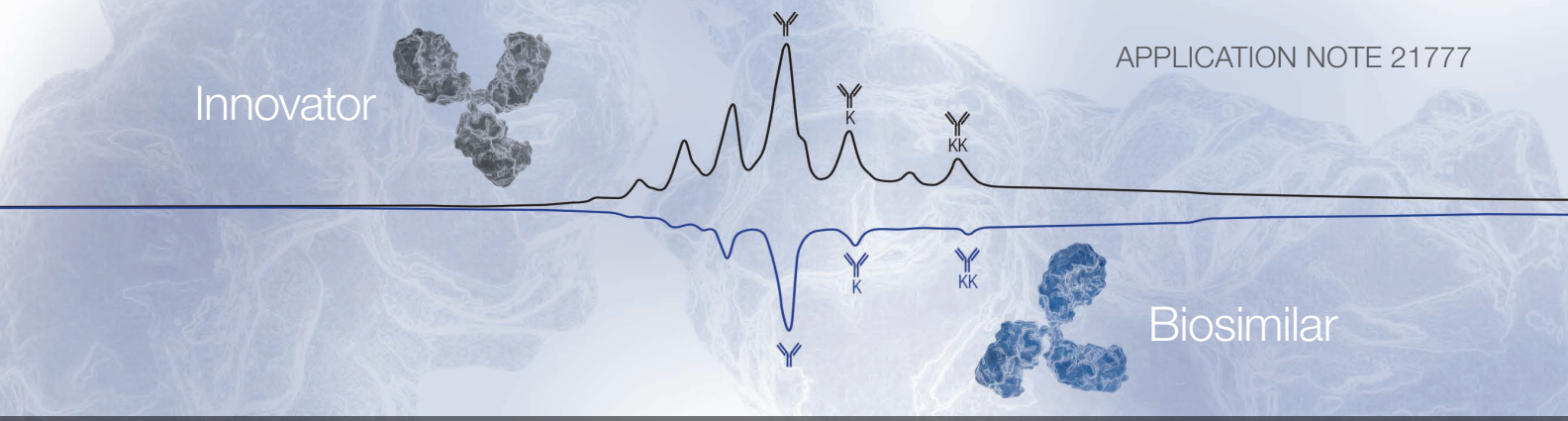
thermo scientific

APPLICATION NOTE 21777



Simple charge variant profile comparison of an innovator monoclonal antibody and a biosimilar candidate

Authors

Silvia Millán, Anne Trappe, Amy Farrell, and Jonathan Bones
Characterization and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland.

Keywords

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

Application benefits

- Confident charge variant evaluation of biosimilars obtained in a single analysis
- Quick assay optimization: no need for mobile phase evaluation
- Reduced risk of method variability

Goal

To demonstrate the effectiveness of a simple pH gradient/ion-exchange chromatography workflow approach to the characterization of the different charge variants profiles of an innovator molecule (cetuximab) and a candidate biosimilar. To show the assay is simple, reproducible, easily optimized and resolves variants effectively.

Introduction

Therapeutic proteins are large, heterogeneous molecules that are subject to a variety of enzymatic and chemical modifications during their expression, purification, and long-term storage. These changes include several possible modifications, such as oxidation, deamidation, glycosylation, aggregation, misfolding, or adsorption, leading to a potential loss of therapeutic efficacy or unwanted immune reactions.

nibrt National Institute for Bioprocessing Research and Training

ThermoFisher SCIENTIFIC

Biosimilars are therapeutic proteins that are similar to originator protein therapeutics but are obtained using a different bioprocess. The two products can vary due to the cell line in which the monoclonal antibody is expressed, from small changes in the purification process or from a different composition of the final formulation. As a growing number of biosimilars have been introduced to the market, robust and reliable analytical techniques to confidently evaluate similarities and differences between the biosimilar and its originator are required. For biosimilar approval, regulatory bodies require detailed therapeutic protein characterization including lot-to-lot and batch-to-batch comparisons, stability studies, impurity profiling, glycoprofiling, determination of related proteins and excipients, as well as determination of protein aggregates.[1]

Charge heterogeneity analysis is critical for monoclonal antibody (mAb) characterization as it provides valuable information regarding product quality and stability. Heterogeneity is complex and can be caused by such molecular adaptions as C-terminal lysine modification,[2] deamidation, and other post translational modifications (PTMs).

Protein charge depends on the number and type of ionizable amino acids present. Lysine, arginine, and histidine residues are basic, whereas glutamic acid and aspartic acid residues are acidic. Each ionizable group has its own pKa; therefore, the number and type of ionizable amino acid groups dictates the overall number of charges on a particular protein at a given pH. Considering the structure in its entirety, each protein has a pI value corresponding to the pH value where its surface has no net charge.

The number of possible charge variants increases with the molecular weight of the protein and changes in charge may be additive or subtractive, depending on any PTMs. Numerous variants are commonly observed when mAbs are analyzed by charged-based separation techniques. These variants are generally referred to as “acidic” or “basic’ species when compared with the main protein isoform.

Ion-exchange chromatography (IEX) is a widely used, powerful reference technique for the characterization of therapeutic proteins.[3,4,5] It is applied to both the qualitative and quantitative evaluation of charge heterogeneity. IEX separates charge variants by differential interactions on a charged support. Cation-exchange chromatography (CEX) is considered the gold standard, but method development is often complex as parameters such as column type and mobile phase composition (pH and salt concentration) are often required to be optimized for each individual protein analyzed.[6]

CEX chromatographic separation may be performed using either a salt- or pH-based gradient. While both approaches may be optimized to provide good peak resolution, use of a pH gradient is advantageous due to the simplicity of using commercially available mobile phases, which allows easy generation of a linear pH gradient. Indeed, buffers used for salt gradient elution are usually difficult to prepare in a reproducible way. Salt gradients can cause corrosion to metal HPLC systems and care must be taken to avoid stainless steel or regularly passivate the HPLC system to avoid this.

With good buffer choice to develop a linear pH change across the HPLC gradient, the use of a pH gradient facilitates more reproducible mobile phase preparation resulting in consistent method selectivity and greater retention time precision when compared to salt gradient buffers.[7] In addition, a pH gradient is readily optimized and can be applied more universally for mAb variant analysis of diverse proteins. Nevertheless, for an optimum separation of monoclonal antibodies acid and basic variants, the possibility to obtain a steady pH change across the gradient is important to improve selectivity and resolution.

Monoclonal antibodies typically possess pI values between 6 and 10. When the pH is at the pI of the protein, there will be no ionic interaction between the protein and the IEX stationary phase; when using a pH gradient there will be no interaction between the protein and the stationary phase so the protein will elute from the column. This requires the starting pH of a mobile phase to be below the pI of the lowest pI proteoform, while the final pH has to be somewhat higher than the pI of the highest pI proteoform. Considering the behavior of charge variants in cation-exchange chromatography, acidic variants elute before the main peak and basic variants after the main peak.

This application note presents a CEX approach to comparing biomolecule charge variant profiles using the Thermo Scientific™ pH Gradient Buffer platform in

combination with Thermo Scientific™ MAbPac™ SCX-10 column coupled to a Thermo Scientific™ Vanquish™ UHPLC system. This provides a solution that is simple, allows fast assay development, and reduces the risk of method variability.

The pH buffer platform 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, delivering robust, reproducible gradients applicable to a wide range of mAbs. It is ready to use with existing LC columns and systems, without the need to formulate complex mobile phases. For method optimization, the separation can be simply modified by use of different mobile phase gradients.

The MAbPac SCX-10 LC column works in conjunction with the pH buffer platform to allow high-resolution, high-efficiency analysis of mAbs and their associated variants. The unique nonporous pellicular resin provides the resolving power to separate mAb variants that differ by as little as one charged residue. A hydrophilic layer surrounds the polymeric beads and eliminates hydrophobic interactions with the resin, resulting in very efficient peaks. A proprietary grafted cation-exchange surface provides pH selectivity control for high-resolution separations.

The Thermo Scientific™ Vanquish™ Flex Quaternary system is a biocompatible UHPLC system that delivers new benchmarks in accuracy, precision, and sensitivity with a state-of-the-art quaternary low pressure mixing pump. Thermo Scientific™ Viper™ Fingertight Fitting connections throughout the system make setup easy.

For this application, the cetuximab innovator product, a chimeric monoclonal IgG1 antibody produced in a mammalian cell line (Sp2/0) by DNA technology, has been chosen to compare with a biosimilar candidate produced in CHO cell line (Thermo Scientific™ ExpiCHO™ Expression System), as model analytes with the required charge variant complexity to highlight their different profiles.

A comparison of the charge variant profile of the candidate biosimilar mAb and the commercially available chimeric IgG1 innovator product was made using a MAbPac SCX-10 HPLC column with a pH gradient on a Vanquish Flex Quaternary UHPLC equipped with a UV detector.

Experimental

Chemical and reagents

- Deionized (DI) water, 18.2 MΩ·cm resistivity
- Thermo Scientific™ CX-1 pH Gradient Buffer A, pH 5.6 (P/N 085346)
- Thermo Scientific CX-1 pH Gradient Buffer B, pH 10.2 (P/N 085348)
- Thermo Scientific™ Chromacol™ vials (1.2-UHRSV)
- Thermo Scientific Chromacol caps and septa (9-SCX(B)-ST1X)
- Carboxypeptidase B (150 units/mg; Roche Diagnostics™ P/N 10103233001)

Buffer preparation

- Pump Eluent A: dilute Thermo Scientific CX-1 buffer A pH 5.6 ten times with DI water.
- Pump Eluent B: dilute Thermo Scientific CX-1 buffer B pH 10.2 ten times with DI water.

Sample preparation

Samples were diluted to 5 mg/mL using deionized water. To verify C-terminal lysine content, samples were digested with carboxypeptidase B by incubation at 37 °C for 2 hours at 500 rpm.

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
Autosampler temperature	10 °C
Injection volume:	10 µL
DAD detector settings:	280 nm
pH gradient:	Table 1

Table 1. pH gradient conditions

Time (min)	% Eluent B	
	Gradient A	Gradient B
0.0	0	10
30.0	100	40
30.1	0	10
40.0	0	10

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.4

Results and discussion

Figures 1 and 2 demonstrate cetuximab innovator product and biosimilar candidate separations, respectively. Figures 1a and 2a show the separation on MAbPac SCX-10 HPLC column the using gradient A, over the total pH range of 5.6 to 10.2. Figures 1b and 2b show a smaller pH range using a shallow gradient. While both separations provided fast separations of charge variants, as expected, higher resolution was achieved with the shallower gradient allowing better identification of variants.

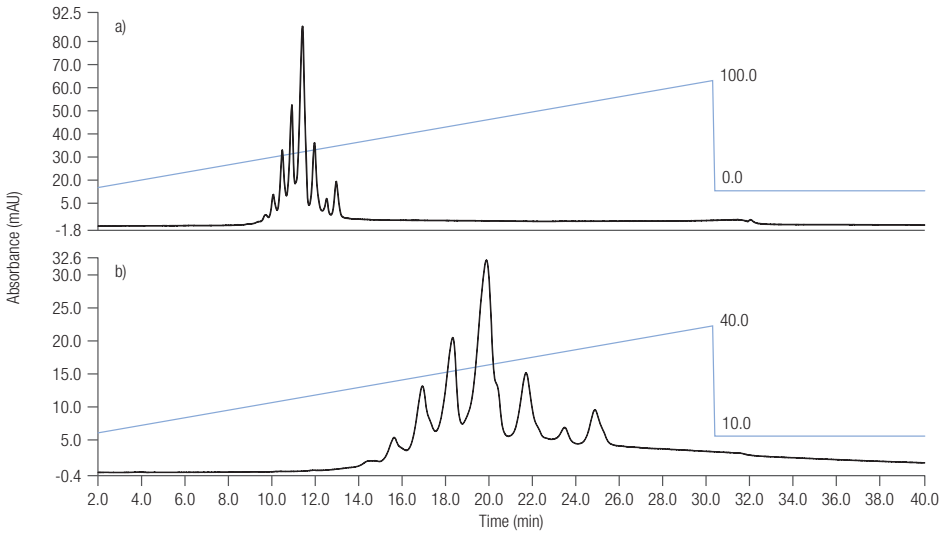


Figure 1. Chromatographic separation of commercial chimeric IgG1 mAb (cetuximab) variants using (a) pH gradient A, (b) pH gradient B, on a MAbPac SCX-10, 10 µm, 4 × 250 mm column

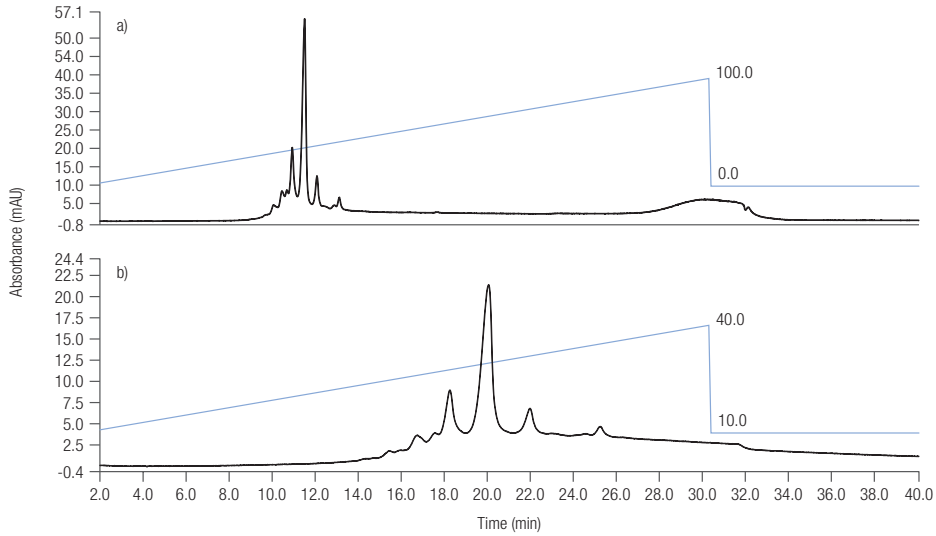


Figure 2. Chromatographic separation of cetuximab biosimilar candidate variants using (a) pH gradient A, (b) pH gradient B, on a MAbPac SCX-10, 10 µm, 4 × 250 mm column

Figure 3 shows the comparison of charge variants profile for the cetuximab innovator (black trace) and a cetuximab biosimilar candidate produced in house (blue trace) using the optimized pH-based gradient B. It illustrates good resolution of C-terminal lysine truncation variants, and other acidic and basic variants. In both samples ten variants forms were identified; the main isoform was assigned to peak 6/F. Differences in charge variant profile between analytes were observable, particularly in peaks 3, 4, and 9 of the commercial product.

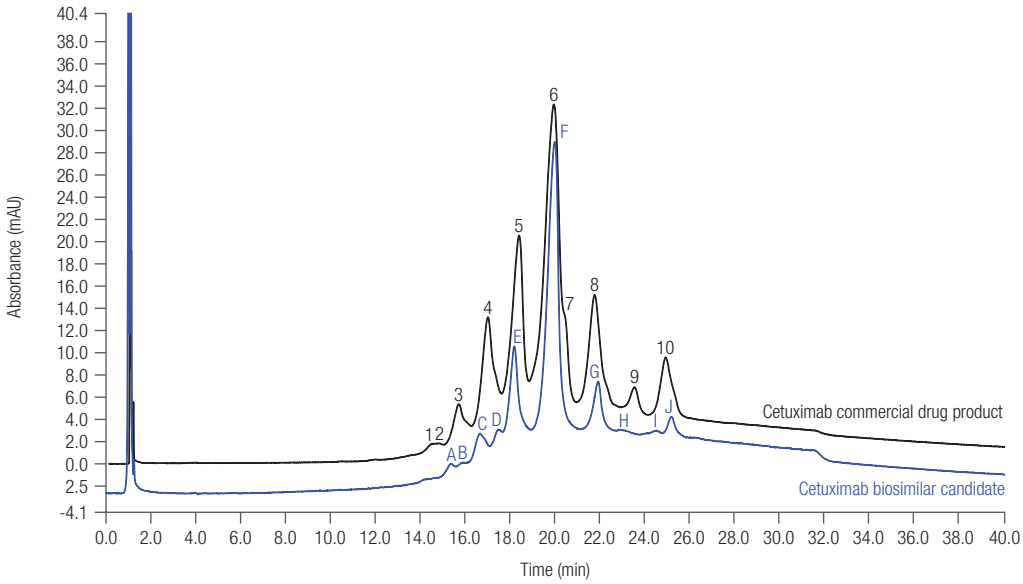


Figure 3. Charge variant chromatographic profile comparison of commercial chimeric IgG1 mAb (black trace) and cetuximab biosimilar candidate (blue trace) obtained with MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH-based gradient mode.
Peak labeling corresponds to the number of peaks in each trace and does not indicate peak identification.

Retention time precision (RSD) is essential for consistent peak identification in charge variants analysis. Retention time precision was measured for repeated injections of the cetuximab innovator and biosimilar candidate (Figures 4 and 5). Ten peaks were evaluated and precision is shown in Tables 2 and 3. The average relative standard deviation (RSD) for retention time variation was calculated at 0.14% including all peaks. The largest variation observed for a single peak was 0.65% for an early eluting low abundance variant.

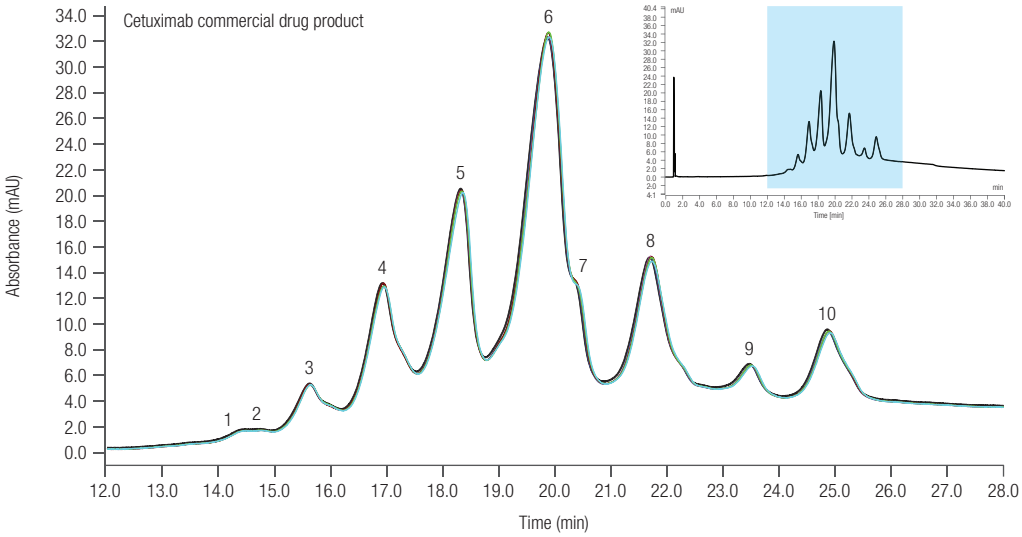


Figure 4. Overlay of six repeat injections of cetuximab innovator

Table 2. Retention time precision (n=6) for charge variant peaks from cetuximab innovator drug product shown in Figure 4

Charge Variant Peak	1	2	3	4	5	6	7	8	9	10
Retention Time (min)	14.50	14.75	15.64	16.94	18.33	19.89	20.42	21.72	23.48	24.89
Retention Time Precision (RSD %)	0.44	0.13	0.05	0.08	0.08	0.07	0.28	0.08	0.19	0.05

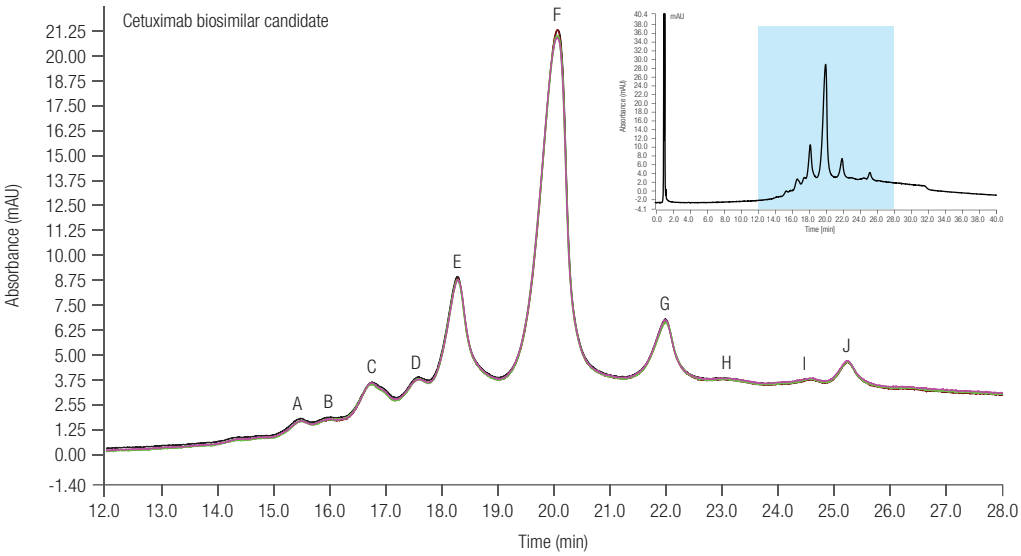


Figure 5. Repeated injections of cetuximab biosimilar candidate on MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH-based gradient separation

Table 3. Retention time precision (n=6) for charge variants peaks from cetuximab biosimilar candidate shown in Figure 5

Charge Variant Peak	A	B	C	D	E	F	G	H	I	J
Retention Time (min)	15.38	16.18	16.65	17.51	18.19	19.97	21.91	22.94	24.52	25.17
Retention Time Precision (RSD %)	0.10	0.65	0.08	0.13	0.04	0.03	0.01	0.20	0.11	0.04

It was postulated that Peaks 7 to 10 (G to J) for both mAbs correspond to C-terminal lysine truncation variants of the main peak (Figures 4 and 5). To verify that the different retention times of these peaks were due to the different heavy chain C-terminal lysine content, the mAbs were treated with carboxypeptidase B (CPB), an exopeptidase that specifically cleaves C terminal lysine residues. This treatment resulted in the quantitative disappearance of peaks 7–10 (G–J) (Figures 6 and 7) in both samples. For cetuximab drug product, peaks 8 and 10 contain 1 and 2 terminal lysine residues, respectively. Other minor variants with lysine truncation are present as peaks 7 and 9. For biosimilar candidate, peaks G and J contain 1 and 2 terminal lysine residues, respectively. Minor variants with lysine truncation are evident in peaks H and I.

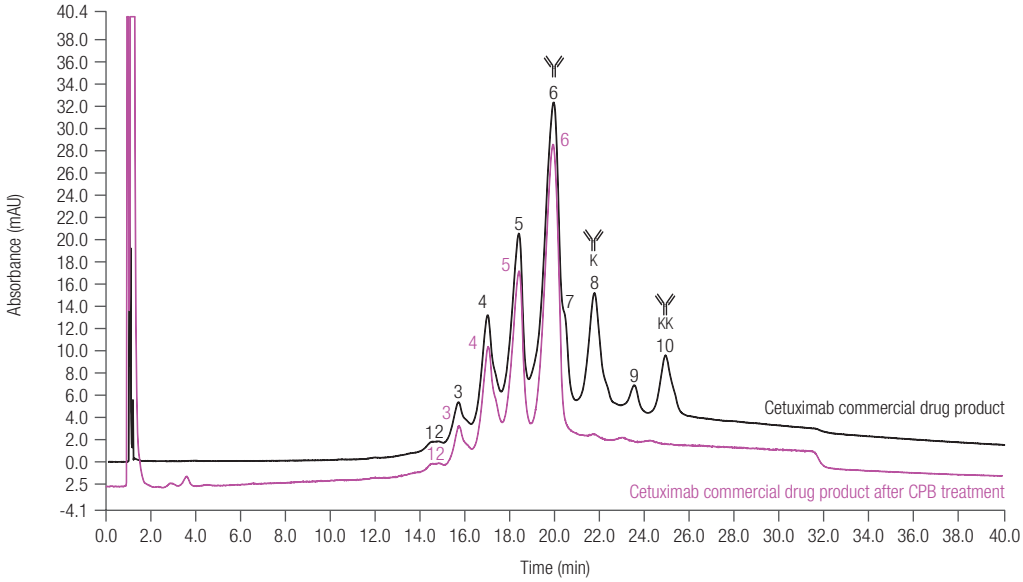


Figure 6. Characterization of cetuximab drug product C-terminal lysine truncation variants using MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH gradient mode.

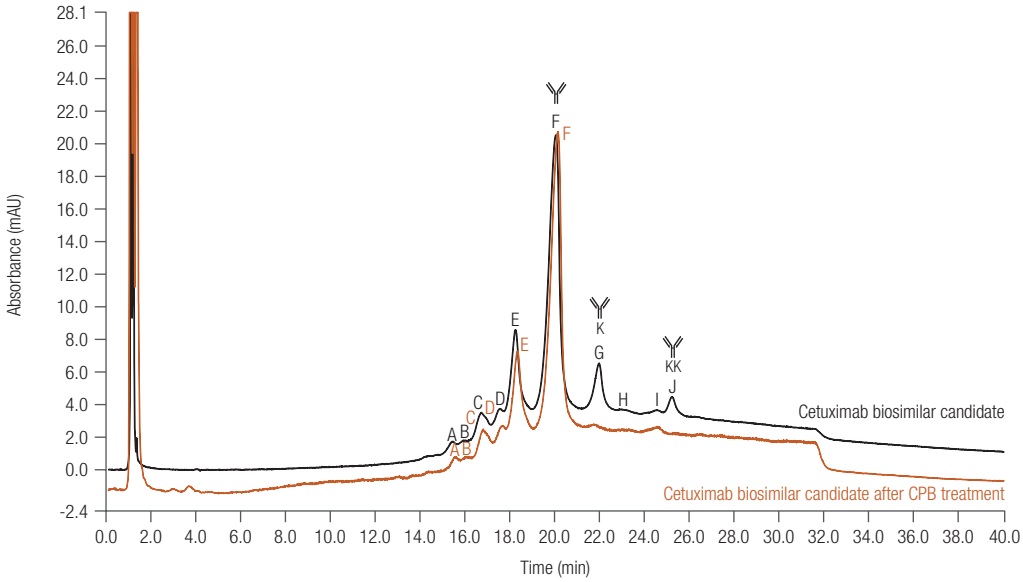


Figure 7. Characterization of cetuximab biosimilar candidate C-terminal lysine truncation variants using MAbPac SCX-10 column (4.0 × 250 mm, 10 µm) and pH gradient mode.

Conclusions

- The approach detailed here has been successfully employed to evaluate the charge heterogeneity differences between an innovator and biosimilar mAb.

- pH-based gradients are effective for simplified ion-exchange analysis of charge variants. The Thermo Scientific CX-1 pH gradient buffer method meets the speed and repeatability requirements for a platform method. Additionally, method development and optimization are also facile.

- The combination of the Thermo Scientific CX-1 pH gradient buffer, MAbPac SCX-10 column, and the Vanquish Flex UHPLC provides excellent separation of charge variants from bio-therapeutics with excellent retention time precision.

References

1. Beck, A.; Wagner-Rousset, E.; Ayoub, D. et al. Characterisation of Therapeutic Antibodies and Related Products. *Analytical Chemistry*, **2013**, *85*, 715-736.
2. Zhang, X.; Tang, H.; Sun, Y.T. et al. Elucidating the effects of arginine and lysine on a monoclonal antibody C-terminal lysine variation in CHO cell cultures. *Biotechnological Products and Process Engineering*, **2015**, *99*, 6643-6652.
3. Zhang, T.; Bourret, J.; Cano, T. Isolation and characterization of therapeutic antibody charge variants using cation exchange displacement chromatography. *Journal of Chromatography A*, **2011**, *1218*, 5079-5086.
4. Teshima, G.; Li, M.X.; Danishmand, R. et al. Separation of oxidized variants of monoclonal antibody by anion-exchange. *Journal of Chromatography A*, **2011**, *1218*, 2091-2097.
5. Fekete, S.; Beck, A.; Veuthey, J.L. et al. Ion-exchange chromatography for the characterization of biopharmaceuticals. *Journal of Pharmaceutical and Biomedical Analysis*, **2015**, *113*, 43-55.
6. Rea, J.C.; Moreno, G.T.; Lou, Y. et al. Validation of a pH gradient-based ion-exchange chromatography method for high-resolution monoclonal antibody charge variant separations. *Journal of Pharmaceutical and Biomedical Analysis*, **2011**, *54*, 317-323.
7. Thermo Scientific Application Note 21788: Evaluation and application of salt and pH-based ion-exchange chromatography gradients for analysis of therapeutic monoclonal antibodies, 2018.

Find out more at **thermofisher.com/chargevariants**

ThermoFisher SCIENTIFIC