Application Note 21092

High-Resolution Charge Variant Analysis for Top-Selling Monoclonal Antibody Therapeutics Using a Linear pH Gradient Separation Platform

Shanhua Lin and Chris Pohl, Thermo Fisher Scientific, Sunnyvale, CA, USA

Key Words

Rituxan, rituximab, Herceptin, trastuzumab, Humira, adalimumab, Avastin, bevacizumab, CX-1 pH Gradient Buffer Kit, MAbPac SCX-10, mAb charge variant analysis

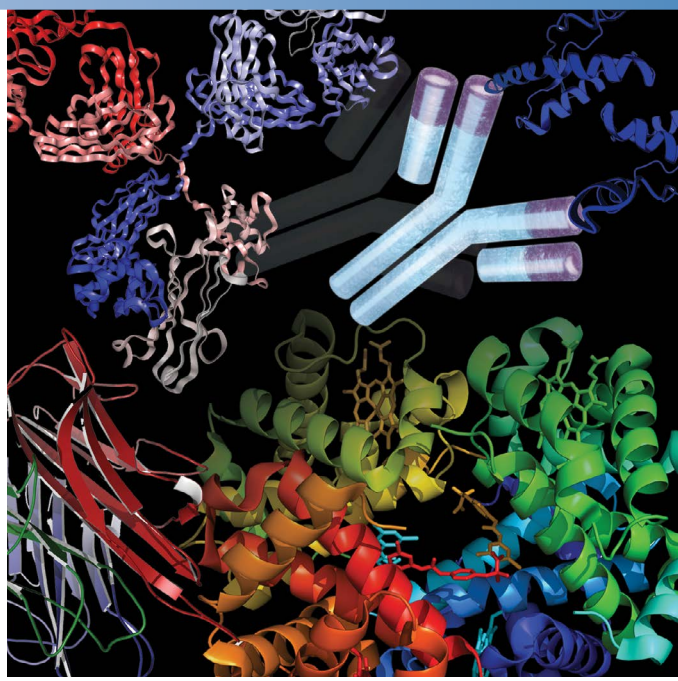
Goal

To develop a high-resolution charge variant analysis for Rituxan® (rituximab), Herceptin® (trastuzumab), Humira® (adalimumab), and Avastin® (bevacizumab) using a linear pH gradient separation method.

Introduction

Monoclonal antibody (mAb) therapeutics is a quickly growing market. In 2013, of the top ten bestselling pharmaceutical products, five were monoclonal antibodies (Humira, Remicade, Rituxan, Herceptin, and Avastin). Humira sales revenue alone exceeded $11 billion.[1] With the patent protection for these blockbuster drugs expiring in the next few years, many companies are entering the development of biogenerics, also known as the biosimilars. In order to demonstrate the safety and efficacy of the biosimilars and gain approval of the regulatory agency, it is essential to detect, characterize, and quantify impurities as well as structural variants of these biosimilars. Some structural variants, such as charge variants, have been demonstrated as critical quality attributes (CQAs).

Charge variants of mAbs are due to modifications such as sialylation, deamidation, and C-terminal lysine truncation. Traditionally, salt gradient cation-exchange chromatography has been used with some success in characterizing mAb charge variants.[2] However, significant effort is often required to tailor the salt gradient method for each individual mAb. In the fast-paced drug development environment, a quick 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 meet these platform method requirements.[3] The 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.



In this study, the charge variants of Rituxan (rituximab), Herceptin (trastuzumab), Humira (adalimumab), and Avastin (bevacizumab) are analyzed on a Thermo Scientific™ MAbPac™ SCX-10 column with a linear pH gradient separation method. The linear gradient from pH 5.6 to pH 10.2 is 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). The results demonstrate the general applicability of the pH gradient method on monoclonal antibody charge variant analysis. The data also show that the pH gradient method delivers higher-resolution power than the traditional salt method. The methods described here can be widely used in the development of the biosimilars of these top-selling mAbs.

Thermo SCIENTIFIC

Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- MES hydrate [≥99.5%]
- Sodium chloride [NaCl, ≥99.5%]
- Rituxan, Herceptin, Humira, and Avastin were a gift from a biotechnology company.

Sample Handling Equipment

Polypropylene, 0.3 mL vials (P/N 055428)

Columns and Buffers

- MAbPac SCX-10, 10 µm, 4 × 250 mm (P/N 074625)
- CX-1 pH Gradient Buffer A (pH 5.6), 125 mL (P/N 083273)
- CX-1 pH Gradient Buffer B (pH 10.2), 125 mL (P/N 083275)

LC Separation

The LC separation conditions were as follows:

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:
	SRD-3400 Solvent Racks with degasser (P/N 5035.9245)
	HPG-3400RS Biocompatible Binary Rapid Separation Pump (P/N 5040.0046)
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a semi-micro flow cell PEEK, 2.5 µL volume, 7 mm path length, PCM-3000 pH and Conductivity Monitor (P/N 6082.2005)
pH gradient mobile phase A	1X CX-1 pH Gradient Buffer A Dilute CX-1 pH Gradient Buffer A 10-fold using deionized water.
pH gradient mobile phase B	1X CX-1 pH Gradient Buffer B Dilute CX-1 pH Gradient Buffer B 10-fold using deionized water.
Salt gradient mobile phase A	20 mM MES (pH 5.6) + 60 mM NaCl Add 3.9 g of MES (Sigma) to 900 mL of deionized water. Adjust the pH with NaOH to 5.6 and add 17.53 g of NaCl, adjust volume to 1 L and filter through a 0.22 µm filter before use.
Salt gradient mobile phase B	20 mM MES (pH 5.6) + 300 mM NaCl Add 3.9 g of MES (Sigma) to 900 mL of deionized water. Adjust the pH with NaOH to 5.6 and add 58.44 g of NaCl, adjust volume to 1 L and filter through a 0.22 µm filter before use.

Full pH gradient pH 5.6 to pH 10.2

Time (min)	%A	%B
0.0	100	0
1.0	100	0
31.0	0	100
34.0	0	100
34.1	100	0
45.0	100	0

Half pH gradient pH 5.6 to pH 7.9

Time (min)	%A	%B
0.0	100	0
1.0	100	0
31.0	0	50
34.0	0	50
34.1	100	0
45.0	100	0

Full salt gradient 60 mM NaCl to 300 mM NaCl

Time (min)	%A	%B
0.0	100	0
1.0	100	0
31.0	0	100
34.0	0	100
34.1	100	0
45.0	100	0

Half salt gradient 60 mM NaCl to 180 mM NaCl

Time (min)	%A	%B
0.0	100	0
1.0	100	0
31.0	0	50
34.0	0	50
34.1	100	0
45.0	100	0

Flow rate	1 mL/min
Run time	40 min
Temperature	30 °C
UV detector wavelength	280 nm
Injection volume	5 µL
Samples	Rituximab, 5 mg/mL; Trastuzumab, 5 mg/mL; Adalimumab, 5 mg/mL; Bevacizumab, 1 mg/mL

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

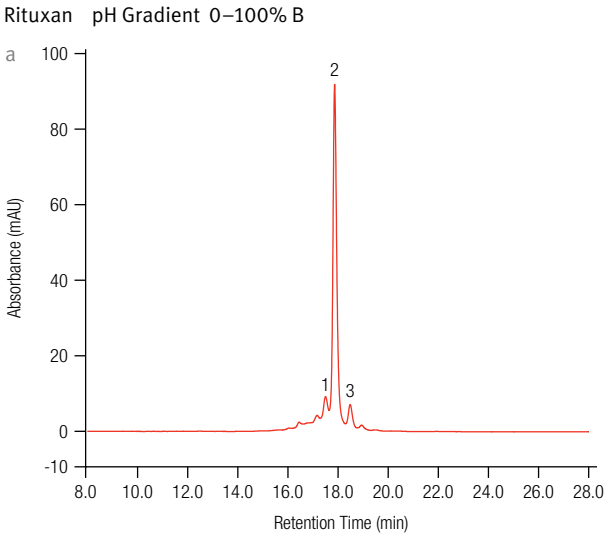
The CX-1 pH gradient buffer kit generates a linear pH gradient when a linear pump gradient is run from 100% CX-1 buffer A to 100% buffer B. This pH gradient method serves as a platform method for the mAb charge variant analysis, covering the pH range from 5.6 to 10.2. Most of the therapeutic mAbs have pI values falling within this pH range. Rituxiamab (Figure 1a), trastuzumab (Figure 3a), adalimumab (Figure 5a), and bevacizumab (Figure 7a) were analyzed on a MAbPac SCX-10 column using the full pH gradient method. Satisfactory separations of multiple variants were observed with all four samples. After the initial survey runs of the full pH gradient, the subsequent runs were aimed at improving resolution by decreasing the pH range and gradient slope. The fact that the pH gradient was linear made the method optimization simple. Rituximab (Figure 1b), trastuzumab (Figure3b), adalimumab (Figure 5b), and bevacizumab (Figure 7b) were analyzed using a shallower pH gradient with half the pH range. The variants were identified as peaks 1, 2, 3 in the chromatograms. Improved separations of the variants were observed in all cases.

Traditionally, the salt gradient method has been used for mAb charge variants analysis. The salt gradient method development usually requires screening at different pH values using different buffers. In addition, the minimum salt concentration required to elute the mAb off the cation exchange column must be individually determined. For comparison and speed, the same initial conditions and buffers (20 mM MES and 60 mM NaCl at pH 5.6) were used for all the samples in this study. Rituxiamab (Figures 2a and 2b), trastuzumab (Figures 4a and 4b), adalimumab (Figures 6a and 6b), and bevacizumab (Figures 8a and 8b) were each analyzed by two salt gradient methods: one with steeper gradient slope and the other one with shallower gradient slope.

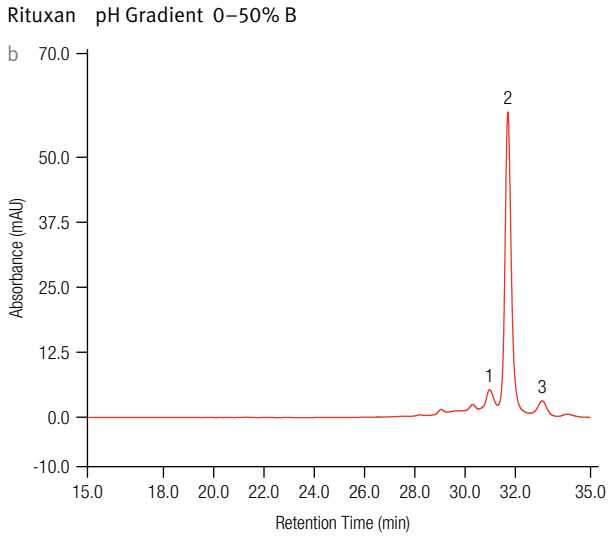
The separation profiles obtained by the pH and salt gradient methods were similar for the same molecule. In order to simplify the comparison, the acidic variant adjacent to the major variant was labeled as peak 1, the major variant was labeled as peak 2, and the basic variant adjacent to the major variant was labeled as peak 3 for each chromatogram (Figures 1–8). In the case of the trastuzumab salt gradient chromatogram, the minor acidic variant was very close to the major peak and could be not be determined, but this was resolved by the pH gradient. Table 1 lists the retention time of peak 1 (RT1), peak 2 (RT2), and peak 3 (RT3) and the difference between RT1 and RT2 (ΔRT1-2), as well as RT2 and RT3 (ΔRT2-3). In the case of rituximab, trastuzumab, and bevacizumab, it is clear that the ΔRTs between variants were greater when using the pH gradient profile. In the case of adalimumab, the ΔRTs were similar between the pH gradient profile and the salt gradient profile.

Table 1. Retention time of mAb charge variants analyzed by linear pH gradient and salt gradient methods.

	Method	Gradient	RT1 (min)	RT2 (min)	RT3 (min)	ΔRT2 (min)	ΔRT3 (min)
Rituxan/rituximab	pH	full	17.497	17.86	18.477	0.363	0.617
		half	30.98	31.71	33.077	0.73	1.367
	salt	full	12.927	13.11	13.504	0.183	0.394
		half	22.44	22.783	23.567	0.343	0.784
Herceptin/trastuzumab	pH	full	15.257	15.744	16.207	0.487	0.463
		half	27.404	27.914	28.818	0.51	0.904
	salt	full	n.a.	12.204	12.477	n.a.	0.273
		half	n.a.	21.494	21.844	n.a.	0.35
Humira/adalimumab	pH	full	16.063	16.26	16.593	0.197	0.333
		half	28.834	29.244	29.907	0.41	0.663
	salt	full	15.354	15.56	15.9	0.206	0.34
		half	26.877	27.267	27.947	0.39	0.68
Avastin/bevacizumab	pH	full	11.537	11.824	12.264	0.287	0.44
		half	19.977	20.553	21.433	0.576	0.88
	salt	full	13.494	13.724	14.11	0.23	0.386
		half	23.36	23.817	24.587	0.457	0.77

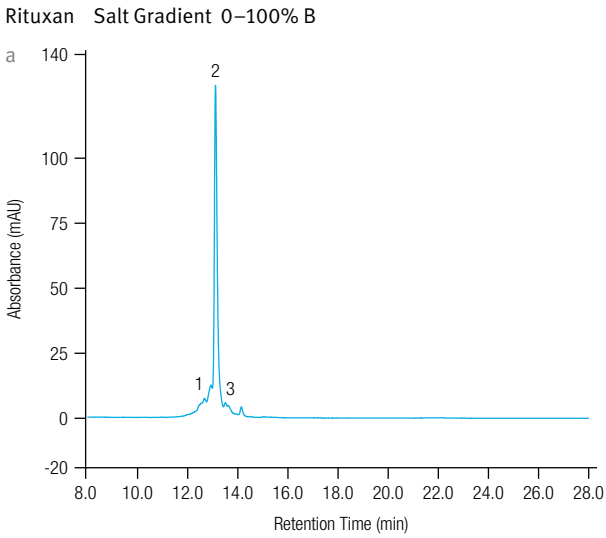


Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Rituxan/rituximab (5 mg/mL)

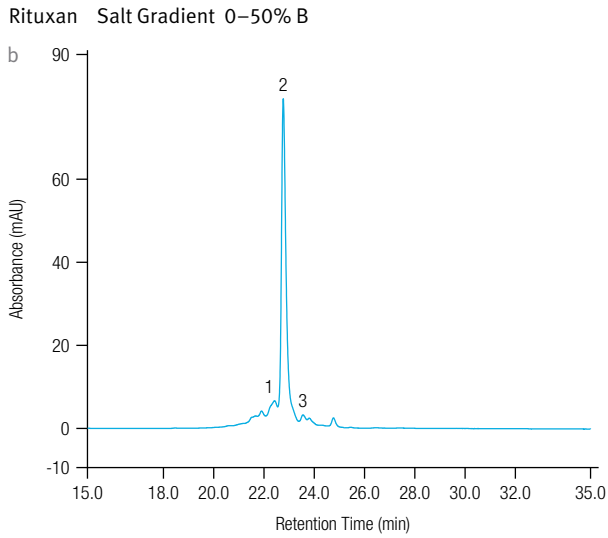


Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Rituxan/rituximab (5 mg/mL)

Figure 1. Rituxan/rituximab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.



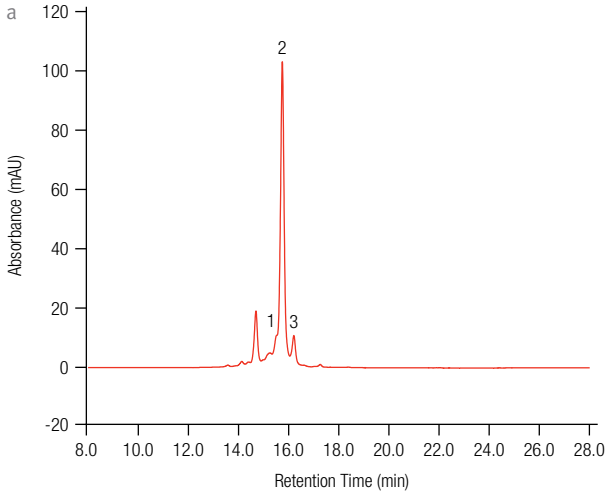
Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Rituxan/rituximab (5 mg/mL)



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Rituxan/rituximab (5 mg/mL)

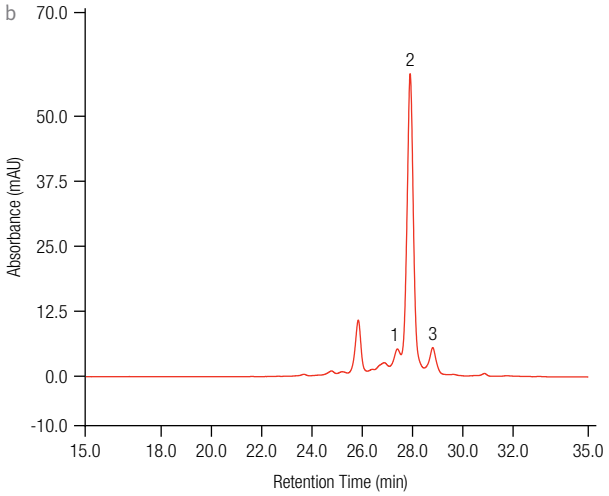
Figure 2. Rituxan/rituximab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.

Herceptin pH Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Herceptin/trastuzumab (5 mg/mL)

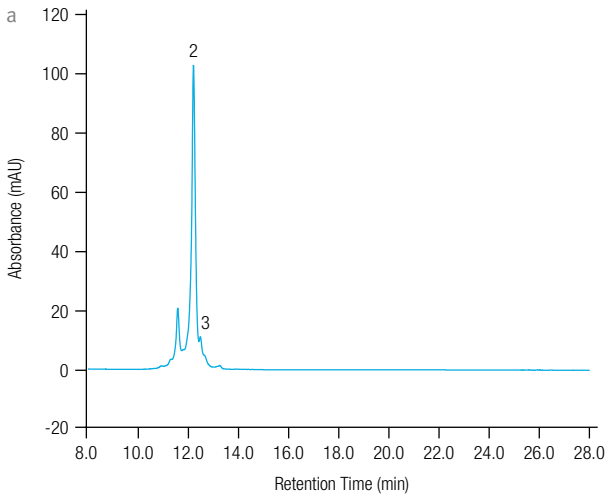
Herceptin pH Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Herceptin/trastuzumab (5 mg/mL)

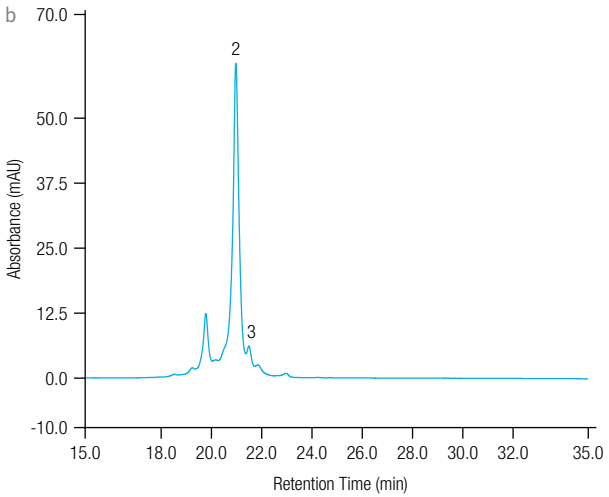
Figure 3. Herceptin/trastuzumab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.

Herceptin Salt Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Herceptin/trastuzumab (5 mg/mL)

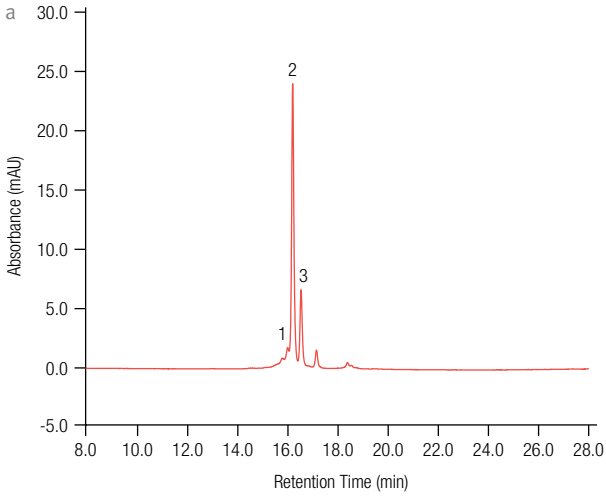
Herceptin Salt Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Herceptin/trastuzumab (5 mg/mL)

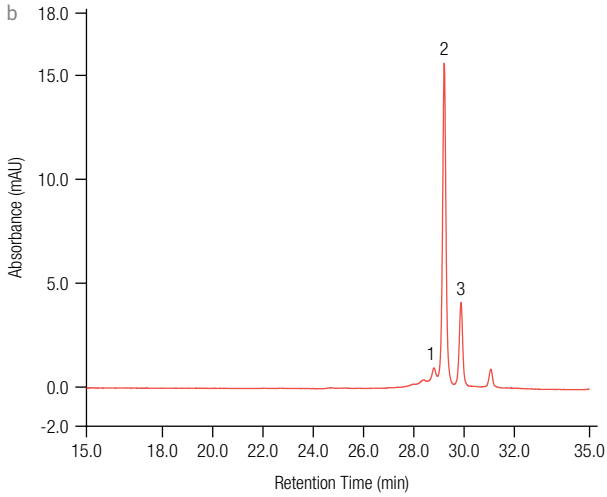
Figure 4. Herceptin/trastuzumab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.

Humira pH Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Humira/adalimumab (5 mg/mL)

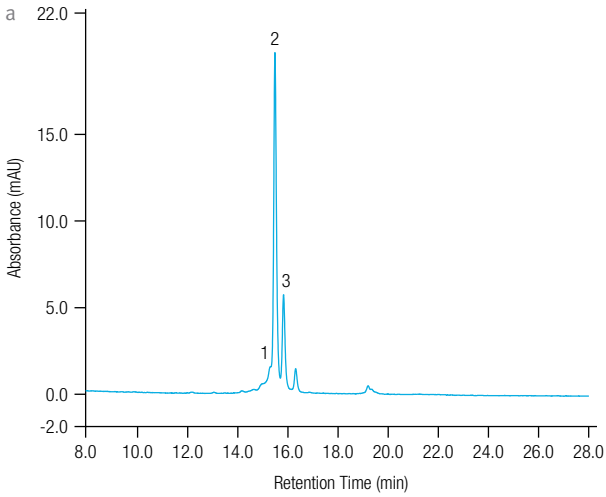
Humira pH Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Humira/adalimumab (5 mg/mL)

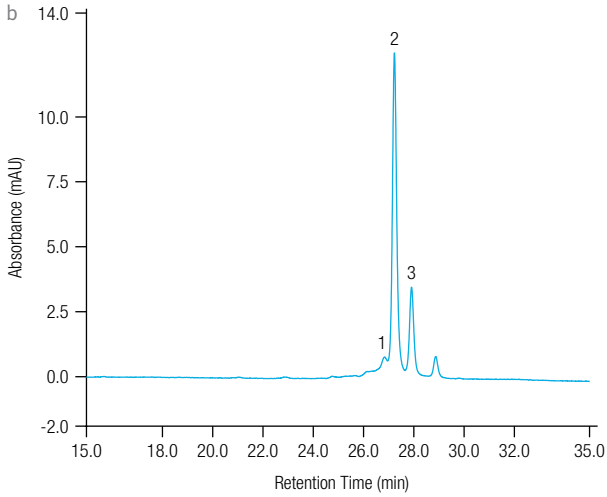
Figure 5. Himira/adalimumab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.

Humira Salt Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Humira/adalimumab (5 mg/mL)

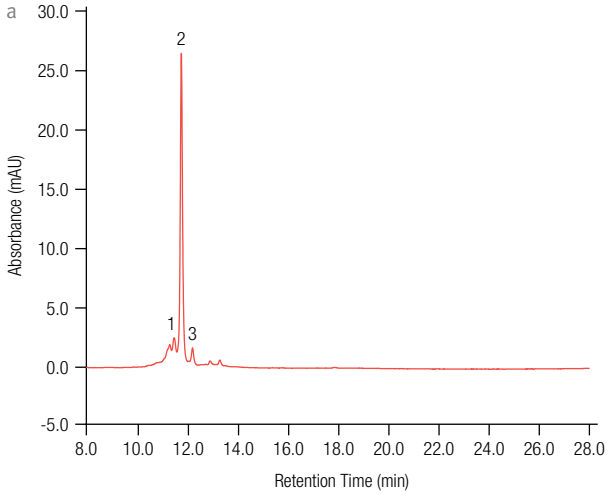
Humira Salt Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Humira/adalimumab (5 mg/mL)

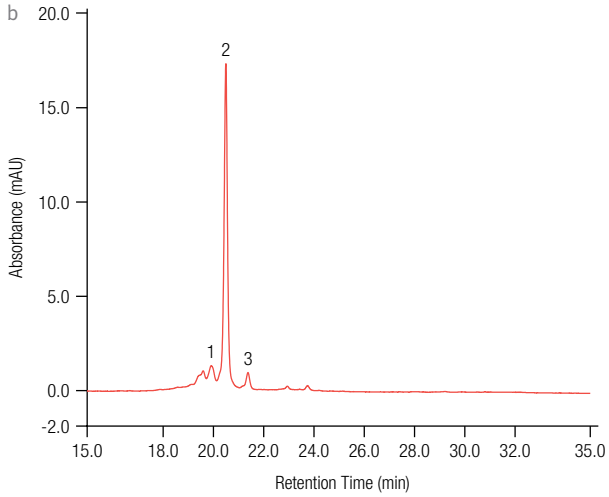
Figure 6. Himira/adalimumab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.

Avastin pH Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Avastin/bevacizumab (1 mg/mL)

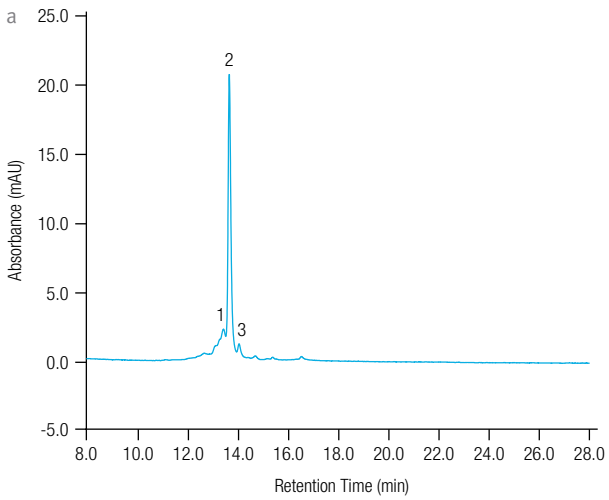
Avastin pH Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	1X CX-1 pH gradient buffer A, pH 5.6
Mobile Phase B:	1X CX-1 pH gradient buffer B, pH 10.2
pH Gradient:	0% B to 50% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Avastin/bevacizumab (1 mg/mL)

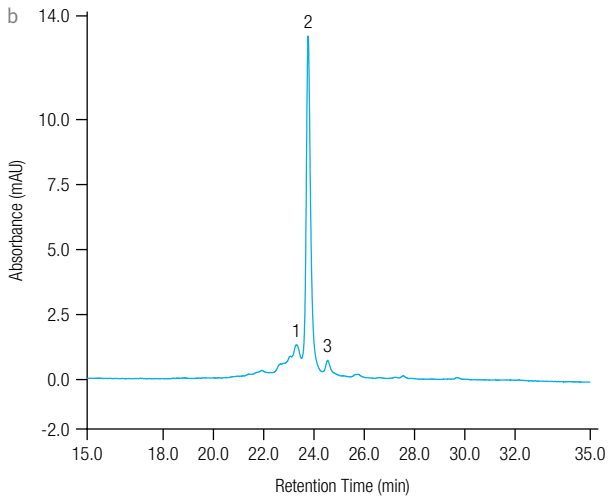
Figure 7. Avastin/bevacizumab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.

Avastin Salt Gradient 0–100% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 100% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Avastin/bevacizumab(1 mg/mL)

Avastin Salt Gradient 0–50% B



Column:	**MAbPac SCX-10**, 10 µm
Format:	4 × 250 mm
Mobile Phase A:	20 mM MES (pH 5.6) + 60 mM NaCl
Mobile Phase B:	20 mM MES (pH 5.6) + 300 mM NaCl
Salt Gradient:	0% B to 0% B from 1 to 31 min
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Temp.:	30 °C
Detection:	UV (280 nm)
Sample:	Avastin/bevacizumab (1 mg/mL)

Figure 8. Avastin/bevacizumab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.

Conclusion

The linear pH gradient method is a platform method for mAb charge variant analysis. It can be easily optimized to improve separation and delivers better charge variant separation than the salt gradient method.

References

1 http://www.pmlive.com/top_pharma_list/Top_50_pharmaceutical_products_by_global_sales

2 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.

3 Vlasak J. and Ionescu R. Heterogeneity of Monoclonal Antibodies Revealed by Charge-Sensitive *Methods, Current Pharmaceutical Biotechnology*, **2008**, *9*, 468-481.

For Research Use Only

thermoscientific.com/bioLC

USA and Canada +1 800 332 3331
Australia 1300 735 292 (free call domestic)
China 800 810 5118 (free call domestic)
400 650 5118
France +33 (0)1 60 92 48 34
Germany +49 (0) 2423 9431 20 or 21
India +91 22 6742 9494
+91 27 1766 2352
Japan 0120 753 670 (free call domestic)
0120 753 671 (fax)
Korea +82 2 3420 8600
United Kingdom +44 (0) 1928 534 110
New Zealand 0800 933 966 (free call domestic)
Singapore +65 6289 1190
All Other Enquiries +44 (0) 1928 534 050

Technical Support

For advice and support, please visit our website: www.thermoscientific.com/chromexpert

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

AN21092-EN0616S