Application Note 20946

A Fast and Robust Linear pH Gradient Separation Platform for Monoclonal Antibody (mAb) Charge Variant Analysis

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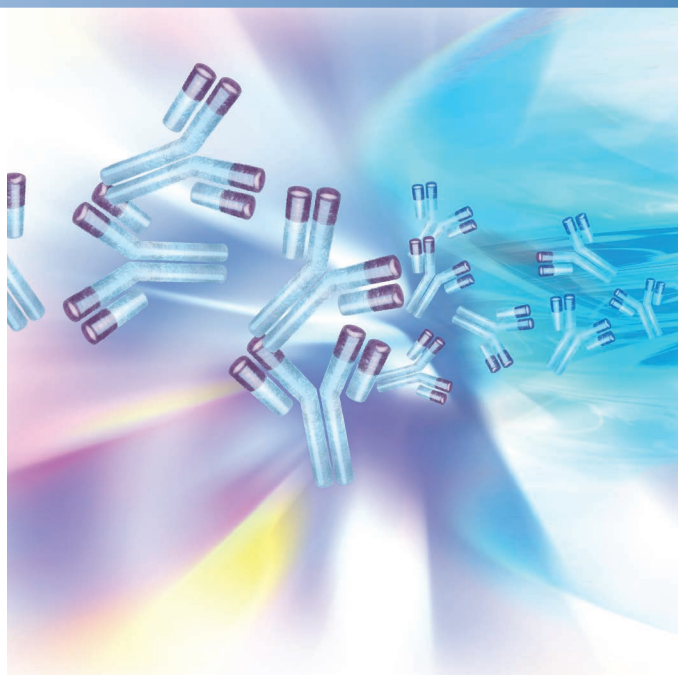
Key Words

CX-1 pH Gradient Buffer Kit, MAbPac SCX-10, mAb charge variant analysis

Abstract

This application note describes a fast, 10 min cycle time separation of mAb charge variants using a linear pH gradient separation method. A gradient from pH 5.6 to pH 10.2 was generated over time by running a linear pump gradient from 100% Thermo Scientific™ CX-1 pH Gradient Buffer A (pH 5.6) to 100% CX-1 pH Gradient Buffer B (pH 10.2). Linear UV response with up to 300 µg of protein loading was achieved. The elution pH values of mAbs are in linear relationship with their corresponding pI values.



Introduction

Recombinant monoclonal antibodies (mAbs) can be highly heterogeneous due to modifications such as sialylation, deamidation, and C-terminal lysine truncation. Salt gradient cation exchange chromatography has been used with some success in characterizing mAb charge variants; however, significant effort is often required to tailor the salt gradient method for an individual mAb. In the fast-paced drug development environment, a fast and robust platform method is desirable to accommodate the majority of the mAb analyses.

Thermo Fisher Scientific recently introduced cation-exchange pH gradient buffers that enable the fast and robust generic platform method requirements [1]. This buffer system consists of a low-pH buffer A at pH 5.6 and a high-pH buffer B at pH 10.2. A linear pH gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% buffer A to 100% buffer B.

Using mAbs with a variety of isoelectric points (pI values) from 7 to 10, we have demonstrated that the linear pH gradient method separates charge variants consistently. In addition, the loading capacity on a Thermo Scientific™ MAbPac™ SCX-10 column when running pH gradient was investigated by injecting increasing amount of protein standards. The study showed that there was a linear signal response up to 300 µg of protein loading. Furthermore, a fast separation of charge variants was achieved in a 10 min cycle time using a 2 mL/min flow rate on a MAbPac SCX-10, 5 µm, 4 × 50 mm column.

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Experimental Details

Consumables		Part Number
Standard proteins were purchased from a reputable supplier. Monoclonal antibodies were a gift from a biotechnology company.		
Columns:	MAbPac SCX-10, 10 µm, 4 × 250 mm	074625
	MAbPac SCX-10, 5 µm, 4 × 50 mm	078656
Buffers:	CX-1 pH Gradient Buffer A (pH 5.6), 125 mL	083273
	CX-1 pH Gradient Buffer B (pH 10.2), 125 mL	083275

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System equipped with SRD-3600 Membrane Degasser, DGP-3600RS Biocompatible Rapid Separation Pump, TCC-3000SD Thermostated Column Compartment with two biocompatible 10-port valves, WPS-3000TBRS Biocompatible Rapid Separation Thermostated Autosampler, VWD-3400RS UV Detector equipped with a Micro Flow Cell, and PCM-3000 pH and Conductivity Monitor.

Eluents

Eluent A and B were prepared by simply diluting the corresponding CX-1 pH Gradient Buffer 10 fold using deionized water. Proteins and mAb were dissolved in deionized water.

Gradients

The linear pH gradient was generated by running a linear pump gradient from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). For pH gradient analysis carried out on the MAbPac SCX-10, 10 µm, 4 × 250 mm columns, the gradient method in Table 1 was used. For pH gradient analysis carried out on the MAbPac SCX-10, 5 µm, 4 × 50 mm columns, the gradient method in Table 2 or Table 3 was used. All methods cover the pH range from pH 5.6 to pH 10.2.

Data Processing

Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8

Results

pH Gradient Loading Capacity

The separation of proteins with different pI values by pH gradient was demonstrated in Figure 1 using the gradient specified in Table 1. The protein mixture contained lentil lectin with three isoforms [lectin-1 (pI 7.8), lectin-2 (pI 8.0), lectin-3 (pI 8.2)], trypsinogen (pI 8.7), ribonuclease A (pI 9.3), and cytochrome C (pI 10.2). The concentrations of these proteins were 3 mg/mL, 2 mg/mL, 3 mg/mL, and 2 mg/mL, respectively. Figure 1 shows the multiple UV traces of chromatograms recorded from a series of injections of the protein mixture at 2 µL, 4 µL, 8 µL, 16 µL, 32 µL, 50 µL, and 100 µL. The peak area and peak width at half height (PWHH) were plotted against the sample loading in Figures 2a, 2b, 2c, and 2d for lentil lectin-1, trypsinogen, ribonuclease A, and cytochrome C, respectively. Trypsinogen, ribonuclease A, and cytochrome C exhibited a linear response up 100 µL of sample loading, while lentil lectin-1 showed linear response up to 50 µL of sample loading. The separation of these proteins was maintained throughout the range of 20 µg to 1,000 µg total protein loading.

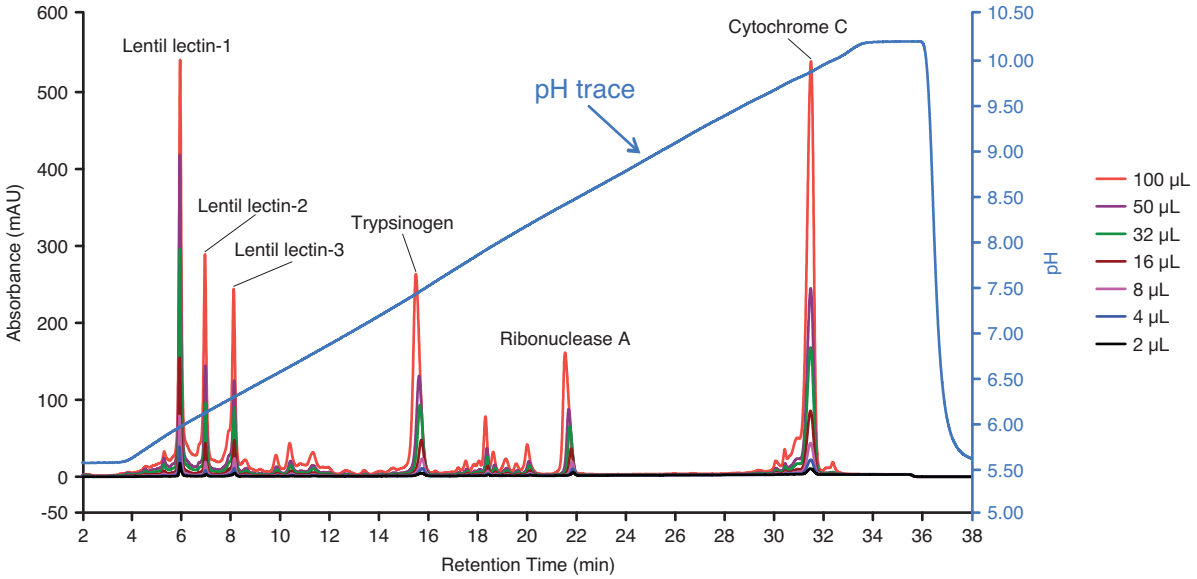

Figure 1: Chromatogram of a protein mixture separated using a 30 min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. Protein names are labelled for each peak. The protein concentrations in the sample mixture were: lentil lectin, 3 mg/mL; trypsinogen, 2 mg/mL; ribonuclease A, 3 mg/mL; cytochrome C, 2 mg/mL. Increasing amounts of samples were loaded onto the column using the following injection volumes: 2 µL (black), 4 µL (blue), 8 µL (pink), 16 µL (brown), 32 µL (green), 50 µL (purple), and 100 µL (red). The pH trace is blue.

Time (minutes)	Flow rate (mL/min)	% A	% B
0–1	1	100	0
1–31	1	100–0	0–100
31–34	1	0	100
34–40	1	100	0

Table 1: A 30 min linear gradient method used with the MAbPac SCX-10, 10 µm, 4 × 250 mm, cation exchange column. Total run time is 40 min. The linear pH range covers from pH 5.6 to pH 10.2.

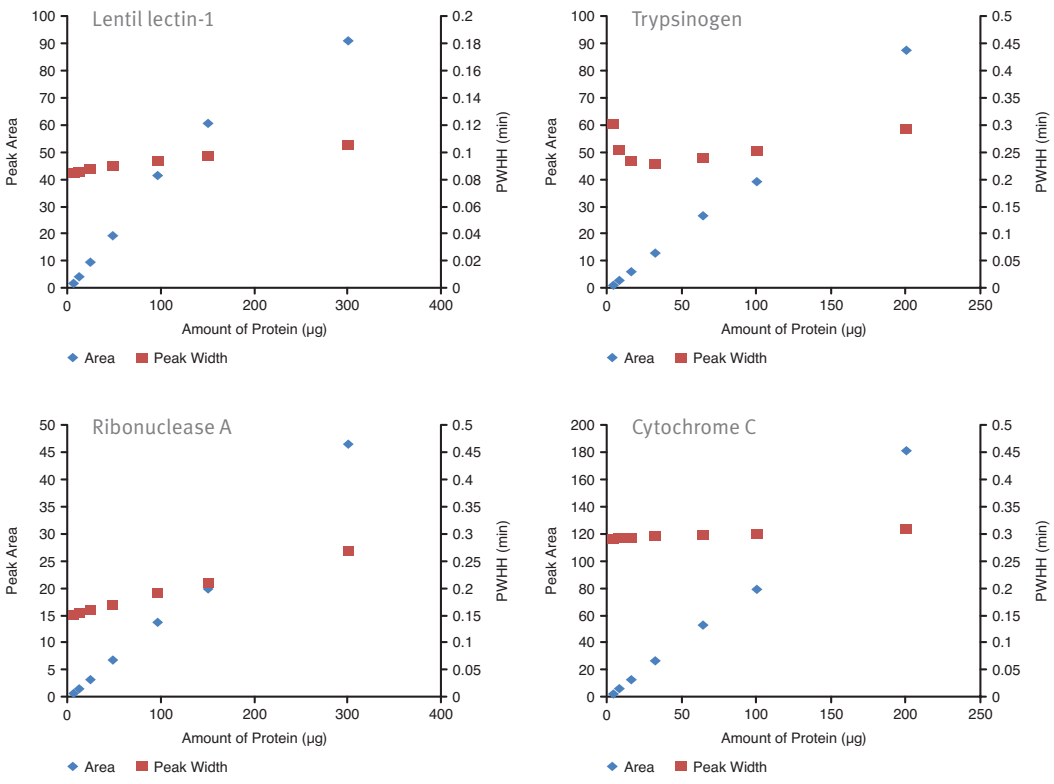

Figure 2: Plots of peak area and PWHH versus sample loading. Peak area is plotted on the primary Y-axis and the PWHH is plotted on the secondary Y-axis.

Linear Correlation of Elution pH Versus Protein pI Value

An analysis was performed to show that there is a correlation between the elution pH versus the corresponding pI values of the protein components. Figure 3 compares the measured pH values for six protein component peaks in Figure 1 as a function of the corresponding pI values. The measured pH values for the six protein component peaks exhibited a strong linear correlation to the literature based pI values, with an R^2 value of 0.9929. Since mAb molecules within the IgG1 class share most of the protein sequence, we expect to see a more linear correlation of the mAb elution pH versus their pI. Figure 4 shows pH gradient separation of charge variants from six mAbs with pI values at 7.2, 7.6, 7.8, 8.3, 9.0, and 10.0. Figure 5 is a plot of the elution pH of each mAb major variant versus the mAb pI value. The linear fit yielded an R^2 value of 0.9988. Thus, after a calibration procedure, this example supports the fact that linear regression coupled with the gradient method described here can be used to estimate the pI of a mAb based on the peak retention time and elution pH.

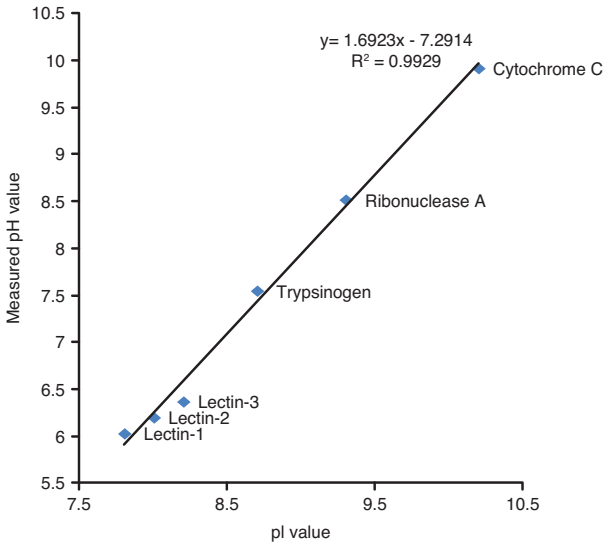

Figure 3: A graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 1.

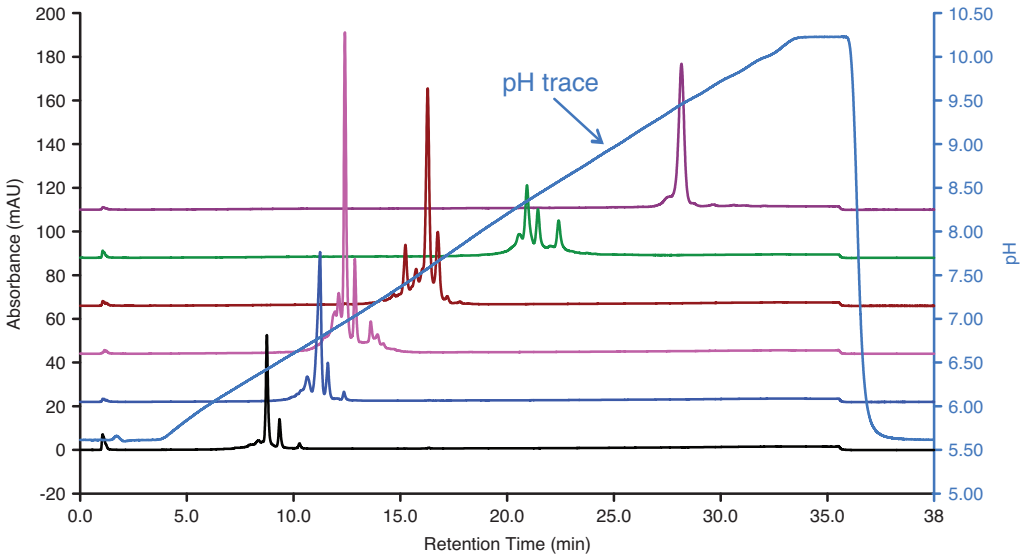

Figure 4: Chromatogram of six mAbs separated using a 30 min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. The UV traces are correspond to the mAbs with the following pI: 7.2 (black), 7.6 (blue), 7.8 (pink), 8.3 (brown), 9.0 (green), and 10.0 (purple). The pH trace is blue.

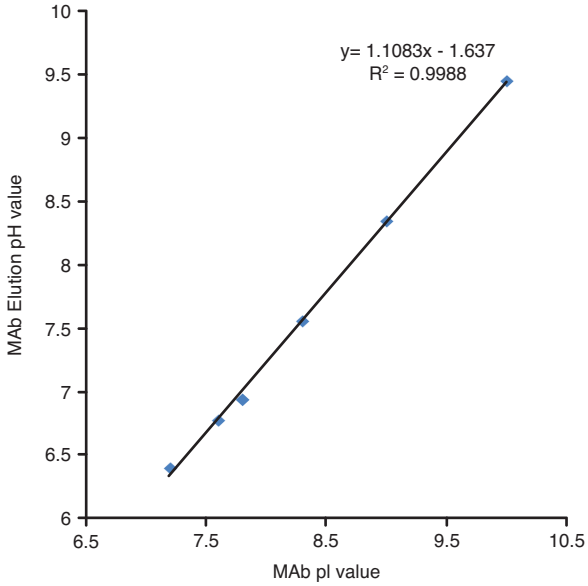

Figure 5: A graph plotting the elution pH values for six mAbs as a function of the corresponding pI values. The measured pH values of the major variant from all six mAbs were exported from the same experiment shown in Figure 4.

Fast pH Gradient Separation

The pH gradient method is fast and rugged. Figure 6 shows pH gradient runs on a MAbPac SCX-10, 5 µm, 4 × 50 mm column. With the gradient specified in Table 2 and a 1 mL/min flow rate, mAb variants separation was achieved within 15 min with a total run time of 20 min (Figure 6). Using the gradient specified in Table 3 and a 2 mL/min flow rate, mAb variants separation was achieved within 7.5 min with a total run time of 10 min (Figure 7). In both cases, the linearity of the pH gradient was maintained. Once the elution pH of the unknown mAb was determined in the scouting run, a bespoke method was set up with increasing % of eluent A as starting point and decreasing % of eluent B as ending point.

Time (minutes)	Flow rate (mL/min)	% A	% B
0–1	1	100	0
1–16	1	100–0	0–100
16–17	1	0	100
17–20	1	100	0

Table 2: A 15 min linear gradient method used with the MAbPac SCX-10, 5 µm, 4 × 50 mm, cation exchange column. Total run time is 20 min. The linear pH range covers from pH 5.6 to pH 10.2.

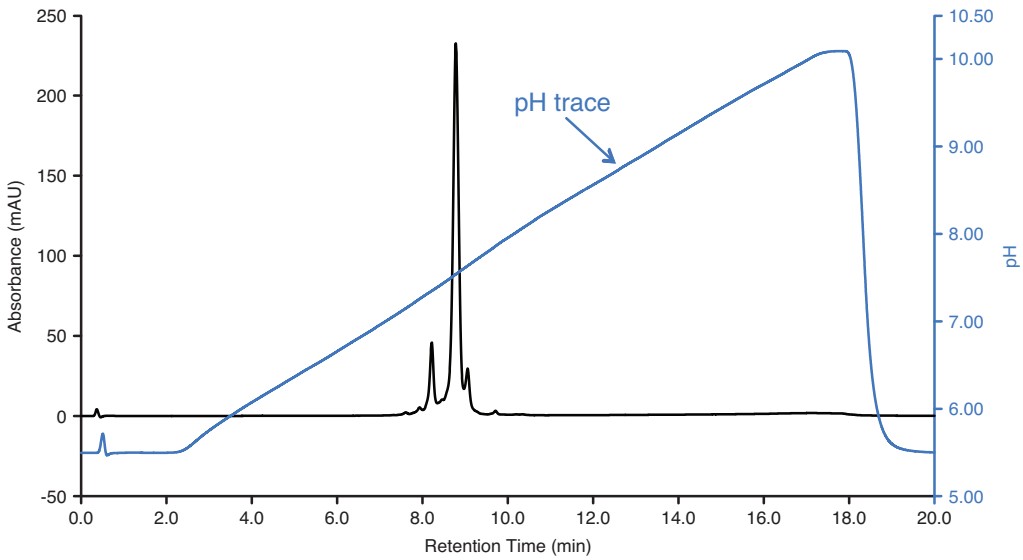

Figure 6: Fast mAb variant separation using pH gradient on a MAbPac SCX-10, 5 µm, 4 × 50 mm column with a 15 min gradient method. The mAb sample concentration was 5 mg/mL.

Time (minutes)	Flow rate (mL/min)	% A	% B
0–0.5	2	100	0
0.5–8	2	100–0	0–100
8–8.5	2	0	100
8.5–10	2	100	0

Table 3. A 7.5 min linear gradient method used with the MAbPac SCX-10, 5 µm, 4 × 50 mm, cation exchange column. Total run time is 10 min. The linear pH range covers from pH 5.6 to pH 10.2.

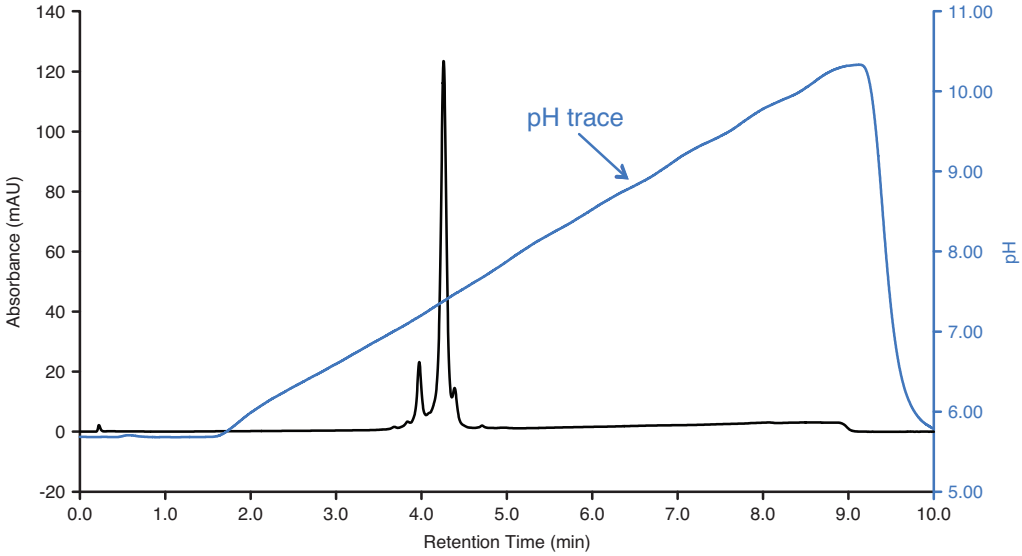

Figure 7: Fast mAb variant separation using pH gradient on a MAbPac SCX-10, 5 µm, 4 × 50 mm column with a 7.5 min gradient method. The mAb sample concentration was 5 mg/mL.

Conclusion

Using the pH gradient method:

- Linear signal response was achieved with up to 300 µg protein loading, such as Ribonuclease A.
- Elution pH values of mAbs exhibited a linear relationship with their corresponding pI values.
- Fast separation of mAb charge variants was achieved within a 10 min cycle time.

Reference

[1] Lin, S.; Baek, J.; Decrop, W.; Rao, S.; Agroskin, Y. and Pohl, C. Development of a Cation-Exchange pH Gradient Separation Platform. Presented at 39th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Amsterdam, The Netherlands, June 16-20, 2013.

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