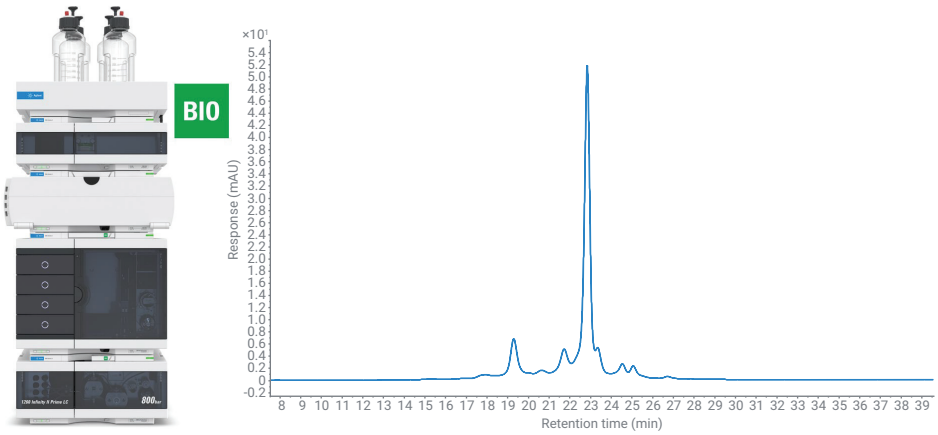
Application Note

Biopharma

Convenient Customization of Your Cation Exchange Analysis

Combining the Agilent 1260 Infinity II Prime Bio LC System, Agilent Buffer Advisor Software, and pH gradients for high-resolving charge variant analysis



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Abstract

Charge variants separation of monoclonal antibodies can be a challenging task for the chromatographer. Due to the microheterogeneity of the analyzed monoclonal antibody, extensive method development can be necessary to find the optimal desired resolution. Outperforming many traditional salt gradients, the resolving power of pH gradients enables the separation of charge variants in a very efficient way. This application note demonstrates high-resolving and reproducible charge variant analysis of two monoclonal antibodies, trastuzumab and NIST mAb, with different types of pH gradients.

The Agilent 1260 Infinity II Prime Bio LC System, with a completely iron-free flow path and featuring an Agilent 1260 Infinity II Bio Flexible Pump, enables the use of Agilent Buffer Advisor Software to facilitate dynamic mixing of solvents from only four stock solutions.

Introduction

Therapeutic monoclonal antibodies (mAbs) are highly heterogeneous molecules and are composed of a large number of variants. These are naturally occurring in this kind of biopharmaceutical and are not necessarily considered impurities. Proteins in solution have mostly polar and charged amino acids at the protein interface to aqueous media, while the hydrophobic residues tend to self-associate due to hydrophobic interactions. These amino acids on the "outside" of the proteins that are in contact with surrounding liquid are more predisposed to modifications.[1]

Variants, also called protein microheterogeneity, might originate from post-translational modifications during antibody production. In addition, modifications after purification processes, formulation and/or storage can be formed.[2] However, if the variants are present in the pharmaceutical protein, their biological activity might differ and immunogenicity might be enhanced.[2] Hence, the microheterogeneity of the mAbs is subject to extensive analytical characterization to ensure safety and efficacy of the biopharmaceutical.

Cation exchange chromatography (CEX) is considered the gold standard for charge variant analysis of monoclonal antibodies.[3] Classic salt gradients have high resolving power once the method is fully optimized. However, the amount of effort required to develop a high-resolving ion exchange method for protein separation can be very high. Salt concentration, mobile phase pH values, and additives are only a few of the parameters to be optimized. In addition, every molecule and especially biological molecules might show different behavior and the developed methods are not tolerant to large changes in experimental parameters, especially with respect to pH values.[1,4]

pH gradient-based CEX, also known as chromatofocusing, enables high-resolving as well as robust methods for the separation of mAb charge variants.[1,4,5] In typical ion-exchange chromatography (IEX), the molecules are eluted from the column by increasing the ionic strength (mostly with salts like NaCl) of the buffer. In contrast, with pH gradients, the bound molecules are eluted with the changing pH of the buffer. This alters their net surface charge to enable the elution of the bound molecules at their isoelectric point (pI), where the molecule is electrically neutral.

Wide pH gradient methods are more generic and can separate variants from different antibodies within a single buffer system.[1] Also, the method development of pH gradient-based methods is more straightforward and significantly shorter compared to conventional ionic strength-based IEX.

The 1260 Infinity II Prime Bio LC System is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in bio chromatography: The sample 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 ferric ions (e.g. oxidation and protein complex formation) can be avoided.

The 1260 Infinity II Bio Flexible Pump, as a quaternary pump, enables the use of Buffer Advisor Software to facilitate dynamic mixing of solvents from only four stock solutions, simplifying the bioanalysis workflow and significantly reducing the time required for buffer preparation. With Buffer Advisor Software, quaternary salt gradients as well as pH gradients can be generated quickly and simply by the calculation of pump timetables for IEX.

This application note presents the analysis of charge variants for trastuzumab and the NIST mAb reference standard with two different pH gradients.

Experimental

Equipment

The Agilent 1260 Infinity II Prime Bio LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow bio-compatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Software

Agilent OpenLab CDS version 2.5 or later versions

Columns

Bio MAb, NP5, 2.1 × 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 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, *tris* (*tris*(hydroxymethyl) aminomethane), imidazole, hydrochloric acid, and piperazine hexahydrate were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

- Agilent-NISTmAb (part number 5191-5744)
- Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8.

Buffer preparation

The buffers were prepared according to the Stock Solution Recipes from Buffer Advisor Software (see Figure 1).

Quaternary phosphate-based buffer system—calculated by Buffer Advisor

A: Water

B: 1,700 mM sodium chloride

C: 44.5 mM sodium phosphate monobasic

D: 55 mM sodium phosphate dibasic

Note: This setup can be used for salt as well as pH gradient elution—enabling direct comparison and increasing the possibilities for method development.

Binary wide pH gradient buffer system—calculated by Buffer Advisor

With Buffer Advisor, it is also possible to create wide range pH gradients, also termed Composite Buffer. In these cases, only the C and D channels are employed to create the gradient. This experiment used a pH gradient described by Farnan and Moreno[1] and inserted the buffer composition as a User Mixture in the stock solution composition of Buffer Advisor (2.4 mM tris, 1.5 mM imidazole, 11.6 mM piperazine, HCl for pH adjustment to pH 6 → C and 10.5 → D). With this option, the user can construct self-made buffer compositions to enable the desired pH range. Buffer Advisor calculates the ionic strength (IS) as well as the buffering capacity (BC) for both buffer mixtures.

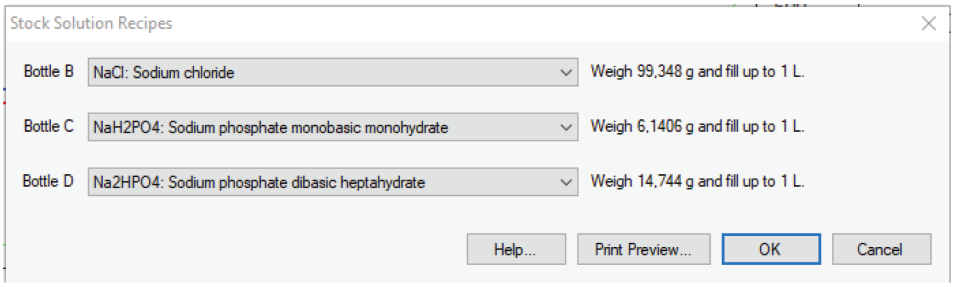


Figure 1. Agilent Buffer Advisor Stock Solution Recipe—quaternary phosphate buffer system.

A: Water

B: n/a

C: pH = 6; IS = 22.5 mM; BC = 6.19 mM

D: pH = 10.5; IS = 0.717 mM; BC = 2.31 mM

Method

Table 1. Quaternary phosphate-based buffer system—salt gradient chromatographic conditions.

Parameter	Value
Solvent	A: Water B: 1,700 mM sodium chloride C: 44.5 mM sodium phosphate monobasic D: 55 mM sodium phosphate dibasic
Gradient	Gradient from 0 to 30 minutes from 10 to 110 mM NaCl in 30 mM phosphate buffer, pH 6.8 with 500 mM NaCl washing step from 30 to 31 minutes Stop time: 31 minutes Post time: 15 minutes
Flow Rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

Table 2. Quaternary phosphate-based buffer system—pH gradient chromatographic conditions.

Parameter	Value
Solvent	A: Water B: 1,700 mM sodium chloride C: 44.5 mM sodium phosphate monobasic D: 55 mM sodium phosphate dibasic
Gradient	Gradient from 0 to 30 minutes from pH 7 to 8.4 in 30 mM phosphate buffer, pH 6.8 with 500 mM NaCl washing step from 30 to 31 minutes Stop time: 31 minutes Post time: 15 minutes
Flow Rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

Note: When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. So, for example, for solvent B in the phosphate-buffered gradient with 1,700 mM NaCl, use *Sodium Chloride 1.5 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 hence using the preconfigured solvent tables enables best pump performance.

Results and discussion

With Buffer Advisor, it is possible to calculate both salt as well as pH gradients. With the quaternary phosphate buffer described in the Experimental section of this application note, it is possible to calculate both versions for the separation of trastuzumab charge variants.

Figure 2 displays the overlay of two chromatograms, separating trastuzumab charge variants with a flat salt gradient (blue) and a phosphate-buffered pH gradient from pH 7 to 8.4 (green). The separations by salt and pH gradient are comparable, with slight improvements in resolution when using the pH gradient.

Table 3. Binary wide pH gradient buffer system/Farnan pH gradient.

Parameter	Value
Solvent	A: n/a B: n/a C: pH = 6; IS = 22,4 mM; BC = 6,14 mM (Farnan Buffer[1]) D: pH = 10,5; IS = 0,717 mM; BC = 2,31 mM (Farnan Buffer[1])
Gradient	Trastuzumab gradient: Gradient from 0 to 50 minutes from pH 8.3 to 10 with a subsequent "wash" step from 51 to 55 minutes at pH 10.5 Stop time: 55 min Post time: 20 min NISTmAb gradient: Gradient from 0 to 45 minutes from pH 8.9 to 10.5 Stop time: 50 min Post time: 20 min
Flow Rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

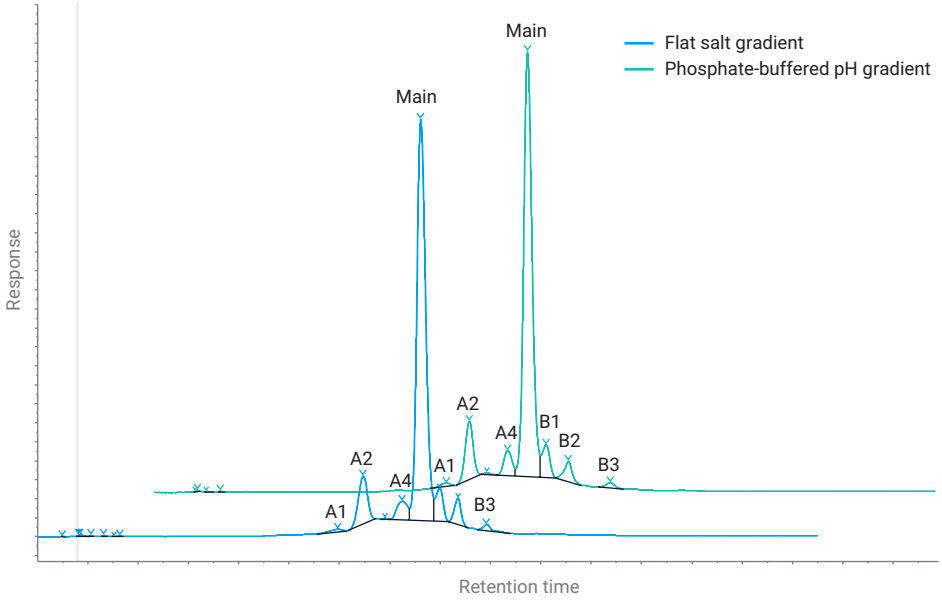


Figure 2. Overlay of two chromatograms for the separation of charge variants with a flat salt gradient (blue) as well as a phosphate-buffered pH gradient from pH 7 to 8.4 (green).

One of the features of Buffer Advisor Software is the improved calculation of linear gradients (salt as well as pH) by adding additional gradient steps within the given gradient to enable perfect linearity without major deviations from the desired/preset pH. To enable this functionality, the **Optimize Gradient** box in the *4. Create % Timetable* section in the Buffer Advisor user interface (UI) needs to be checked (see red circle in Figures 3A and 3B).

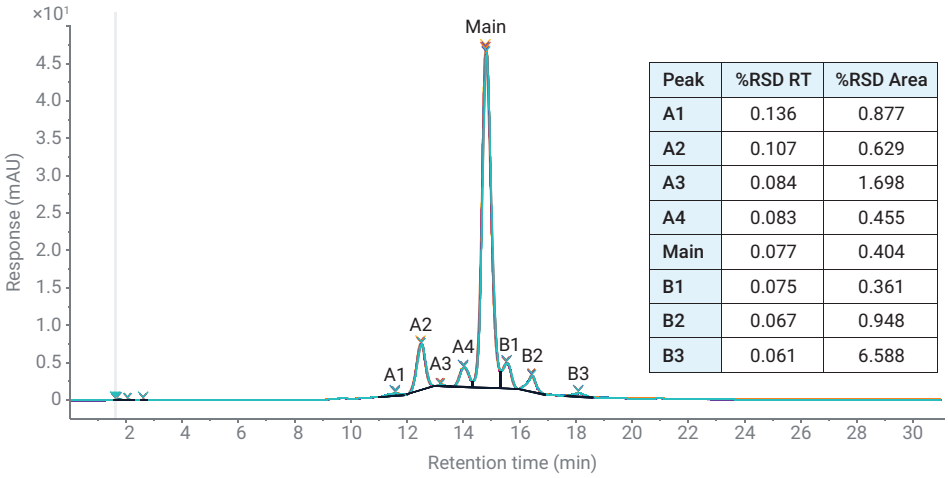
Figures 3A and B showcase the difference between the preset and the actual pH if the box is unchecked (A) and checked (B). With no further optimization from Buffer Advisor, the actual pH can deviate up to 0.4 units from the preset pH, which makes it difficult for the user to rely on the running gradient linearity. By checking the **Optimize Gradient** box (Figure 3B), additional steps are inserted into the original gradient to ensure linearity of the pH gradient. The **Result Pump Gradient Timetable** on the bottom left displays the additional inserted steps resulting in the actual gradient being as close as possible to the preset gradient. This gradient timetable can then be exported into the method in OpenLab for an easy transfer without additional time needed for typing.

Figure 3. Optimize Gradient function in Agilent Buffer Advisor software to enable highly linear pH gradient. A shows no optimization, B displays the optimized gradient.

Figure 4 shows an overlay of seven subsequent runs of the charge variant analysis of trastuzumab with the phosphate-buffered pH gradient from pH 7 to 8.4. Excellent reproducibility was found for retention time (RT) and area with relative standard deviation (RSD) of less than 0.085% apart from the first two peaks. Due to the minimal area as well as height of the variants A3 and B3, the area reproducibility is higher than 1%.

Within the phosphate-buffered system, method development is limited, especially if the pI of the molecules of interest is not in the pH buffering range between 6 and 8. If the pI of the molecule is too high, elution is not possible using this buffer system. For example, the NIST mAb with a pI of 9.18[6] needs a different buffer system to enable elution from the CEX column.

A more generic approach is to use a wide-range pH gradient composed of more than one buffer system. This setup is also incorporated in Buffer Advisor Software under the name *Composite Buffer (Wide Range pH Gradient)*. The pH gradient with pH range 6.0 to 10.5 from Farnan and Moreno (2009)[1] is a suitable buffer system to analyse the charge variants of monoclonal antibodies. This system was further used and the method optimized for trastuzumab and NIST mAb.



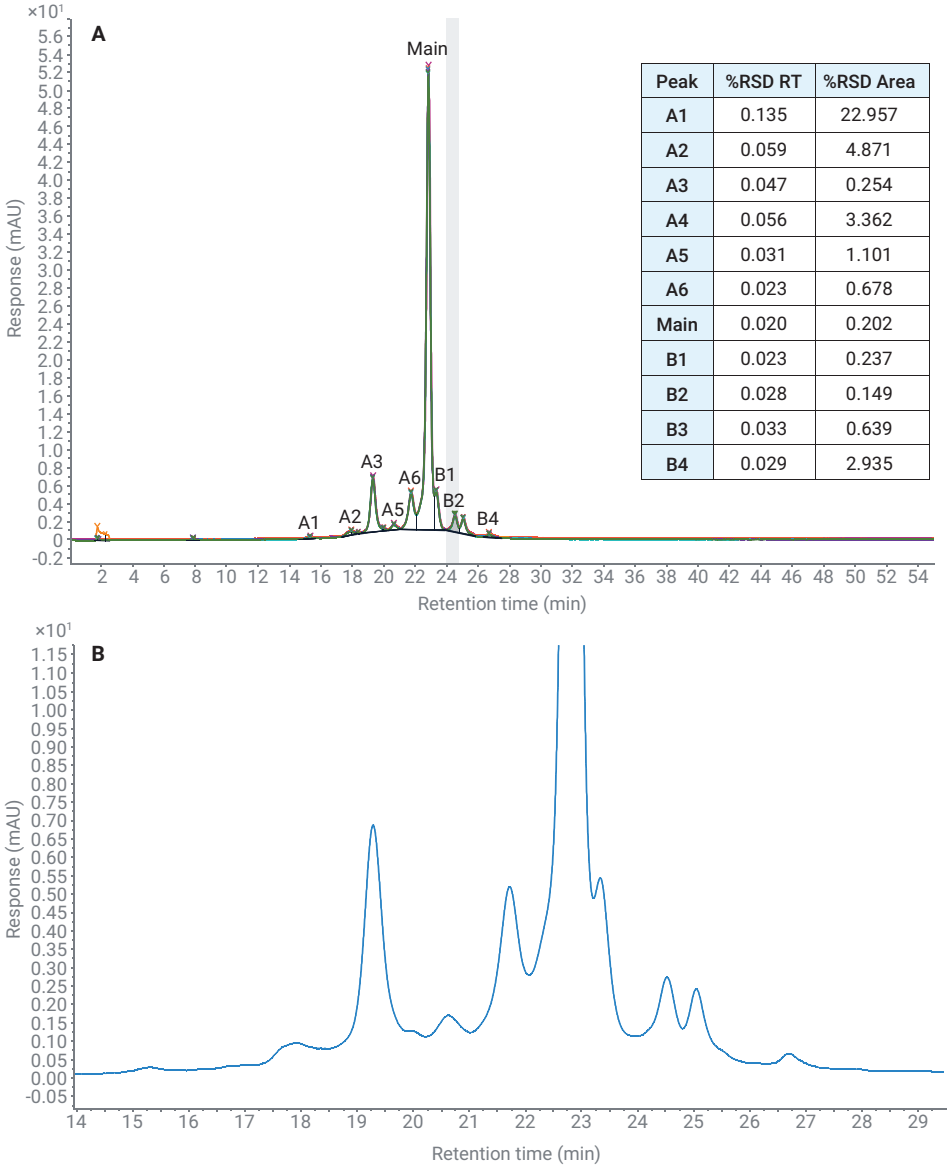
Peak	%RSD RT	%RSD Area
A1	0.136	0.877
A2	0.107	0.629
A3	0.084	1.698
A4	0.083	0.455
Main	0.077	0.404
B1	0.075	0.361
B2	0.067	0.948
B3	0.061	6.588

Figure 4. Overlay of seven consecutive runs of trastuzumab analyzed with phosphate-buffered pH gradient from pH 7 to 8.4 including the precision table for retention time (RT) and area.

Figure 5 shows the separation of trastuzumab charge variants using the wide-range pH gradient, narrowed from pH 8.3 to 10 to achieve optimal resolution. Compared to the phosphate-buffered pH gradient (see Figure 2), it was possible to resolve two more acidic variants—A1 to A6—eluting before, and one more basic variant eluting after the main peak. Especially the zoomed view in Figure 5B shows the excellent resolution of different charge variants around the main peak, with sharper peaks and enhanced resolving power compared to the shallow salt and pH gradient shown in Figure 2.

The precision of RT and area was evaluated for all resolved variants (see peak table in Figure 5). Even for extremely small peaks, the precision of RT was excellent, with values below 0.06% RSD except for the first variant A1. The area precision showed excellent values for most of the peaks except for the extremely small ones.

The pH gradient used by Farnan and Moreno[1] has also proven to be ideal for the analysis of the NISTmAb (see Figure 6). For the NISTmAb, the pH gradient was modified to a different pH range due to the different pI of the NISTmAb. With this developed shallow gradient from pH 8.9 to 10.5, it was possible to separate three acidic and two basic variants.



Peak	%RSD RT	%RSD Area
A1	0.135	22.957
A2	0.059	4.871
A3	0.047	0.254
A4	0.056	3.362
A5	0.031	1.101
A6	0.023	0.678
Main	0.020	0.202
B1	0.023	0.237
B2	0.028	0.149
B3	0.033	0.639
B4	0.029	2.935

Figure 5. Overlay of seven consecutive runs of trastuzumab using a wide pH gradient from pH 8.3 to 10 including the precision table for retention time (RT) and area A. Zoomed view of a single injection B.

Conclusion

The advantage of pH gradients over salt gradients was demonstrated for the analysis of monoclonal antibodies. pH gradients have been shown to outperform even shallow salt gradients with simplifying method development on the one hand and the generation of high-resolution chromatographic results on the other hand. While the potential of salt gradient method development is rather limited—changes in gradient slope just increase peak width with no further changes in resolution[7]—pH gradients reveal possibilities to further increase resolution and maintain sharp peaks. This was showcased by the analysis of trastuzumab and NIST mAb, especially by the use of the wide range pH gradient, based on Farnan and Moreno.[1] Buffer Advisor Software facilitated dynamic mixing of four stock solutions for the phosphate-buffered systems, preventing time-consuming buffer preparation hands-on time in the lab. In addition, the wide-range pH gradient could be easily calculated by Buffer Advisor. Hence, all methods—developed with Buffer Advisor Software and run on the Agilent 1260 Infinity II Prime Bio LC System with Flexible pump, with its completely iron-free sample flow path—delivered highly reliable and reproducible results.

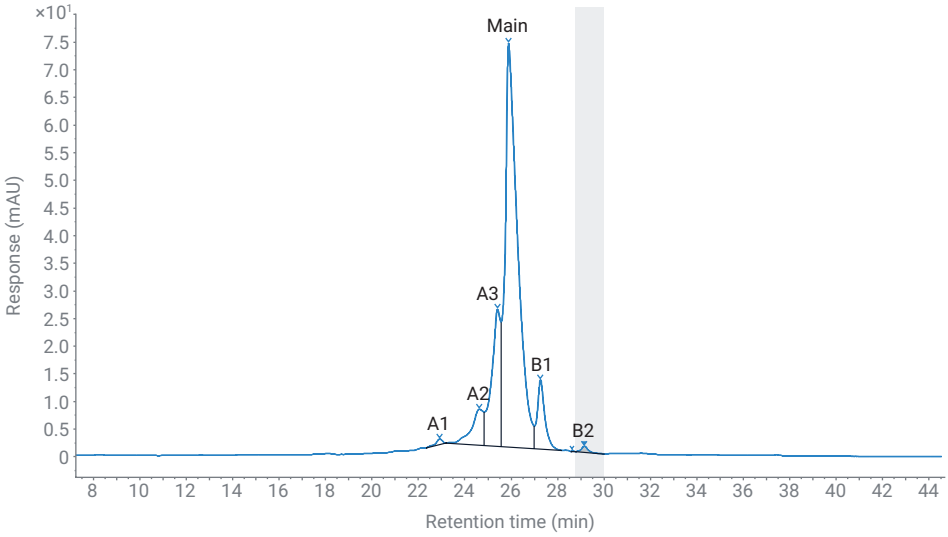


Figure 6. Separation of the NISTmAb with a pH gradient modified according to Farnan and Moreno[1] from pH 8.9 to 10.5.

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