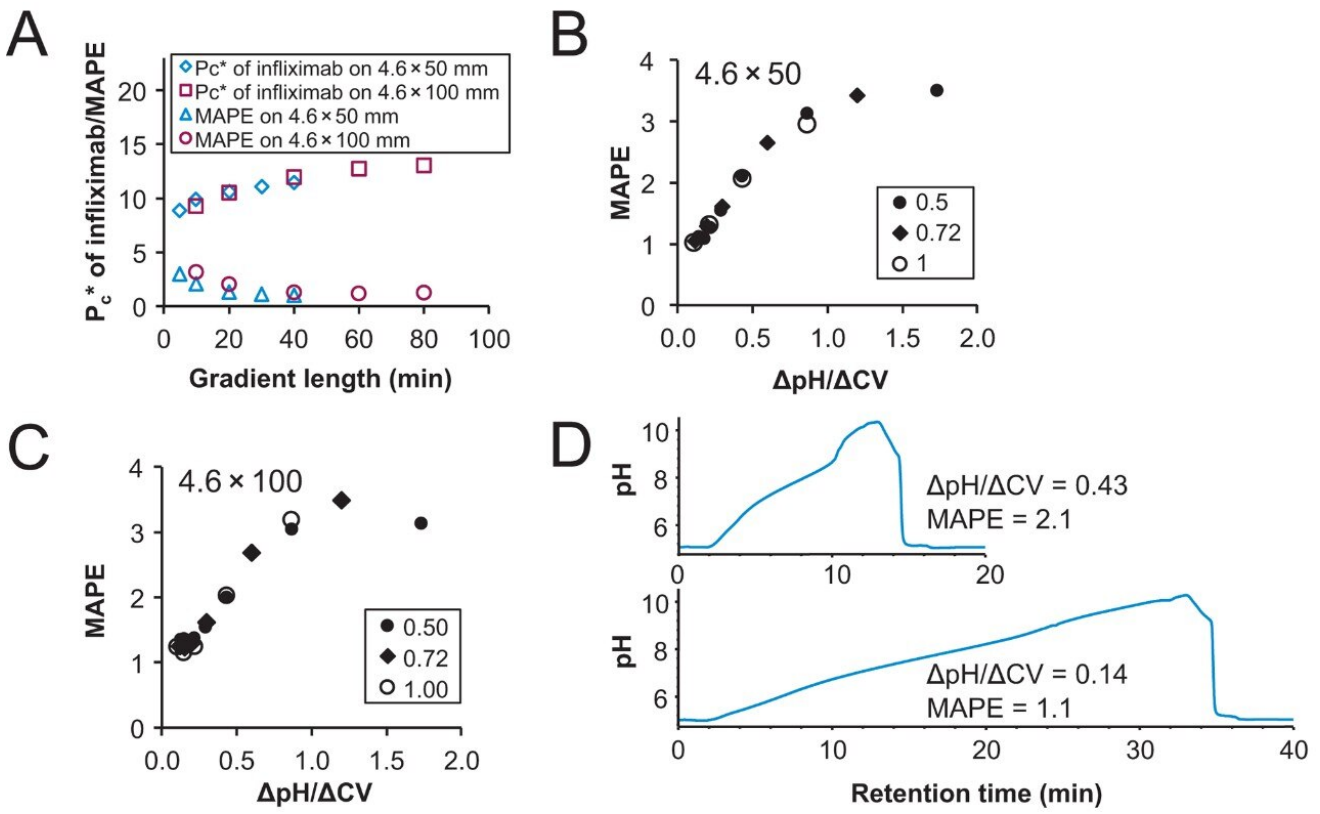


Figure 2. The relationship between resolution, pH linearity, and gradient steepness for pH gradient mAb charge variant separations with BioResolve CX pH Concentrates. (A) The resolution of infliximab and pH linearity/mean absolute percent error (MAPE) as a function of gradient time on a 4.6 × 50 mm or a 4.6 × 100 mm BioResolve SCX mAb Column operated at the flow rate of 1.00 mL/min. The relationship between MAPE and ΔpH/Δcolumn volume (CV) obtained using BioResolve CX pH Concentrates on a 4.6 × 50 mm (B) or a 4.6 × 100 mm (C) BioResolve SCX mAb Column at flow rates of 0.50, 0.72, and 1.00 mL/min. (D) Representative pH traces using BioResolve CX pH Concentrates on a 4.6 × 50 mm BioResolve SCX mAb Column at a flow rate of 1.00 mL/min. Chromatograms were acquired with an ACQUITY UPLC H-Class Bio System, and pH traces were obtained with GE Healthcare Monitor pH/C-900.

Performance, Robustness, and Lifetime

The chromatographic capabilities of BioResolve CX pH Concentrates are best illustrated in a comparison to an alternative commercially available technology. Herein, a study was performed to compare the separation of four different monoclonal antibodies, infliximab, trastuzumab, adalimumab, and NIST mAb, using the combination of BioResolve CX pH Concentrates and BioResolve SCX mAb, 4.6 × 50 mm Columns versus similar offerings from a leading competitor. The chromatograms were acquired with two different batches of mobile

phase concentrates and two different batches of column stationary phases for both the BioResolve and competitor separation technologies (Figure 3A). Resolution metrics corresponding to the average performance have been compiled into a chart and are displayed in Figure 3B. At the same pH gradient slope of 0.17 pH unit/min, the BioResolve separation technologies showed higher resolving power than the leading alternative technologies, as measured in the form of peak capacity, resolution, or peak-to-valley numbers. It was clear that sizeable gains in resolution were achieved for high pI mAbs (e.g., adalimumab and NIST mAb) with the BioResolve separation technologies. Within this comparison, it was only with the BioResolve separation technologies that the acidic variant front shoulder of NIST mAb could be partially resolved from its main peak. In addition, the performance variation of the BioResolve separation technologies was markedly lower and so too was carryover (Figures 3C).

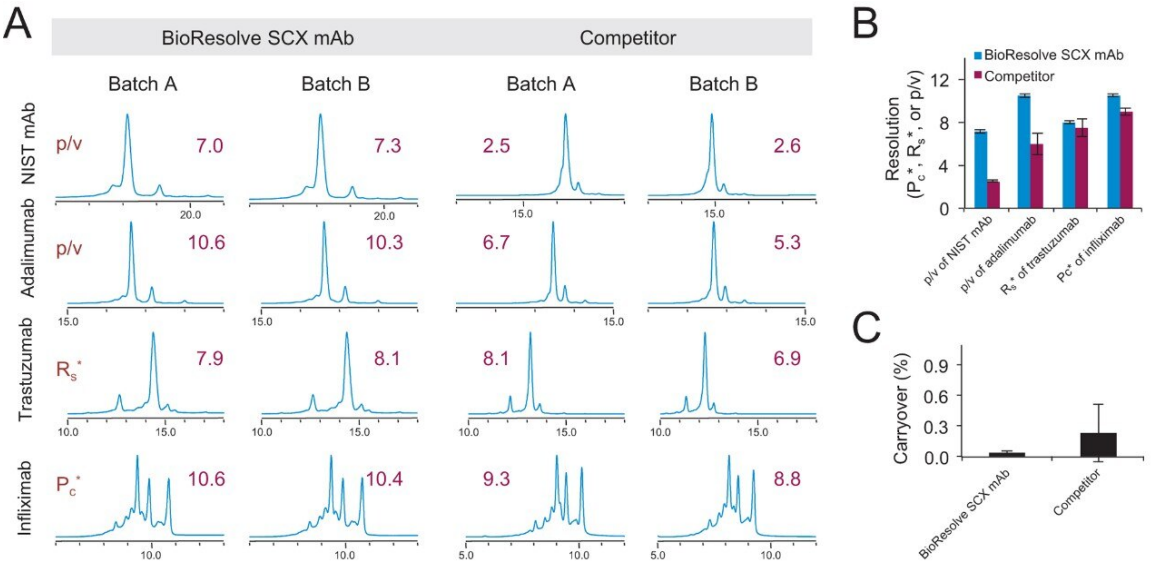


Figure 3. Separations of NIST mAb (RM 8671), adalimumab, trastuzumab, and infliximab as performed with different batches of BioResolve SCX mAb, 3 µm, 4.6 × 50 mm Columns and BioResolve CX pH Concentrates or different batches of competitor SCX, 3 µm, 4.0 × 50 mm columns and competitor CX pH concentrates. UV chromatograms (A) and measured resolution metrics (B) from the separations, including p/v of the major basic variant of adalimumab and NIST mAb, a pseudo resolution value for the separation of an acidic variant (R_s^*) of trastuzumab, and effective peak capacity (P_c^*) of infliximab, are displayed. (C) Percent carryover of NIST mAb observed during a repeat gradient.

On top of this, the combination of BioResolve CX pH Concentrates and BioResolve SCX mAb Columns facilitates performing faster analyses versus a legacy approach to LC-based charge variant profiling. The separation of

NIST mAb was directly compared between a BioResolve SCX mAb, 3 µm, 4.6 × 50 mm Column and a competitor, 10 µm, 4.0 × 250 mm column using the BioResolve and competitor CX pH concentrates, respectively (Figure 4). Superior peak resolution was achieved with the BioResolve separation technologies with a 20-minute gradient versus the competitor setup with a 50-minute gradient. With the same sample load, not only were the major basic variants better resolved from the main peak, but three minor basic variants were also well resolved with the BioResolve separation technologies (Figure 4 insets).

Not only is it important that these separations achieve high resolution, but it is important that they do so with good reliability. Column lifetime testing was performed with BioResolve CX pH Concentrates to examine the robustness of BioResolve SCX mAb Columns with pH gradient separations. Five hundred injections of NIST mAb were repeated on a BioResolve SCX mAb, 4.6 × 50 mm Column at a reasonably high flow rate of 1.00 mL/min. No significant difference was observed in the representative chromatograms of NIST mAb throughout the 500 injections, and the partial resolution of the acidic variant front shoulder was achieved in every chromatogram (Figure 5A). Moreover, consistent retention times and column pressure were observed across the 500 injections, and the resolution of the basic variant only drifted slightly toward the end of the lifetime test (Figure 5B).

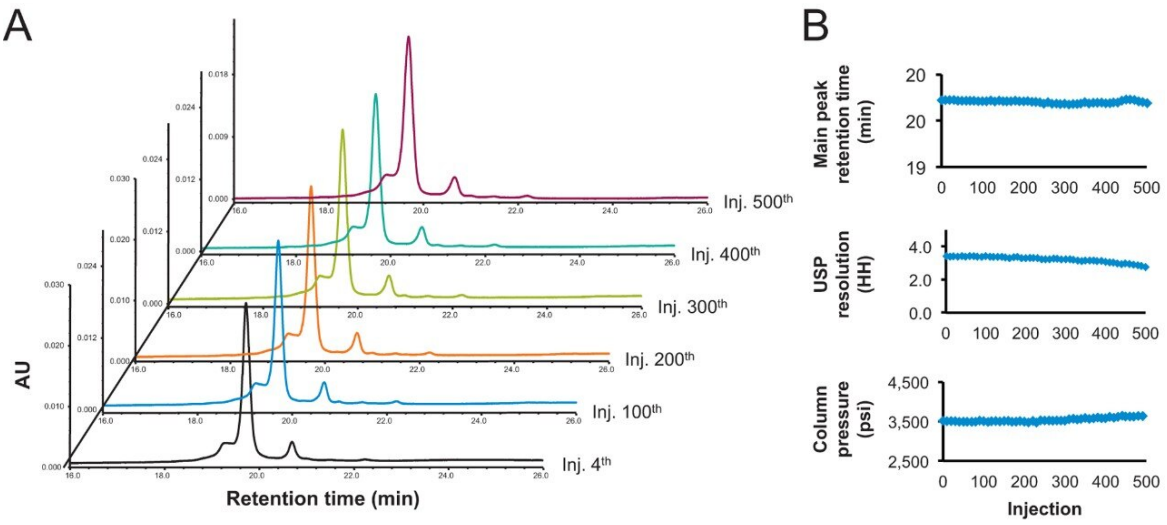


Figure 5. Lifetime testing of a BioResolve SCX mAb Column using BioResolve CX pH Concentrates. (A) UV chromatograms obtained for NIST mAb (RM 8671) with a 4.6 × 50 mm column at a flow rate of 1.00 mL/min. (B) Retention time of main peak, USP half-height resolution, and column pressure across 500 injections.

Method Optimization to Improve Throughput and/or Resolution

The challenge to develop methods for LC-based charge variant profiling has been a resounding and detracting comment about ion-exchange chromatography. Indeed, many factors in a salt gradient method, including the choice of buffer salts, ionic strengths of buffer salts, buffer pH, gradient slope, flow rate, and temperature, need to be optimized for each individual sample. While method development tools, such as Auto · Blend Plus, help assuage these concerns,[7] the BioResolve CX pH Concentrates provide yet another attractive option for quickly obtaining robust and high resolution separations of mAbs with pI values ranging from 6 to 10. To further improve throughput and/or resolution, as little as two injections with a generic 0–100% B gradient method are needed. As demonstrated in Figure 6 for infliximab, the elution window of infliximab can be easily determined. The column void volume can be calculated from the retention time of the void marker, such as tryptophan in the Waters' mAb Charge Variant Standard. After approximating the delay in solvent delivery, the percentages of mobile phase B at the beginning and end of the infliximab charge variant profile can be estimated to be 15 and 30%. With this information, a shortened gradient with a gradient slope of 0.17 pH unit/min can be employed to achieve the same results as the generic method but with quicker turnaround (Figure 6C). Conversely, a focused gradient at a shallower slope of 0.026 pH unit/min can be employed to improve resolution within the same analysis time of the generic method (Figure 6D). Since signal intensity decreases as gradient gets shallower, sample load can be increased accordingly to maintain sensitivity.

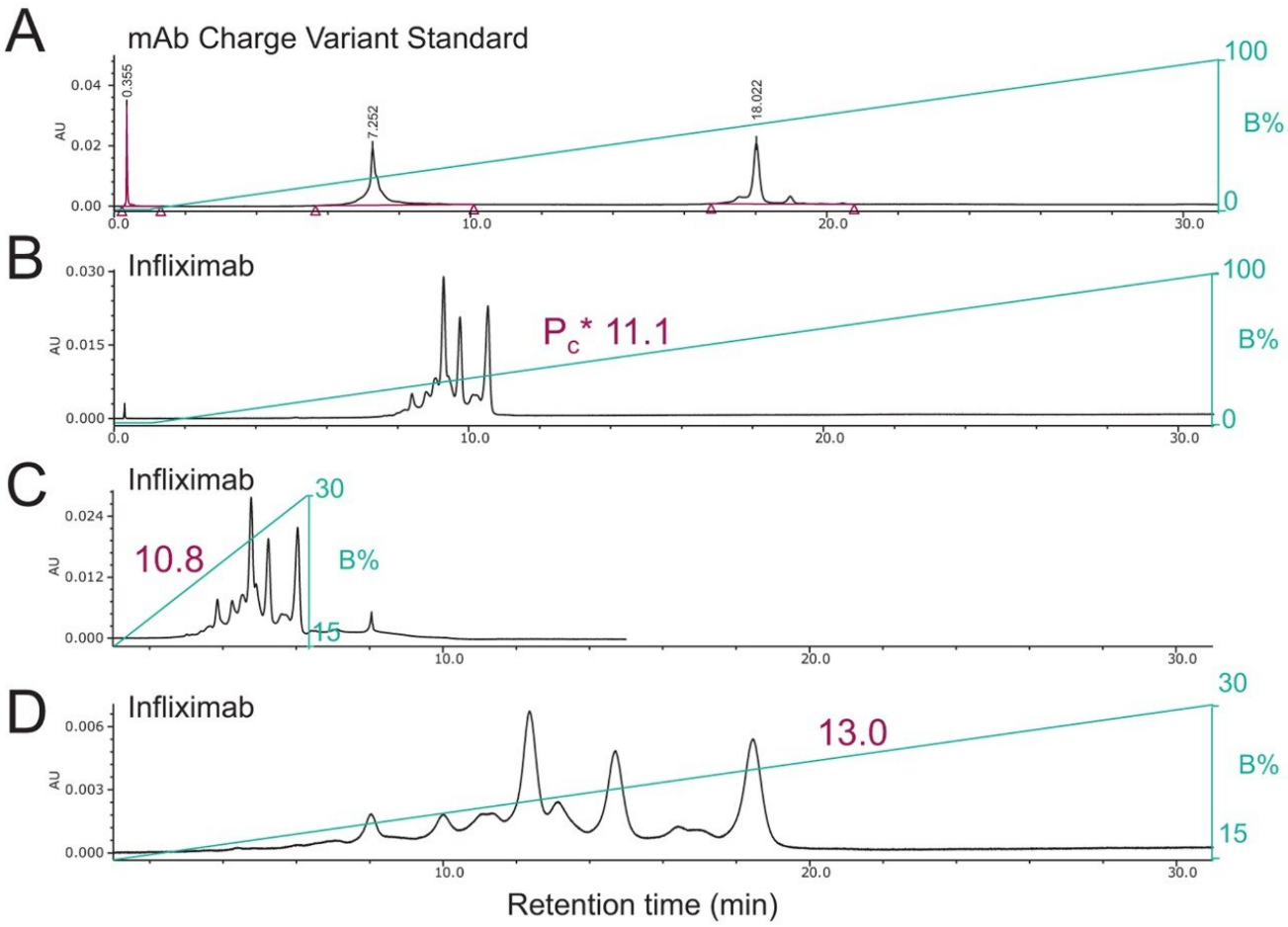


Figure 6. Method optimization to improve throughput or resolution for a particular mAb. Representative chromatograms of the Waters mAb Charge Variant Standard (A) and infliximab (B) on a BioResolve SCX mAb, 3 µm, 4.6 × 50 mm Column using BioResolve CX pH Concentrates with a generic gradient of 0–100% B at a pH gradient slope of 0.17 pH unit/min, a shortened gradient at a slope of 0.17 pH unit/min (C), or a focused gradient at a slope of 0.026 pH unit/min (D) at a flow rate of 1.00 mL/min. The chromatograms were obtained with an ACQUITY UPLC H-Class Bio System.

Conclusion

A new pH gradient mobile phase system, based on BioResolve CX pH Concentrates, has been developed to provide universally applicable cation-exchange separations of mAbs having a wide range of pI values. The composition and concentrations of buffer salts, as well as the pH range, were optimized using theory and

empirical observations to achieve robust and high resolution separation of mAbs. The performance of this method has been confirmed to be reproducible from batch-to-batch and to afford column lifetimes up to and beyond 500 injections. Moreover, it has been found that it is particularly advantageous to employ these mobile phase concentrates with BioResolve SCX mAb Columns. Method implementation and optimization has been simplified while both throughput and resolution have been improved. The BioResolve CX pH Concentrates, together with BioResolve SCX mAb Columns, provide an effective and efficient approach to mAb charge variant analysis.

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