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Application Note

Developing Robust and Efficient IEX Methods for Charge Variant Analysis of Biotherapeutics Using ACQUITY UPLC H-Class System and Auto▪Blend Plus

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Abstract

The objective of this application note is to demonstrate the efficiency and robustness of Auto▪Blend Plus Technology for optimization of an IEX method for charge variant separations. A chimeric monoclonal antibody, infliximab, was used as a model therapeutic protein to showcase the application. The Biopharmaceutical Platform Solution with UNIFI was used to perform the analysis, from acquisition to processing and reporting.

Benefits

- Increased productivity through the automation of analytical techniques
- Robust method development that delivers consistent and reproducible results for confirmation and quantification of biotherapeutic charge variants
- Reproducible and simplified method development without the need to prepare additional buffers

Introduction

Charge variant analysis is critical for characterizing and monitoring quality attributes of therapeutic proteins. Protein modification such as deamidation, N-terminal pyroglutamation, isomerization, sialylated glycans, and C-terminal lysine clipping all contribute to charge variant formation.[1] In some cases, such changes affect binding, biological activity, patient safety, and shelf lifetime of therapeutic proteins.

The biopharmaceutical industry relies on tools such as ion exchange chromatography (IEX) and isoelectric focusing (IEF) gel electrophoresis to characterize charge variants. Ion exchange chromatography has been particularly useful in the development of biotherapeutics due to its ease of use, wide applicability, and high resolution.

In-depth characterization of charge heterogeneity of therapeutic proteins from the biopharmaceutical development process requires robust and efficient IEX methods. Method development involves a thorough evaluation of all possible experimental parameters such as buffer/ionic strength, buffer pH, salt gradient, flow rate, and column temperature. However, systematic evaluation on the impact of individual experimental parameters on the separation performance often requires a time-consuming and iterative process that involves preparing and testing discreet buffers of varying composition.

Variation in buffer preparation can lead to inconsistent results, consequently increasing method development time. Waters Auto▪Blend Plus Technology takes advantage of the ACQUITY UPLC H-Class System's quaternary solvent management capabilities, and uses pure solutions and concentrated stocks to address these challenges. Calculation of the percentage of each stock to blend to achieve the desired pH is performed by the Auto▪Blend Plus Technology, reducing error, consumable use, and development time.

With such integrated features, the Biopharmaceutical Platform Solution with UNIFI is well suited for robust method development and can be easily automated for increased productivity. The objective of this application note is to demonstrate the efficiency and robustness of Auto▪Blend Plus Technology for optimization of an IEX method for charge variant separations. A chimeric monoclonal antibody, infliximab, was used as a model therapeutic protein to showcase the application.

Experimental

Sample description

A Waters Protein-Pak Hi Res SP, strong cation exchange column (4.6 x 100 mm, 7 µm, P/N 186004930) was conditioned as outlined by the manufacturer. MES monohydrate (P/N AC327761000), MES sodium salt (P/N AC397351000), sodium chloride (P/N S640-500) were purchased from Fisher Scientific. The chimeric mAb evaluated in this study was used as received for all experiments at a concentration of 20 µg/µL.

LC conditions

LC system:	ACQUITY UPLC H-Class with Auto•Blend Plus
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Vials:	Total Recovery vial: 12 x 32 mm glass,screw neck, cap, nonslit
Column:	Protein-Pak Hi Res SP, 4.6 x 100 mm, 7 µm
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	3 µL
Flow rate:	0.5 mL/min
Mobile phase A:	100 mM MES monohydrate
Mobile phase B:	100 mM MES sodium salt
Mobile phase C:	1000 mM NaCl
Mobile phase D:	18 MΩ H_2O

Buffer conditions:	20 mM MES, pH 6.8
Gradient:	25 mM to 65 mM NaCl in 25 minutes (see Figure 2)

Informatics for data collection and processing

UNIFI Scientific Information System, v 1.6

Results and Discussion

Auto•Blend Plus Technology

Method development of ion exchange chromatography (IEX) techniques often involves a time-consuming trial and error methodology. The iterative process involves preparing multiple buffers at a specific pH and ionic strength, followed by testing of each buffer system until an adequate separation is achieved. The Auto•Blend Plus Technology system is integrated software that comes standard with an ACQUITY UPLC H-Class System. It is designed to take the guesswork out of method development and increase productivity in the analysis of charge variants. Auto•Blend Plus helps analysts configure the quaternary solvent management system to blend pure solutions and concentrated stocks to achieve a desired gradient (Figure 1). The end user is presented with an easy-to-use gradient table interface, where the gradient is expressed directly in terms of pH and ionic strength. The software automatically calculates the percentage of acid and base required for the specified pH using the known pK_a value of the chosen buffer system or an empirical calibration table (Figure 2).

Auto•Blend Plus Technology allows for multiple buffer compositions to be tested from a single set of pure components and can be easily automated to improve productivity.

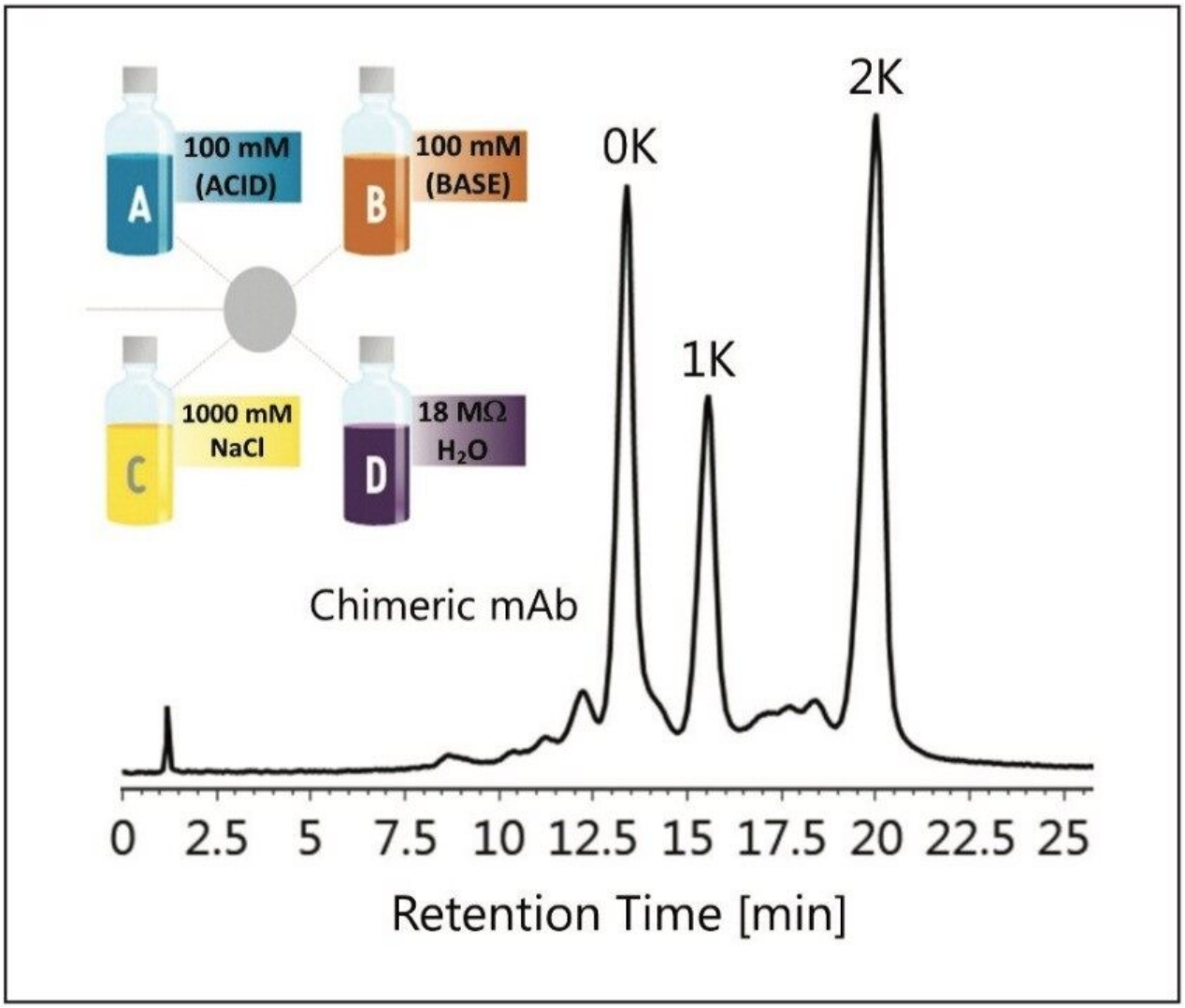
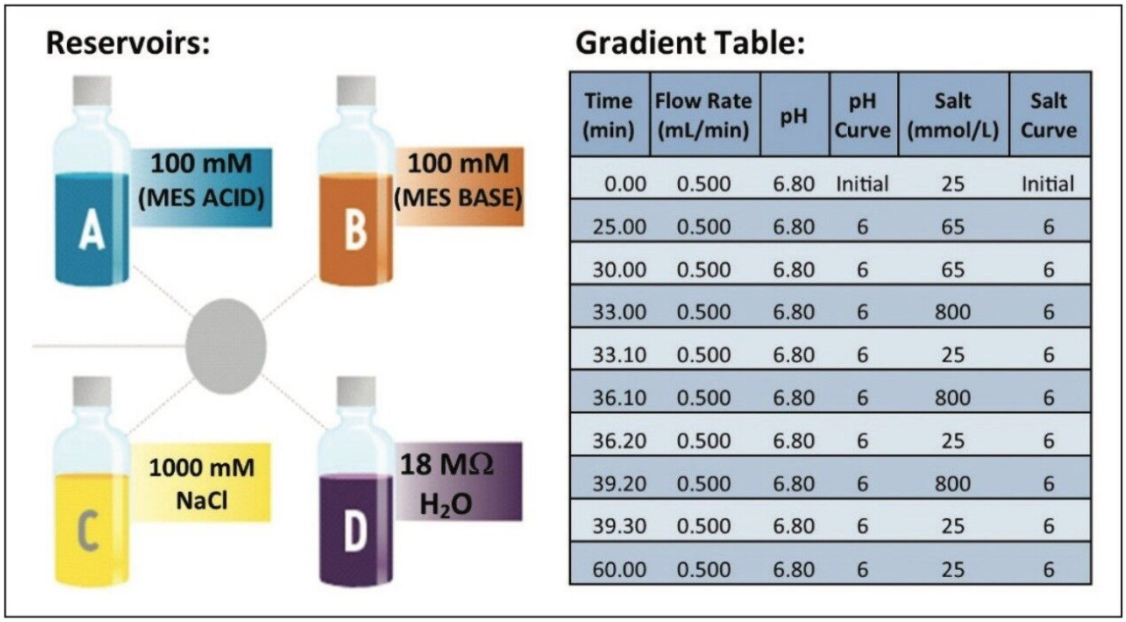


Figure 1. Auto•Blend Plus Technology uses the ACQUITY UPLC H-Class quaternary solvent manager to blend individual pure buffers from the reservoirs to deliver robust separations of charge variants in therapeutic proteins. Here, it is used in the separation of C-terminal lysine truncation variants in a chimeric monoclonal antibody.



Gradient Table:

Time (min)	Flow Rate (mL/min)	pH	pH Curve	Salt (mmol/L)	Salt Curve
0.00	0.500	6.80	Initial	25	Initial
25.00	0.500	6.80	6	65	6
30.00	0.500	6.80	6	65	6
33.00	0.500	6.80	6	800	6
33.10	0.500	6.80	6	25	6
36.10	0.500	6.80	6	800	6
36.20	0.500	6.80	6	25	6
39.20	0.500	6.80	6	800	6
39.30	0.500	6.80	6	25	6
60.00	0.500	6.80	6	25	6

Figure 2. Illustration of a typical Auto•Blend Plus Technology reservoir setup with accompanying gradient table for separation of a chimeric monoclonal antibody.

Robust method development

Robustness is a measure of the ability of a separation method to maintain reproducible results with the introduction of small changes in the system. For ion exchange chromatography, these parameters can include pH, protein mass load, and reproducibility. For pharmaceutical companies a robust method can increase productivity with less time spent on method validation. These parameters were explored to evaluate the robustness of method development using the Auto•Blend Plus Technology.

Protocol for validating and qualifying Auto•Blend Plus

Auto•Blend Plus Technology enables easy system validation and qualification when transferring methods between instruments, analysts, and labs.

Three separate MES buffer systems were prepared and tested using the outlined protocol, below. From Table 1, it can be readily seen that the experimental pH from each buffer system is in good agreement with the desired test pH. The precision among the three separate buffer systems results in reproducible chromatograms as shown in Figure 3. Auto•Blend Plus Technology can readily be adapted to qualification protocols, minimizing time spent on system validation.

Test pH	Buffer mix 1	Buffer mix 2	Buffer mix 3	Mean	Std. Dev.	%RSD
5.13	5.10	5.02	5.10	5.07	0.05	0.91
6.12	6.19	6.05	6.19	6.14	0.08	1.32
7.10	7.23	7.06	7.23	7.17	0.10	1.37

Table 1. Experimental pH results for three MES buffer preparations.

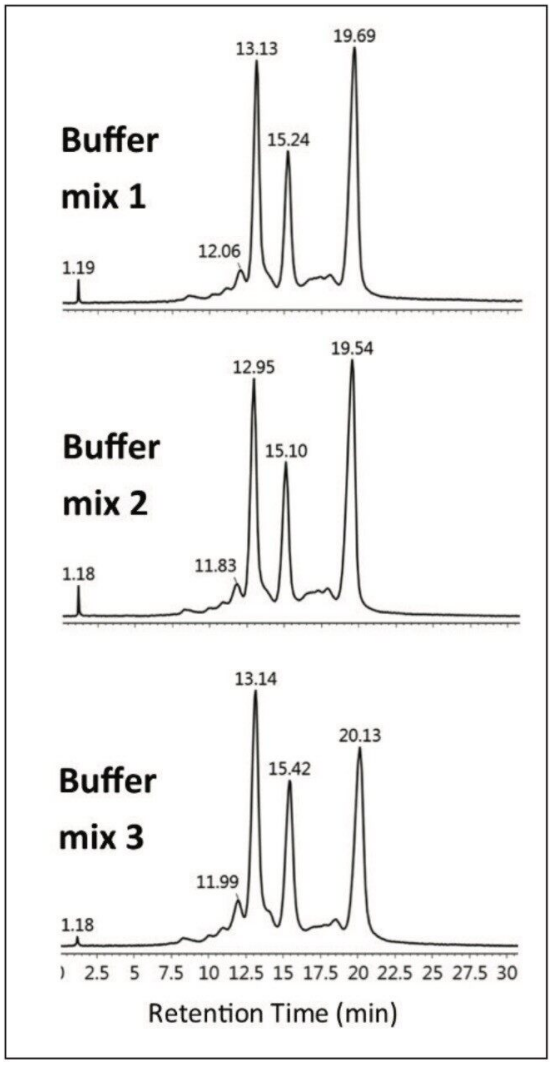


Figure 3. Separation of C-terminal lysine truncation variants with three different preparations of MES buffer over a two-week time period using Auto•Blend Plus Technology.

Install solutions

- A: 100 mL of 1.0 M MES monohydrate in 900 mL HPLC grade H_2O
- B: 100 mL of 1.0 M MES sodium salt in 900 mL HPLC grade H_2O
- C and D: HPLC grade H_2O

Cross-calibrate pH meter

- Low pH reference: Mix 1.8 mL from A, 0.2 mL from B, 8 mL from C
- Medium pH reference: Mix 1 mL from A, 1 mL from B, 8 mL from C
- High pH reference: Mix 0.2 mL from A, 1.8 mL from B, 8 mL from C
- Record pH

Test solutions

- Low: 0.5 mL/min at low pH reference (pH 5.13); Salt concentration: 0
- Medium: 0.5 mL/min at medium pH reference (pH 6.12); Salt concentration: 0
- High: 0.5 mL/min at high pH reference (pH 7.10); Salt concentration: 0

Collect samples

- Run to waste for 10 minutes
- Collect effluent in scintillation vial for 20 minutes
- Repeat for all three test solutions

Measure pH

- Confirm pH meter calibration
- Measure and record pH for each test solution

Maintaining consistent separation performance with increasing sample concentration

Retention time and column performance can be affected by the amount of protein being injected onto the IEX column.

The effects of protein mass load on column performance were tested by injecting between 1-10 µL of the chimeric mAb stock solution in 1 µL intervals. Total peak area was integrated from 5-30 minutes for each

injection. Reproducible retention times were observed over a 9-fold increase in mass load ranging from 20-180 µg of protein as shown in Figure 4. Coupled with the Auto▪Blend Plus Technology, the ACQUITY UPLC H-Class System provides a high degree of fidelity for accurate quantification and characterization of charge variants in biotherapeutics.

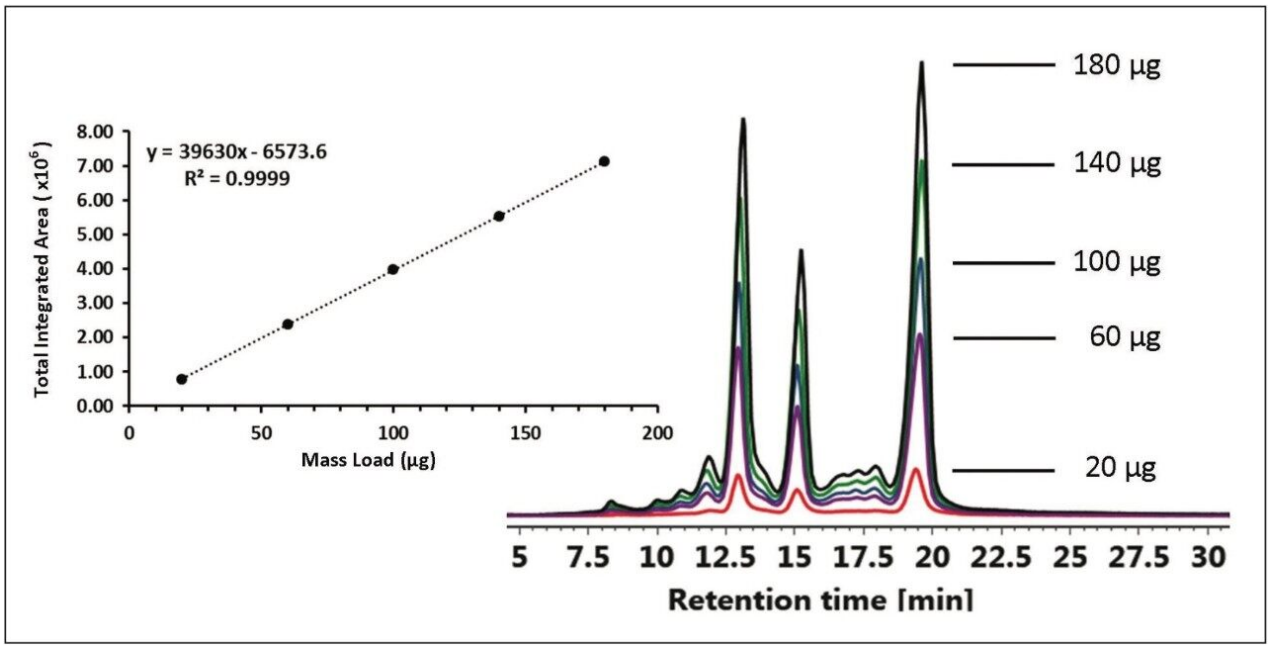


Figure 4. Chromatogram overlays of a chimeric monoclonal antibody with increasing protein concentration. The total peak area was integrated as a measure of precision as shown in the plot of integrated area vs. mass load.

Achieving highly reproducible separations across analysis replicates

Automation of analytical techniques can minimizes error in method development as well as increase productivity.

Auto▪Blend Plus Technology was evaluated over 40 injections to simulate an unattended analysis over three days. Chromatograms are shown at injectiond 1, 20, and 40. The 60-minute separations as outlined in Figure 2 are comprised of a 30-minute separation gradient and a 30-minute cleaning and reconditioning phase. Integration intervals of five peak areas including the three main C-terminal lysine truncation variants are represented by the vertical drop lines in each chromatogram. Calculated areas of each peak area and total area are listed in Table 2.

It can be seen that Auto▪Blend Plus Technology offers reproducible results well within U.S. FDA guidelines[2]

with covariance of the individual peaks below 12% and the total peak area covariance below 9 %. The ability to automate Auto▪Blend Plus Technology, combined with its reproducibility, offers a reliable approach to robust method development for the characterization of charge variants in biotherapeutics.

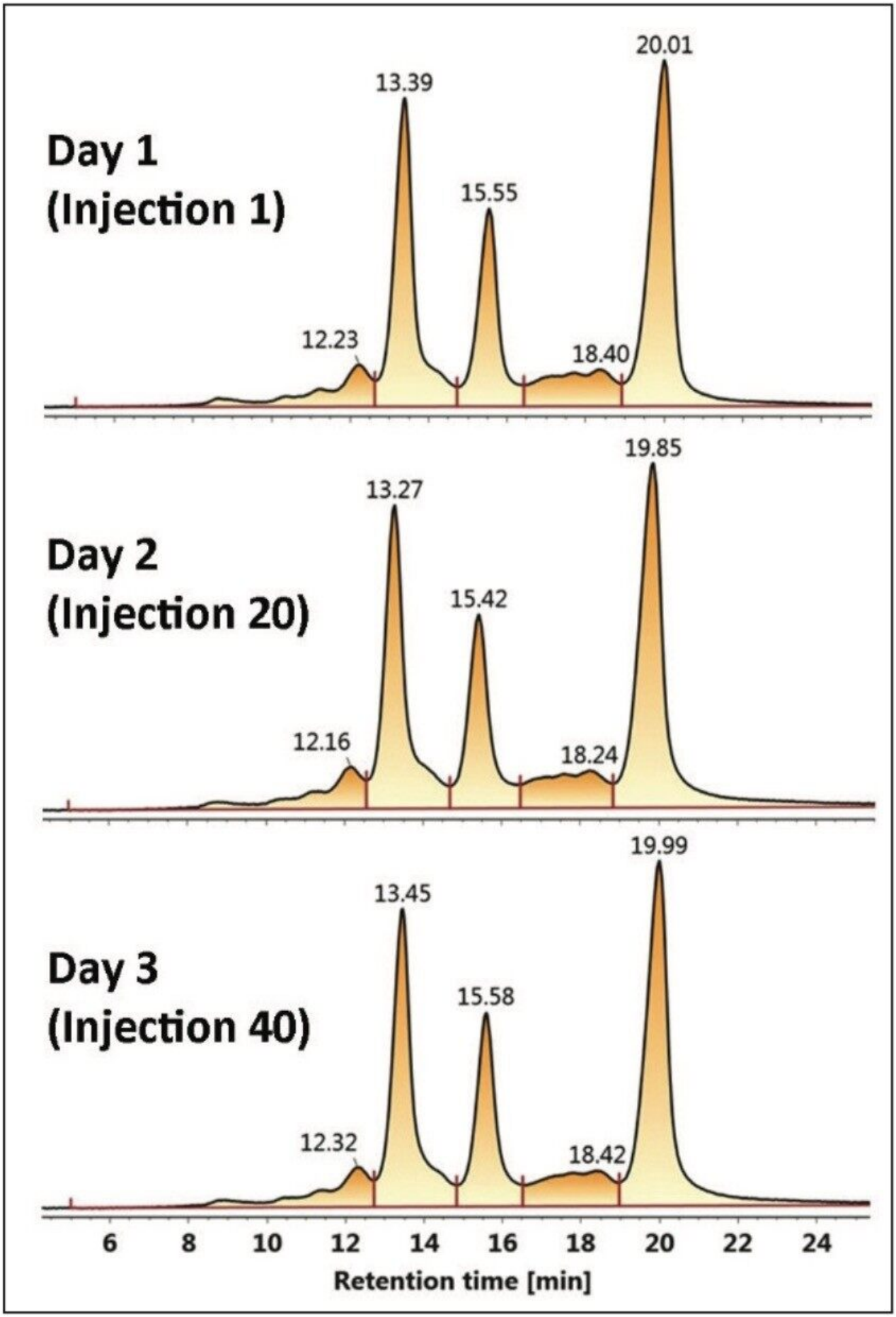


Figure 5. Separation of charge variants of a chimeric monoclonal antibody at three different time intervals o

Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies. *mAbs*. 2012 Sep-Oct; 4(5):578-85.

2. Guidance For Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Rockville, MD, May 2001.

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