

HPLC Separation of Monoclonal Antibody Charge Variants: Comparison of the Agilent Bio MAb, NP10 and a Competitor's 10 μm Particle WCX Column using the Agilent 1260 Infinity Bio-inert Quaternary LC System

Application Note

Biopharmaceuticals

Authors

Phu T Duong, Faizy Ahmed, Taegen Clary
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA
and
Agilent Technologies, Santa Clara,
California
USA

Abstract

Monoclonal antibody (mAb) therapeutics have been shown to have heterogeneity due to modifications that occur during manufacturing. These changes include differences in glycosylation, deamidation, oxidation, incomplete C-terminal processing and other post translational modifications. Monitoring these potential changes to a mAb therapeutic is critical to ensure the safety and efficacy of the product. This stability data is expected by regulatory agencies such as the FDA and EMA. The data presented here focus on the use of analytical weak cation exchange columns for the separation of the charge variants that occurred throughout the manufacturing of a Chinese Hamster Cell (CHO) derived mAb. The separation performance of the Agilent Bio MAb, NP10 (WCX, non-porous, 10 μm particle) and a competitor's 10 μm particle WCX are compared. The data presented here were generated using the Agilent 1260 Infinity Bio-inert Quaternary LC. Methods were optimized for both columns taking buffer pH, salt concentrations, gradient selectivity, flow rates and separation times into consideration. The sensitivity improvements of the 1260 Infinity Bio-inert Quaternary LC for this application is also presented.



Agilent Technologies

Introduction

Highly sensitive analytical techniques are necessary for monitoring the purity and stability of monoclonal antibodies (mAbs) during manufacturing, purification, formulation and storage. The HPLC separation of mAbs from their impurities can be achieved using a variety of chemistries including ion exchange chromatography. The data presented here focuses on the use of analytical weak cation exchange (WCX) for the separation of acidic and basic charge variants of a Chinese Hamster Cell (CHO) derived mAb. Methods are developed on the Agilent Bio MAb, NP 10 (WCX, non-porous, 10 µm particle) HPLC column and a competitor's 10 µm particle WCX HPLC column using the Agilent 1260 Infinity Bio-inert Quaternary LC. The Agilent Bio MAb columns are packed with non-porous polystyrene-divinylbenzene (PS-DVB) particles that have a hydrophilic coating to inhibit nonspecific binding and a dense WCX layer. The competitor's column uses a non-porous PS-DVB particle coated with a hydrophilic layer and functionalized WCX groups. Both columns use 10 µm diameter particles, but differ in their hydrophilic coatings and the density of their WCX functionality. A comparison of the Agilent 1260 Infinity Bio-inert Quaternary LC and the Agilent 1100 LC with regards to signal sensitivity is shown. The significant sensitivity improvements seen with the 1260 Infinity Bio-inert Quaternary LC provides better visibility of low level variants and improves overall sensitivity for this application.

Experimental

HPLC system

The Agilent 1260 Infinity Bio-inert Quaternary LC operates at a maximum pressure of 600 bar and the sample flow path is completely free of metal components. This ensures that the mAb sample does not come into contact with metal surfaces, with as little potential nonspecific binding as possible. Solvent delivery is free of any stainless steel components preventing iron release due to corrosion. The LC system consists of the following modules:

G5611A	Agilent 1260 Infinity Bio-inert Quaternary LC pump
G5667A	Agilent 1260 Infinity Bio-inert High Performance Autosampler
G1330B	Agilent 1200 Series Thermostat
G1316C #19	Agilent 1260 Infinity Thermostatted Column Compartment containing Bio-inert click-in heating elements
G4212A #33	Agilent 1260 Infinity Diode Array Detector with Max-Light 60 mm, high sensitivity flow cell
Software	ChemStation B.04.02

Chemicals and reagents

CHO-cell derived, humanized monoclonal antibody – Creative Biolab, Pennsylvania, PA.

Sodium chloride, monobasic and dibasic sodium phosphate – Sigma-Aldrich (St Louis, MO).

HPLC Columns

Agilent Bio MAb, NP10, 4.6 mm × 250 mm, PEEK (nonporous, 10 µm particle)

Competitor WCX, 10 µm particle, 4 mm × 250 mm, PEEK

LC method

Injection volume: 5 µL (1 mg/mL affinity purified monoclonal antibody)

Mobile phase A: A combination of monobasic and dibasic sodium phosphate 0.025 M and 0.05 M, pH 5.5, 6.0, 6.5, 7.0

Mobile phase B: Mobile phase A plus 0.4 M sodium chloride.

Flow rate: 0.8 mL/min, room temperature.

Detection: Monitoring at 214 nm, 220 nm and 280 nm.

Reference: 360 nm

Results

Buffer pH selectivity- Separation of monoclonal antibody

Buffer optimization is an important step for mAb analysis by cation exchange chromatography. In order to maximize the column's ability to absorb and release an mAb, buffer pH must be carefully optimized. As a general practice, sodium phosphate and sodium chloride are used. Using columns with the same buffer conditions initially helped to understand and compare performance. The method for each column was then modified to achieve optimal separation conditions for each column. Buffers at pH 5.5, 6.0 and 6.5 were used because the mAb pI was unknown but assumed to be between 5 and 7. Dibasic and monobasic sodium phosphate was mixed to 0.025 M and 0.05 M buffer concentrations with desired pHs for mobile phase A. Mobile phase B buffer contained a mixture of mobile phase A buffer plus 0.4 M NaCl. A 5 µg amount of mAb (5 µL) was injected onto the columns and the chromatograms were analyzed at the 214, 220 and 280 nm wavelengths using the Agilent 1260 Infinity Bio-inert Quaternary LC. The initial run time was 60 min with a flow rate of 0.8 mL/min and linear gradient of 0% to 100% mobile phase B from 0 to 60 min at room temperature. Figure 1 shows that the peak retention time increased when the buffer pH decreased (compare Figure 1 panel A to panel C) for both columns. The retention time of the major mAb peak is at ~15 min using pH 5.5, whereas the major mAb peaks elute earlier at pH 6.0

(9.7 min) and 6.5 (8.1 min), respectively. This buffer scouting suggests that pH 5.5 buffer provides the most absorption capacity for these columns. While the peak resolution looked good at pH 6.0 and pH 6.5, much material did not get absorbed by the column and the sample eluted as flowthrough, too close to the void volume (Figure 1, Panels A and B). At pH 5.5,

the columns retained the sample longer, but peak resolution was lost for both columns. A pH of 5.5 optimized the retention time. Based on this initial method optimization, the Agilent Bio MAb column provided better overall separation and peak resolution than the competitors' WCX column in all three buffer conditions.

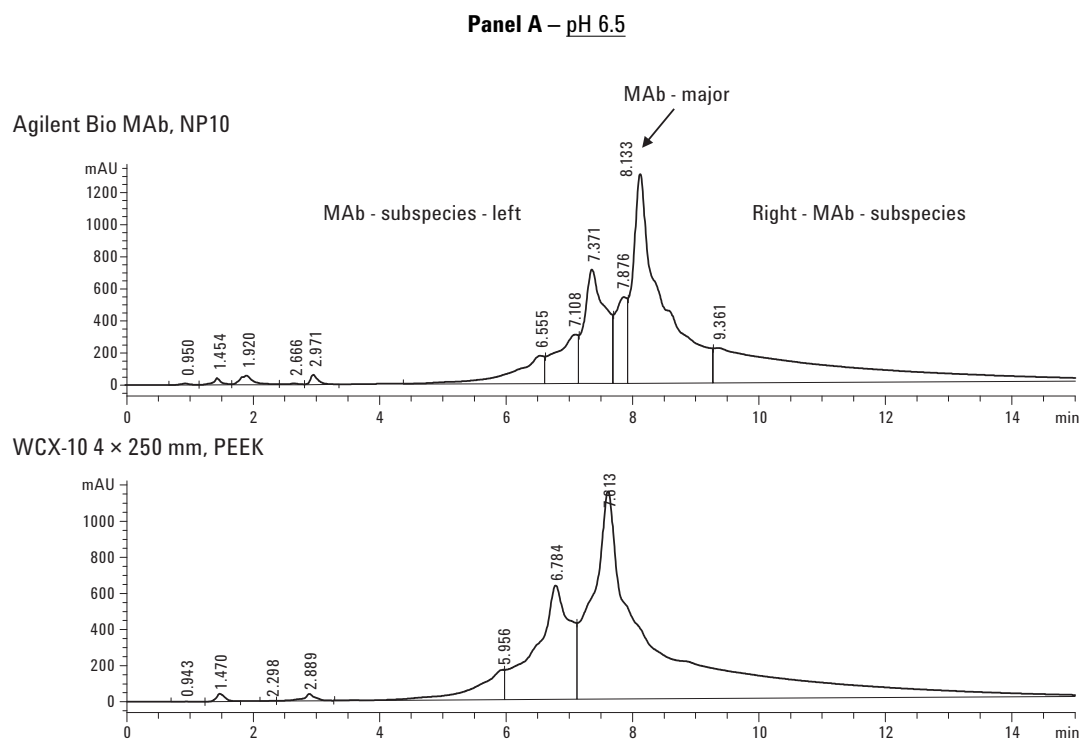
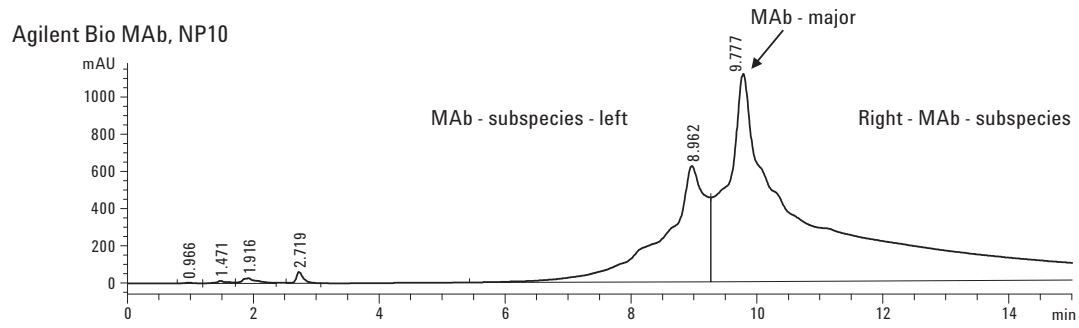
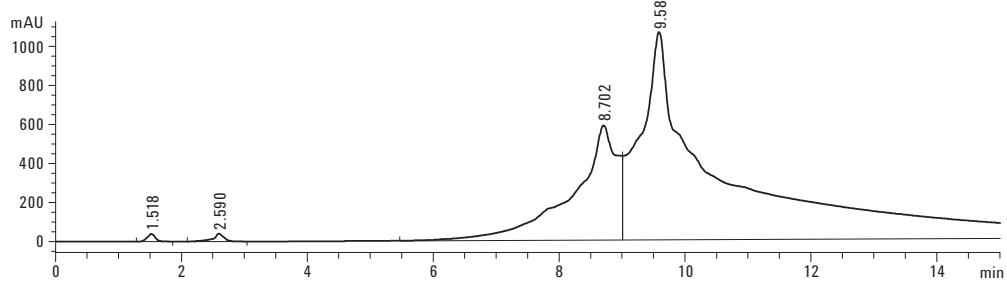


Figure 1. Buffer pH selectivity-mAb separation using Agilent Bio MAb, NP10 and WCX-10 4 × 250 mm, PEEK columns. (Continued)

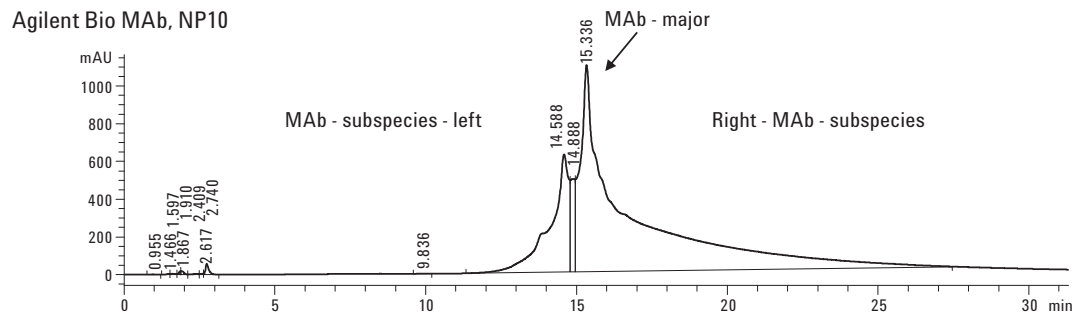
Panel B – pH 6



WCX-10 4 × 250 mm, PEEK



Panel C – pH 5.5



WCX-10 4 × 250 mm, PEEK

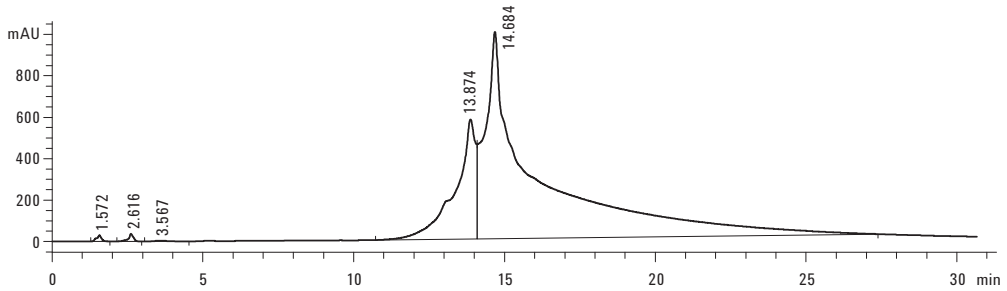


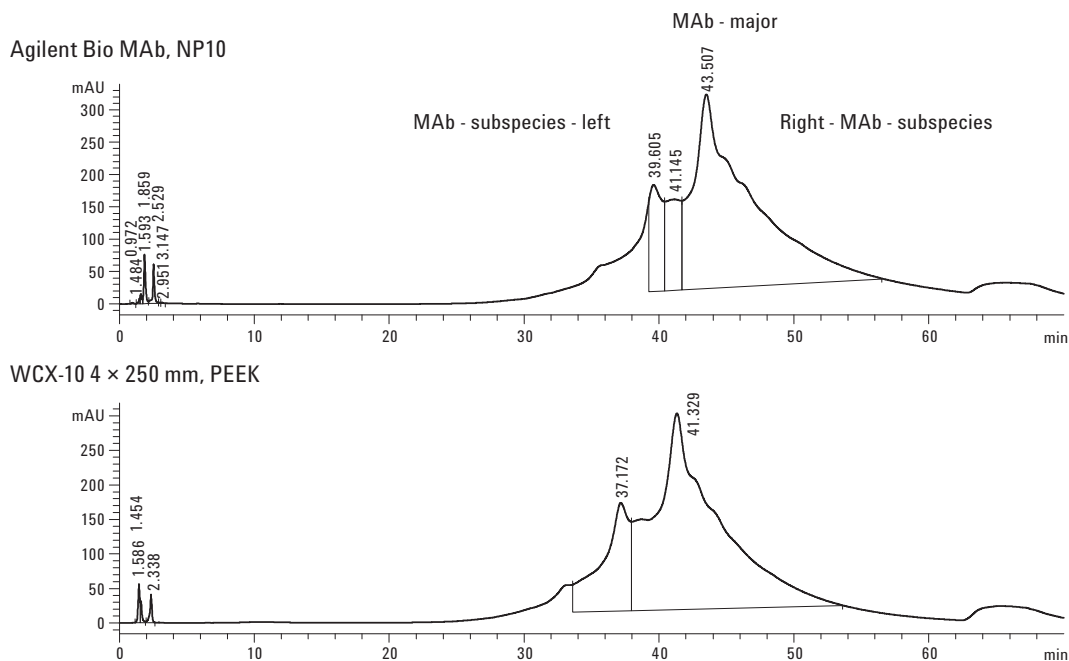
Figure 1. Buffer pH selectivity-mAb separation using Agilent Bio MAb, NP10 and WCX-10 4 × 250 mm, PEEK columns.

Gradient optimization- 60 min run time for separation monoclonal antibody

A variety of gradient conditions were used with both columns. The two most optimal buffer conditions for both the Agilent and competitor columns were, 10 to 35% and 15 to 35% mobile phase B in 60 min. Both gradient systems were conducted in 0.025 M and 0.05 M sodium phosphate buffer, pH 5.5. Buffer conditions remained at pH 5.5 (exact method conditions shown in Figure 2 legend). Figure 2, Panel A shows results from 0.025 M sodium phosphate buffer with linear gradient system 10% to 35% mobile phase B. Both columns generated very similar data. The main mAb peak is well resolved from its acidic and basic charge variants. The Agilent Bio MAb, NP10 column provides more resolved peaks and shows

better capacity due to the slightly later retention time. Similarly, the linear gradient at 15% to 35% mobile phase B shows similar data to the linear gradient system 10% to 35% mobile phase B. However, at 15% to 35%, the columns provide better resolution for both the acidic and basic variants (compare Figure 2 Panel A to Panel B). By starting with a slightly higher initial salt concentration, the shallower gradient allows the columns to better resolve some of the variant peaks. Comparison data between the two columns indicate that Agilent Bio MAb, NP10 column produces significantly better peak resolution than the competitor's WCX column in both gradient systems and the main mAb peak has less tailing using the Agilent Bio MAb, NP10 column, providing better resolution of the basic variants.

Panel A: Linear gradient 10% to 35% mobile phase B, 60 min run time



Panel B: Linear gradient 15% to 35% mobile phase B, 60 min run time

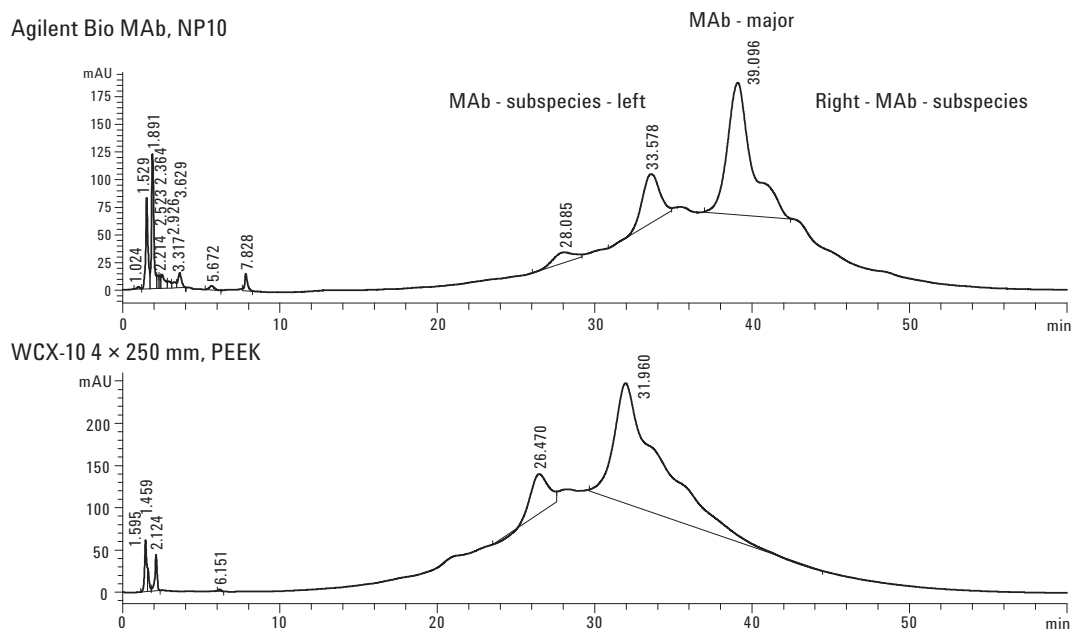


Figure 2. Optimization of linear gradient. Panel A: 10% to 35% mobile phase B, 60 min run time. Panel B: 15% to 35% mobile phase B, 60 min run time. A 15% to 35% B was started at 0 to 60 min. 100% B was started at 60.1 min to 70 min. A 5 µg mAb/run, 0.8 mL/min, 60 min run time, ran at temperature and detected at 220 nm wavelength.

Gradient optimization- 30 min run time for separation of monoclonal antibody

Ion exchange methods usually require long cycle times and long equilibration times. Decreasing run times can often decrease resolution, so speeding up these methods can be difficult. The previous 60-min methods were decreased to 30 min. Methods were again optimized for each column, and gradients of 05 to 30% and 15% to 30% mobile phase B linear gradient system were chosen for the competitor WCX column and the Bio MAb, NP10 column, respectively (complete method shown in Figure 3 legend). The goal of this method optimization was to maintain the separation profile and the resolution. Figure 3 indicates that the chromatograms from the optimized gradient for the 30 min run time are very comparable to the ones from the 60 min run time. The same peaks are observed, and the separation and resolution are well

defined. For the Bio MAb column there is slight loss of resolution in the basic forms, while the loss in resolution for these peaks is more visible for the competitor's WCX column. The major difference in these methods is the peak retention times. Peaks eluted sooner in the 30 min methods (compare Figure 2 to Figure 3). Different gradient systems were chosen for the two different columns, the competitor's WCX column required a shallower gradient. The peak retention times were longer compared to those from the Agilent Bio MAb, NP10 column, which used a steeper gradient system. This comparison shows that the Agilent Bio MAb, NP10 column has a better separation and resolution for this mAb separation in a 30-min run time. The Agilent BioMAB, NP10 column also generated a more stable baseline (Figure 3).

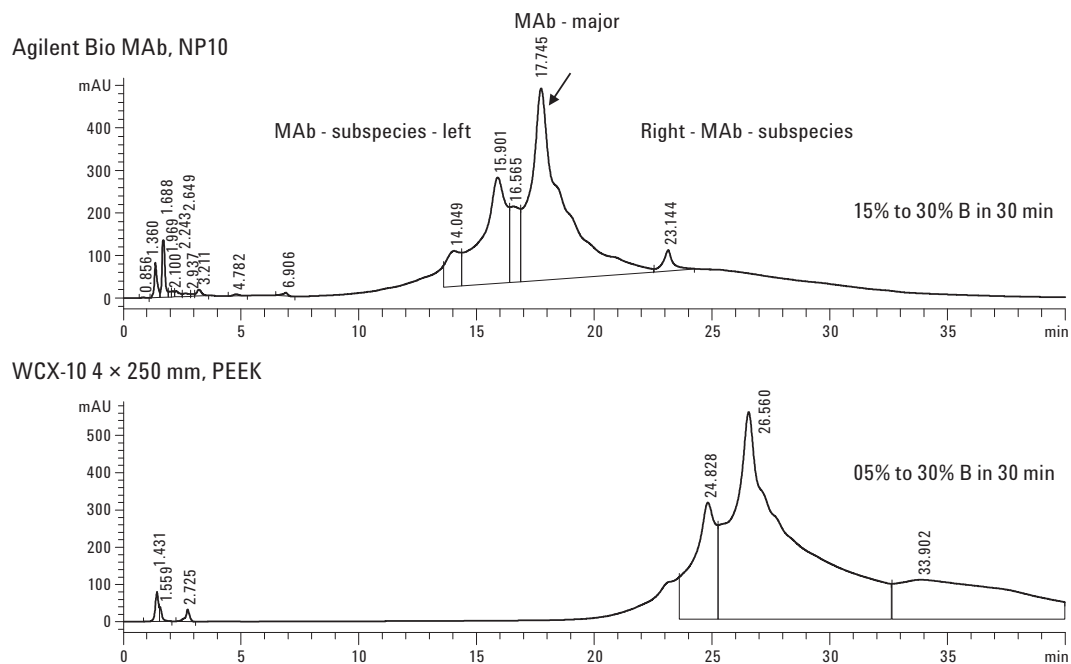


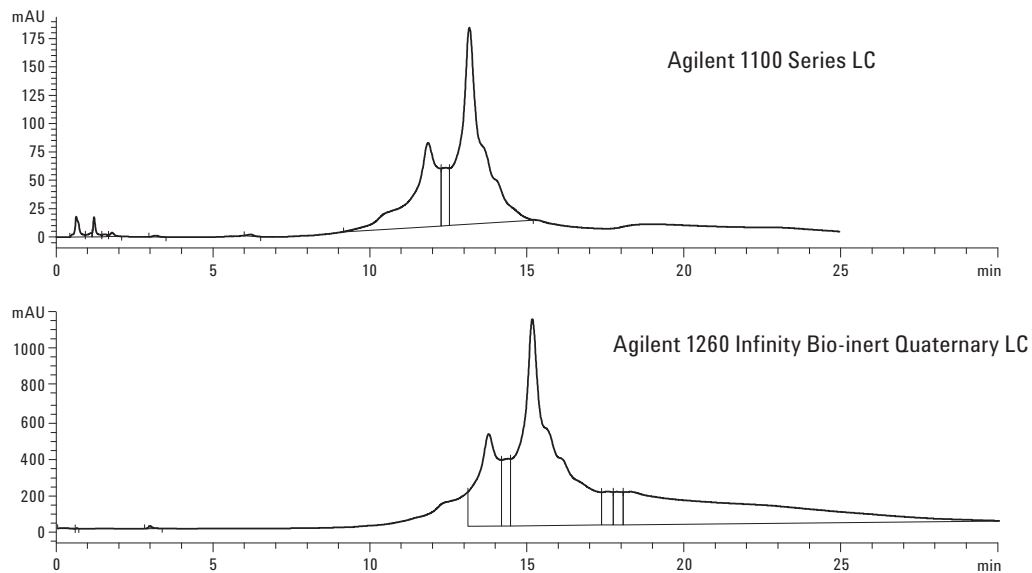
Figure 3. A 30-min runtime for monoclonal antibody separation. A 05% to 30% or 15% to 30% phase B gradient was started at 0 to 30 min. Phase B at 100% was started at 30.1 min to 40 min. A 5 µg mAb/run, 0.8mL/min, 60-min runtime, ran at temperature and detected at 220 nm wavelength.

Comparison of the Agilent 1100 Series LC to the Agilent 1260 Infinity Bio-inert Quaternary LC

The Agilent 1260 Infinity Bio-inert Quaternary LC uses a high sensitivity DAD and the system is constructed to prevent biological samples from coming in contact with metal surfaces. It separates biologics, such as monoclonal antibodies, with small injection volumes and maximum sensitivity. The superior performance of this new LC system was compared to the commonly used Agilent 1100 Series LC. The Agilent Bio MAb, NP10 column was used on both LC systems to separate the mAb as described in previous experiments (Figure 2, panel B). The same method, same injection volumes and concentration were used. The chromatogram from the Agilent 1260 Infinity

Bio-inert Quaternary LC showed a higher overall sensitivity (Figure 4, panel B), and a significantly better resolution of the mAb separation (Figure 4, panel A) compared to the separation on the Agilent 1100 Series LC. With the same sample concentration and injection volume, the signal from the Agilent 1100 Series LC is only at 175 mAU, while the signal from the Agilent 1260 Infinity Bio-inert Quaternary LC is more than 1000 mAU. Also, the basic peaks (right of the main mAb peak) on the Agilent 1260 Infinity Bio-inert Quaternary LC were better defined. These results suggest that the Agilent 1260 Infinity Bio-inert Quaternary LC is very suitable for analytical bioseparations.

Panel A: Different scale comparison of two LC systems using Agilent Bio MAb, NP10 4.6 mm × 250 mm column



Panel B: Same scale comparison of two LC systems using Agilent Bio MAb, NP10 4.6 mm × 250 mm column

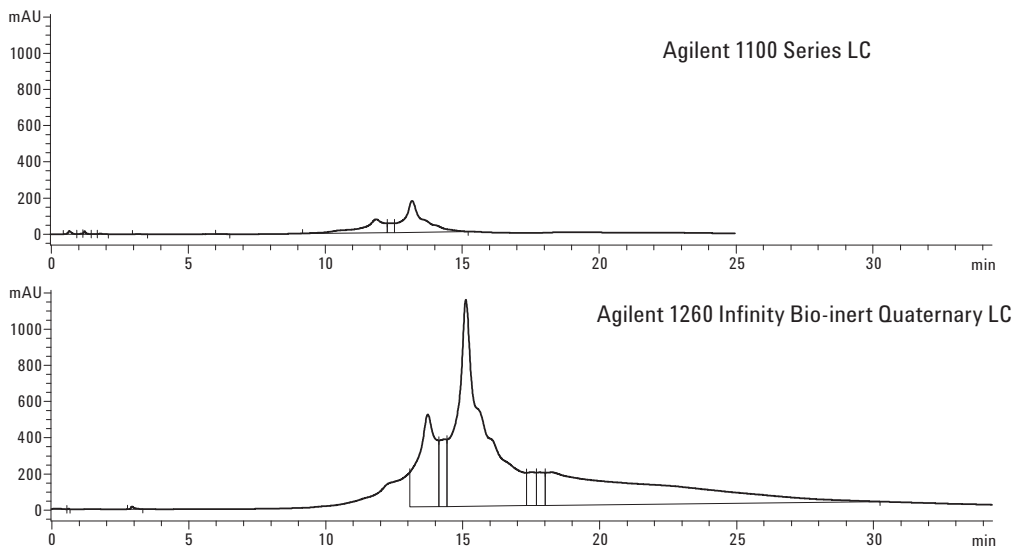


Figure 4. The Agilent 1100 Series LC versus the Agilent 1260 Infinity Bio-inert Quaternary LC on the same and different scale using the same operation method.

Conclusion

As the monoclonal antibody therapeutic market continues to grow, the need for high resolution monitoring of their stability and purity will continue to be critical. Choosing the best column and correct method conditions are critical for high resolution and a stable method. The data presented here shows that the Agilent Bio MAb, NP 10 column is very well suited for the separation of mAbs and their charge variants. These columns have the ability to work across a wide range of buffer pH (from 5.5 to 6.5) and at various salt concentrations, (0.025 M and 0.05 M, data not shown for 0.05 M). The superior performance of the Agilent 1260 Infinity Bio-inert Quaternary LC with high sensitivity DAD and 600 bar operating pressure allowed the Bio MAb column to separate and resolve the various acidic and basic charge variants of the mAb used in this study. The combination of the Bio MAb column and this highly sensitive instrument provides an excellent solution for the development of ion exchange methods for monitoring the purity and stability of therapeutic monoclonal antibodies.

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