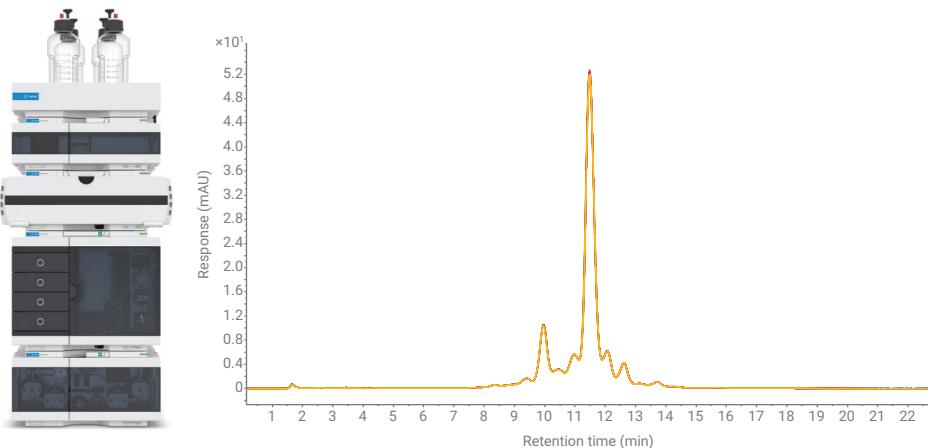


## How Shallow Can You Go?

Suitable for Agilent  
1290 Infinity II LC

Refining charge variant analysis of mAbs with the  
Agilent 1290 Infinity II Bio LC System



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### Abstract

Charge variant analysis is a demanding application for applied liquid chromatography systems due to the use of highly corrosive buffer salts in combination with very shallow gradients for optimal separation. The evaluation of different salt gradients was performed on the Agilent 1290 Infinity II Bio LC System and analyzed for resolution as well as reproducibility.

The 1290 Infinity II Bio LC including High-Speed Pump, with its completely iron-free flow path, is optimally suited for the conditions used in biochromatography—avoiding potentially corrosive damage to the system. Excellent reproducibility even for highly challenging shallow gradients was determined, confirming the 1290 Infinity II Bio LC as the next generation of Agilent high-end liquid chromatography systems for high confidence in generated data.

## Introduction

Monoclonal antibodies (mAbs) are large and highly heterogeneous macromolecules, with a size of around 150 kDa, that are typically generated by recombinant production methods. They are generated in a complex biosynthetic process in which plenty of modifications can occur, leading to hundreds of different variants. Deamidation, oxidation, disulfide bridges, N-glycosylation, N- and C-terminal processing are some of the most common post-translational modifications (PTMs). All these modifications can occur during generation, but also manufacturing and storage contribute to the complexity of these macromolecules. PTMs form a complex isoform profile that needs to be extensively analyzed and monitored, as modifications in the final pharmaceutical might be associated with a loss of biological activity, affected half-life, or immunogenicity.<sup>1</sup> Some of the PTMs result in charge variants of the molecule, which are typically analyzed using ion-exchange chromatography (IEX).<sup>2</sup> Charge variants are considered one of the most important critical quality attributes (CQAs) and therefore strict acceptance criteria and quality controls are to be considered. It is of utmost importance to confirm that the product is correctly manufactured, and to identify and quantify any impurities.

Shallow gradient elution is very common in IEX of proteins. A typical salt gradient in ionic strength mode for the elution of proteins would be approximately 1 to 3 mM/min with a pH value set to a tolerance of  $\pm 0.02$  pH units.<sup>3</sup>

The 1290 Infinity II Bio LC is equipped with a high-performance High-Speed Pump. The major advantage of binary pumps is that solvent mixing is much more accurate and precise when mixing small proportions of one of the solvent components compared to low-pressure mixing pumps (e.g., quaternary pumps). This type of mixing gives highly precise solvent compositions at the start and the end of a solvent gradient.<sup>4</sup> This is a basis for the generation of reproducible and accurate shallow gradients (below 1%/min from each channel).

The 1290 Infinity II Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in biochromatography: high salt concentrations such as 2 M NaCl, up to 8 M urea, and high- and low-pH solvents such as 0.5 M NaOH or 0.5 M HCl. The complete flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of iron ions (e.g. oxidation, protein complex formation) can be avoided.

This application note presents the analysis of charge variants for trastuzumab and the NISTmAb reference standard. Different salt gradient slopes were tested to find the best resolution possible. The best performing gradient slopes were then evaluated for reproducibility.

## Experimental

### Equipment

The Agilent 1290 Infinity II Bio LC System comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with the standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2  $\mu$ L

### Software

Agilent OpenLab CDS Version 2.5

### Columns

Bio MAb, NP5, 2.1  $\times$  250 mm, PEEK (part number 5190-2411)

### Chemicals

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22  $\mu$ m membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and sodium chloride were obtained from Sigma-Aldrich (Steinheim, Germany).

## Samples

- Agilent-NISTmAb (p/n 5191-5744)
- Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland)
- The trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8

## Buffer preparation

For 2 L of 30 mM phosphate buffer, pH 6.8, 4.45 g of sodium phosphate monobasic monohydrate and 7.44 g of sodium phosphate dibasic heptahydrate was weighed and added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water (→buffer A). 29.22 g of sodium chloride, for a total concentration of 500 mM, was added to an empty, amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer A (→buffer B). The pH values of both prepared buffers were checked and adjusted, if necessary, to pH 6.8 (the addition of high amounts of salt can change the pH). Both prepared buffers were filtered using a 0.2 µm membrane filter.

## Method

See Table 1.

**Note:** When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for Solvent B, including 500 mM sodium chloride, use "Sodium Chloride 0.5 M" rather than *Generic Aqueous* or *Water* in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

## Results and discussion

### Method development

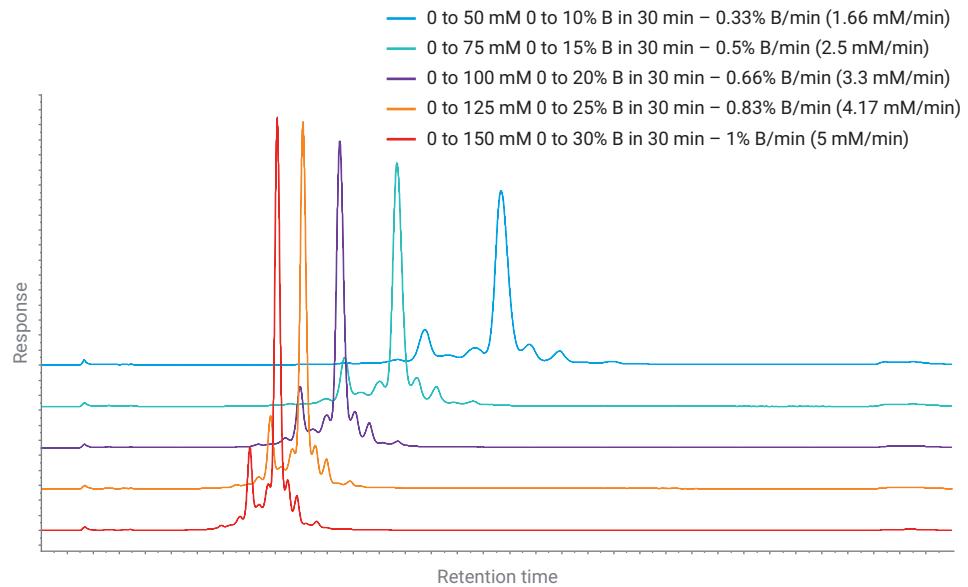
To achieve the desired resolution and enable optimal separation, extensive method development is necessary for charge variant analysis. Two parameters are essential to be successful: finding the optimal pH as well as the optimal gradient slope. Both factors can have a major impact on the separation. First, pH scouting is recommended to find the optimal pH for the separation. In earlier experiments, the pH of the used buffers was analyzed from pH 6.4 to 7.4 and was found to be optimal at pH 6.8 for both samples used: trastuzumab and the NISTmAb reference standard (data not shown). The next step is the determination of the ideal gradient slope to enable the efficient separation.

**Table 1.** Salt gradient chromatographic conditions.

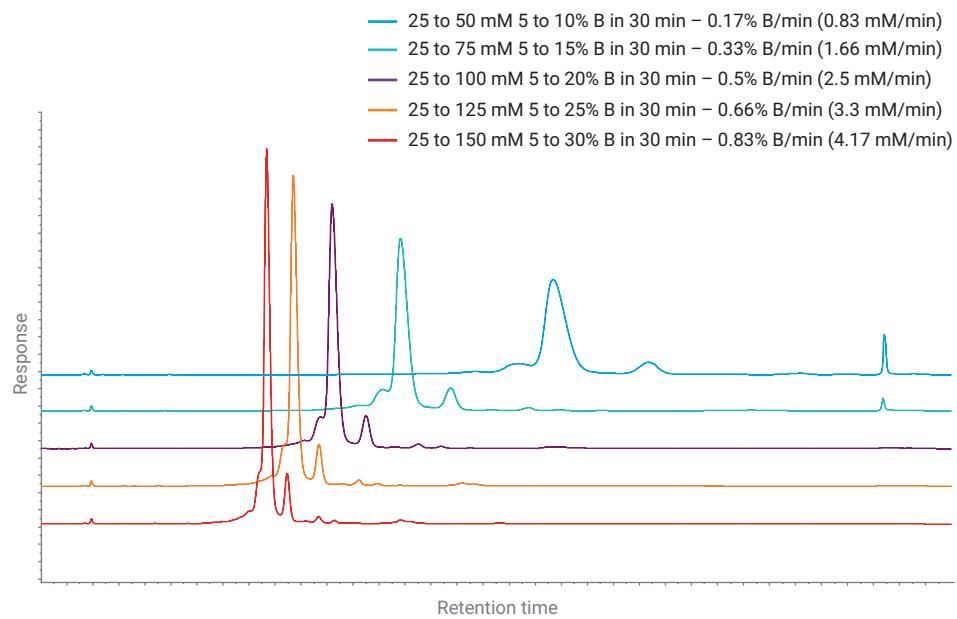
Parameter	Value
Solvent	A) 30 mM phosphate buffer, pH 6.8, B: 30 mM phosphate buffer, pH 6.8, 500 mM sodium chloride
Gradient	0 or 25 mM–150 mM NaCl in 30 minutes—different shallow gradients for method development 0 mM (trastuzumab) and 25 mM (NIST) to 100 mM NaCl in 30 minutes for reproducibility 25 to 50 mM NaCl in 30 minutes for reproducibility (very shallow gradient) 31 minutes–500 mM NaCl wash Stop time: 35 minutes Post time: 15 minutes
Flow Rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 3 µL for trastuzumab and 2 µL for NIST Sample temperature: 10 °C Needle wash: 3 s in water

Figure 1 shows an overlay of charge variant analysis of trastuzumab at different gradient slopes ranging from 1% B/min (5 mM/min) down to 0.33% B/min (1.66 mM/min). The shallower the gradient, the higher the requirements to the pump performance. To deliver highly precise solvent compositions during the gradient, the pump needs to work accurately and precisely when mixing small proportions of the solvent components. It has to be considered, though, that for salt gradients, very shallow gradients do not always result in higher resolution, but simply increase peak width (e.g. 0.33% B/min in Figure 1). Therefore, the chosen gradient slope for reproducibility studies was found in the middle of the tested gradients with 0.66% B/min and 3.3 mM/min, which can still be considered shallow.

A similar method development procedure was carried out for the separation of charge variants of the NISTmAb reference standard (see Figure 2). The starting conditions for NIST contained a slightly higher amount of salt due to the higher isoelectric point (pI) of the NIST antibody (pI of ~9.2), compared to trastuzumab with a pI of ~9. For more effective separation of the charge variants of the NISTmAb reference standard, the gradients were slightly shallower compared to trastuzumab, so the most shallow gradient was at 0.17% B/min (0.83 mM/min), which is a challenging task for the pump. For further reproducibility studies, the 0.5% B/min (2.5 mM/min) gradient slope was chosen.



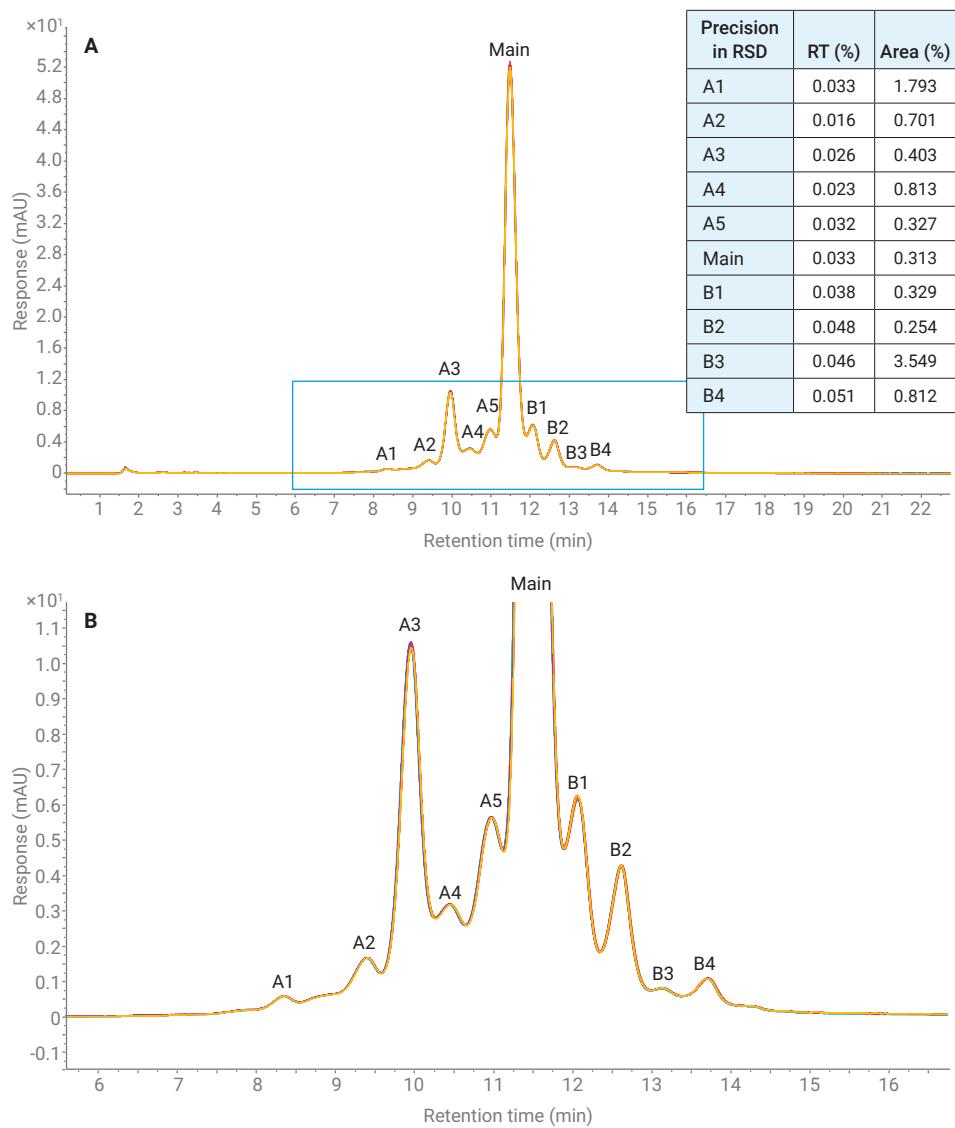
**Figure 1.** Method development for the separation of trastuzumab with different salt gradient slopes.



**Figure 2.** Method development for the separation of the NISTmAb reference standard with different salt gradient slopes.

## Reproducibility for trastuzumab charge variant separation

Figure 3 displays reproducibility studies for charge variant separation of trastuzumab (A) with a 0.66% B/min (3.3 mM/min) gradient slope. Figure 3B shows a zoomed view for better visualization of the separated variants. Variants marked with A represent the acidic variants eluting before the main peaks, whereas the basic variants B elute after the main peak. Five acidic variants were resolved before the main peaks and four basic variants eluted after the main peak. All variants and the main peak were evaluated for the precision of retention time (RT) and area. Both RT as well as area precision are excellent, with values below 0.052% relative standard deviation (RSD) for RT and below 0.82% RSD for area except for the two very small variant peaks A1 and B3.



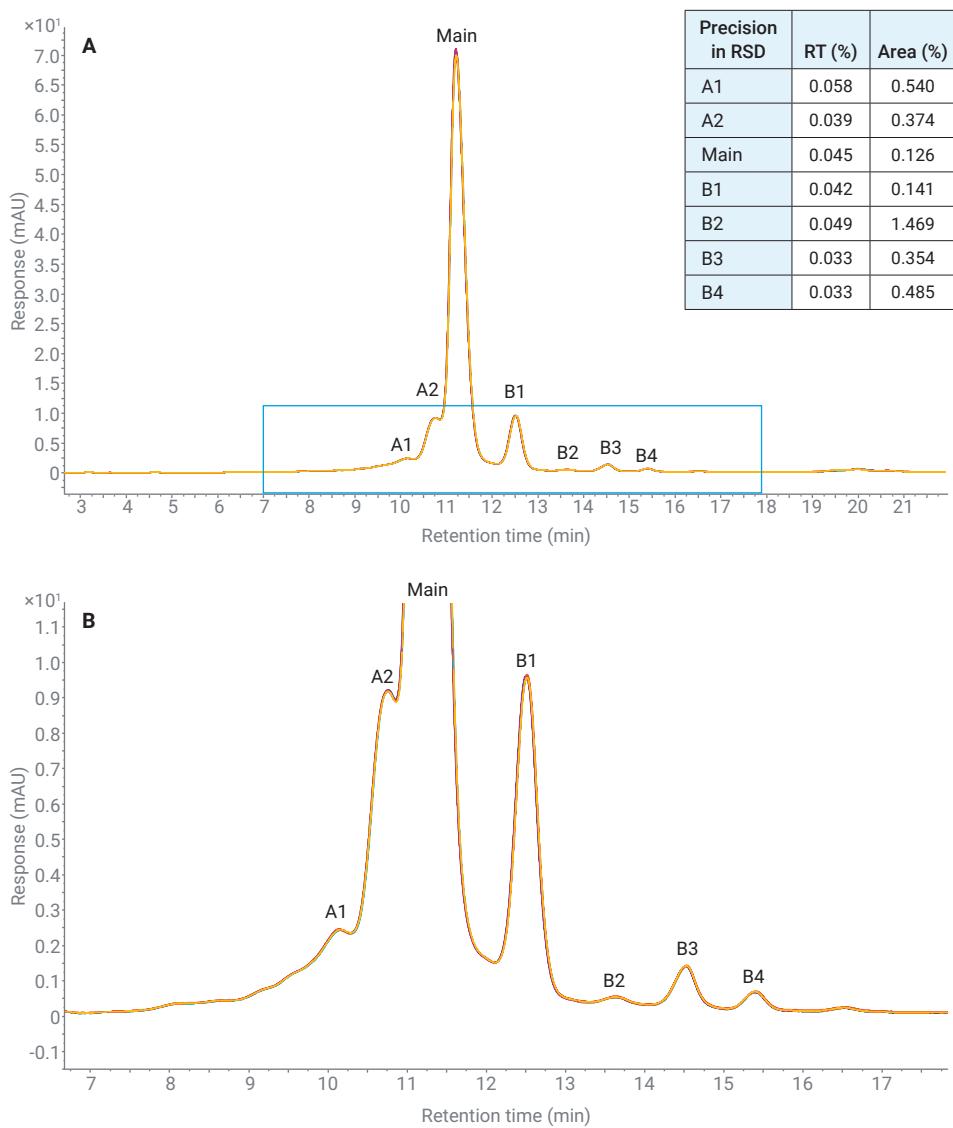
**Figure 3.** Reproducibility studies with seven subsequent runs for charge variant separation of trastuzumab (A) with 0.66% B/min (3.3 mM/min) gradient slope. (B) Zoomed view.

## Reproducibility for NISTmAb charge variant separation

Figure 4 displays reproducibility studies for charge variant separation of the NISTmAb reference standard (A) with 0.5% B/min (2.5 mM/min) gradient slope.

Figure 4B displays the zoomed view with two acidic variants and four basic variants. Again, all variants and the main peak were evaluated for precision of retention time (RT) and area. Both RT as well as area precision are excellent, with values below 0.06% RSD for RT and below 0.55% RSD for area except for one very small variant peak, B2.

As shown in Figure 2, the shallowest gradient with 0.17% B/min (0.83 mM/min) does not deliver a better resolution compared to gradients such as 0.5% B/min (2.5 mM/min) (still shallow—used in the reproducibility studies).

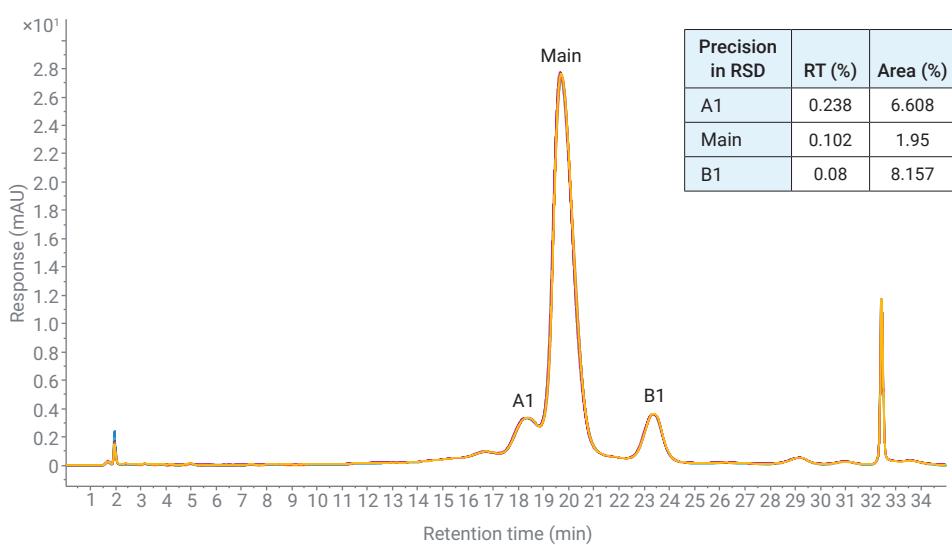


**Figure 4.** Reproducibility studies with seven subsequent runs for charge variant separation of the NISTmAb reference standard (A) with 0.5% B/min (2.5 mM/min) gradient slope. (B) Zoomed view.

However, the 1290 Infinity II Bio High-Speed Pump managed the challenging gradient slope, which is displayed in Figure 5. RT precision over seven subsequent runs was very good (below 0.25% RSD), although the peaks became quite broad with the applied gradient. With increasing peak width, the peak height decreases, which negatively affects the area precision.

## Conclusion

Different salt gradient slopes were evaluated for resolution and reproducibility of the separation of charge variants for trastuzumab and NISTmAb on the 1290 Infinity II Bio LC. At first glance, shallower gradients seemed to improve resolution. However, for both mAbs, the evaluated most shallow gradients did not give the best resolution, as with decreasing slope, the peaks only began to broaden, which led to no further improvement of resolution. The methods with the best combination of high resolution and sharp peak shapes were further evaluated for reproducibility. For a gradient slope of 3.3 mM/min (trastuzumab) and 2.5 mM/min (NISTmAb), excellent reproducibility for RT but also area was documented. The RT precision was below 0.06% RSD for all evaluated peaks. The most shallow gradient tested for NISTmAb was also evaluated for reproducibility, and even for a super shallow gradient with 0.83 mM/min gradient slope, very good RT precision was found (<0.25% RSD). These data show that the 1290 Infinity II Bio LC with its completely iron-free flow path is optimally suited for the conditions used in biochromatography, leading to highly reproducible results.



**Figure 5.** Reproducibility studies with seven subsequent runs for charge variant separation of the NISTmAb reference standard with 0.17% B/min (0.83 mM/min) gradient slope.

## References

1. Dick Jr., L. W. *et al.* Identification and Measurement of Isoaspartic Acid Formation in the Complementarity Determining Region of a Fully Human Monoclonal Antibody. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2009**, 877(30), 3841–3849.
2. Zhang, L. *et al.* Improving pH Gradient Cation-Exchange Chromatography of Monoclonal Antibodies by Controlling Ionic Strength. *J. Chromatogr. A* **2013**, 1272, 56–64.
3. Farnan, D.; Moreno, G. T. Multiproduct High-Resolution Monoclonal Antibody Charge Variant Separations by pH Gradient Ion-Exchange Chromatography. *Anal. Chem.* **2009**, 81(21), 8846–8857.
4. The LC Handbook Guide to LC Columns and Method Development. *Agilent Technologies*, publication number 5990-7595EN, **2016**.
5. Goyon, A. *et al.* Determination of Isoelectric Points and Relative Charge Variants of 23 Therapeutic Monoclonal Antibodies. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2017**, 1065–1066, 119–128.
6. Xie, L. *et al.* Demonstrating Analytical Similarity of Trastuzumab Biosimilar HLX02 to Herceptin with a Panel of Sensitive and Orthogonal Methods Including a Novel Fc $\gamma$ RIIIa Affinity Chromatography Technology. *BioDrugs* **2020**, 34(3), 363–379.

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DE.9640277778

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Printed in the USA, October 15, 2024  
5994-2692EN