pH Gradient Elution for Improved Separation of Monoclonal Antibody Charge Variants

Application Note

Biopharmaceuticals

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Abstract

Ion exchange is a very useful technique to separate protein mixtures under mild conditions, most commonly by applying a shallow gradient of increasing salt concentration as eluent. pH gradient elution is used much less frequently, except in specialist applications such as chromatofocusing, and can require complex buffer systems. However, today's modern quaternary HPLC systems are ideally suited to generating pH gradient elution profiles from conventional buffer salts. In this application note, we demonstrate the benefits of using pH gradient elution for separation of charge isoforms of monoclonal antibodies using Agilent Bio MAb columns on an Agilent 1260 Infinity Bio-inert Quaternary LC system with Agilent Buffer Advisor software.

Agilent Technologies

Introduction

Proteins are complex biomolecules containing numerous amino acids possessing acid and basic side-chain functionality. These functionalities range from carboxylic acids (aspartic acid and glutamic acid) and phenol (tyrosine) to amines (lysine and histidine) and guanidine (arginine), as well as other neutral polar or hydrophobic residues. At a given pH, proteins will possess an overall charge (except at their isoelectric point where the net charge is 0), and will interact with sorbents with a complementary charge. Basic proteins are therefore retained on cation-exchange columns.

Conventional ion-exchange methods use an eluent of increasing ionic strength (salt concentration) in order to compete with and ultimately overcome the interaction between protein and sorbent and cause the molecule to elute from the column. However, pH gradients may also be used so that, with cation-exchange chromatography, an increase in pH will cause the protein to become neutral or negatively charged and will elute from the column.

This approach has begun to receive greater attention as more complex proteins, such as monoclonal antibodies that may possess charge variants arising from subtle changes during biomanufacture, are studied and analyzed. These differences include loss of C-terminal lysine, deamidation and sialylation (making the molecule more acidic), or succinimide formation and amidation of COOH side chains (making the molecule more basic), as shown in Figure 1.

We have shown methods to create pH gradients that allow separation of charge variants of monoclonal antibodies that are otherwise difficult to resolve using conventional salt gradient elution.

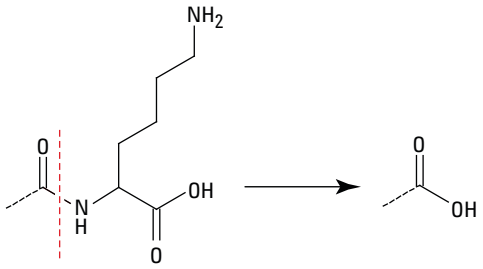
Materials and Methods

Agilent Bio IEX columns are packed with rigid polymeric, nonporous particles grafted with a functionalized hydrophilic polymer layer. The rigid particles provide high resolution and high separation efficiency by reducing the band broadening effects resulting from diffusion limitations with totally porous particles. The chemically bonded hydrophilic coating significantly reduces the effects of non-specific binding and results in greater levels of recovery.

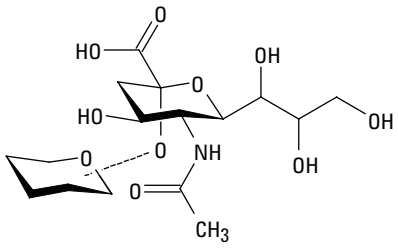
Buffer compositions of known ionic strength and pH, through the use of the quaternary channel capabilities of the Agilent 1260 Infinity Bio-inert Quaternary LC pump, were prepared by combining different proportions of sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate solutions. This buffer system is commonly used in laboratories across a wide pH range (typically pH 5.7 to 8.0).

To facilitate this process, a prototype software program, Buffer Advisor, from Agilent was used (Figures 2a, 2b, and 2c). By entering the appropriate buffer conditions and the composition of the stock solutions, the software calculates the necessary gradient timetable for use with a quaternary pump.

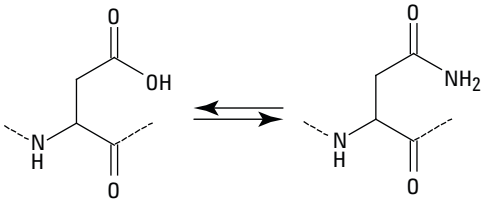
(a) Loss of C-terminal lysine



(b) Sialylation of glycans



(c) Amidation/deamidation



(d) Succinimide formation

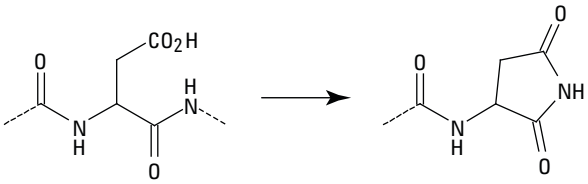


Figure 1. Reactions leading to charge variants.

Conditions

Columns	Agilent Bio MAb, 5 µm, 4.6 × 250 mm
Mobile phase	A: water B: 1.6 M NaCl C: 100 mM NaH_2PO_4 D: 100 mM Na_2HPO_4 By combining predetermined proportions of C and D, buffer solutions at the desired pH range were produced at the selected buffer strengths.
Gradient	pH 6.0 to 8.0, 0 to 20 minutes 0 to 800 mM NaCl, 20 to 25 minutes 800 mM NaCl, 25 to 30 minutes
Flow rate	1.0 mL/min
Temperature	ambient
Injection volume	10 µL
Sample	IgG monoclonal antibody
Sample concentration	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)
Detection	UV, 220 nm
Instrument	Agilent 1260 Infinity Bio-inert Quaternary LC system

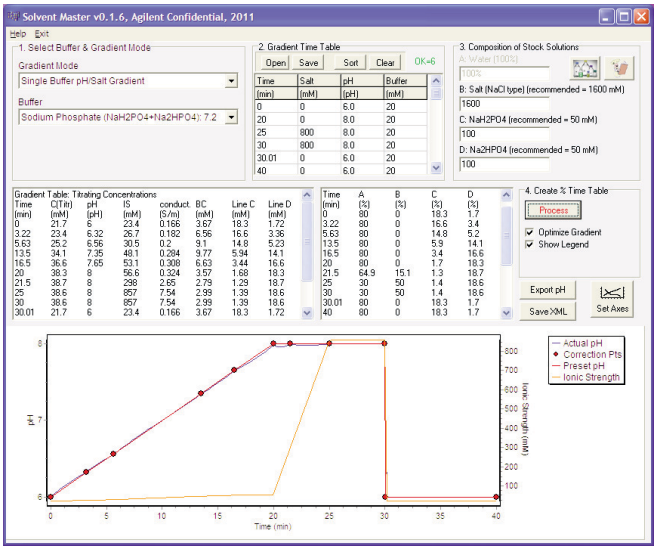


Figure 2a. Screenshot from prototype Buffer Advisor software (showing additional interim gradient steps calculated to maintain a linear pH gradient: 3.22, 5.63, 13.5, 16.5 and 21.5 minutes).

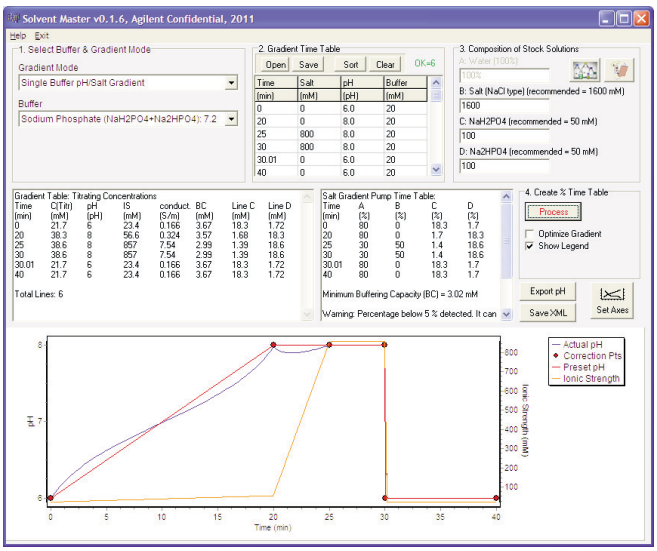


Figure 2b. Screenshot from prototype Buffer Advisor software (non-optimized, without interim gradient steps, showing non-linearity of gradient).

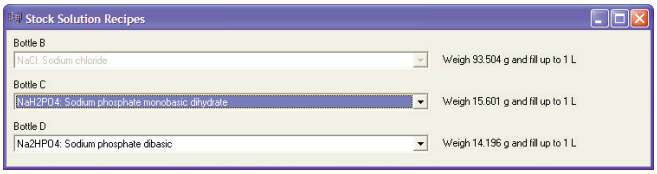


Figure 2c. Screenshot from prototype Buffer Advisor software (showing recipe for preparing buffer stock solutions).

Results and Discussion

We have shown the importance of pH in the use of weak cation-exchange columns for protein separations, and the effect it has on retention times when working with salt gradients (Agilent application note 5990-9628EN). Slight changes in pH can be used to alter selectivity. However, the behavior of a weak cation-exchange column is also affected by ionic strength (Figure 3). It is therefore unsurprising that ionic strength also plays an important part in pH gradient experiments.

Figure 4 shows a series of chromatograms of an IgG monoclonal antibody. Each was run from pH 6.0 to 8.0 (0 to 20 minutes), followed by a conventional salt gradient clean-up (20 to 25 minutes) and re-equilibration (25.01 to 35 minutes). Each chromatogram was obtained at different buffer concentration

and resulting ionic strengths (20 to 50 mM). For this task, the Buffer Advisor software was used to create the necessary gradient timetable from the same stock solutions.

In all instances, the IgG elutes during the latter part of the pH gradient (between pH 7.0 and 8.0), and in fact at 20 mM, the IgG sample does not elute until the salt clean-up portion of the gradient, and therefore the method can be refined to optimize the separation.

Figure 4 shows that with 30 mM buffer strength elution conditions, a greater degree of resolution is observed. However, this may be due to the fact that the IgG elutes later in the gradient. Therefore, the Buffer Advisor software was used to program a shallower pH gradient for both the 30 mM and 50 mM buffer strength separations, from pH 7.0 to 8.0, 0 to 20 minutes and from pH 6.5 to 7.5, 0 to 20 minutes, respectively.

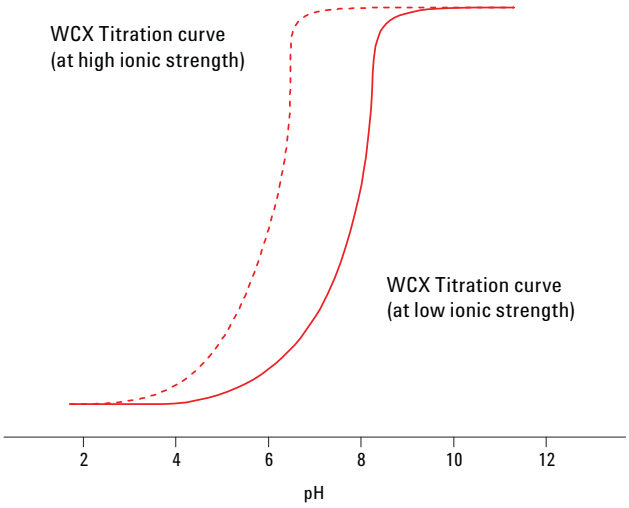


Figure 3. Typical titration curve for weak cation-exchanger.

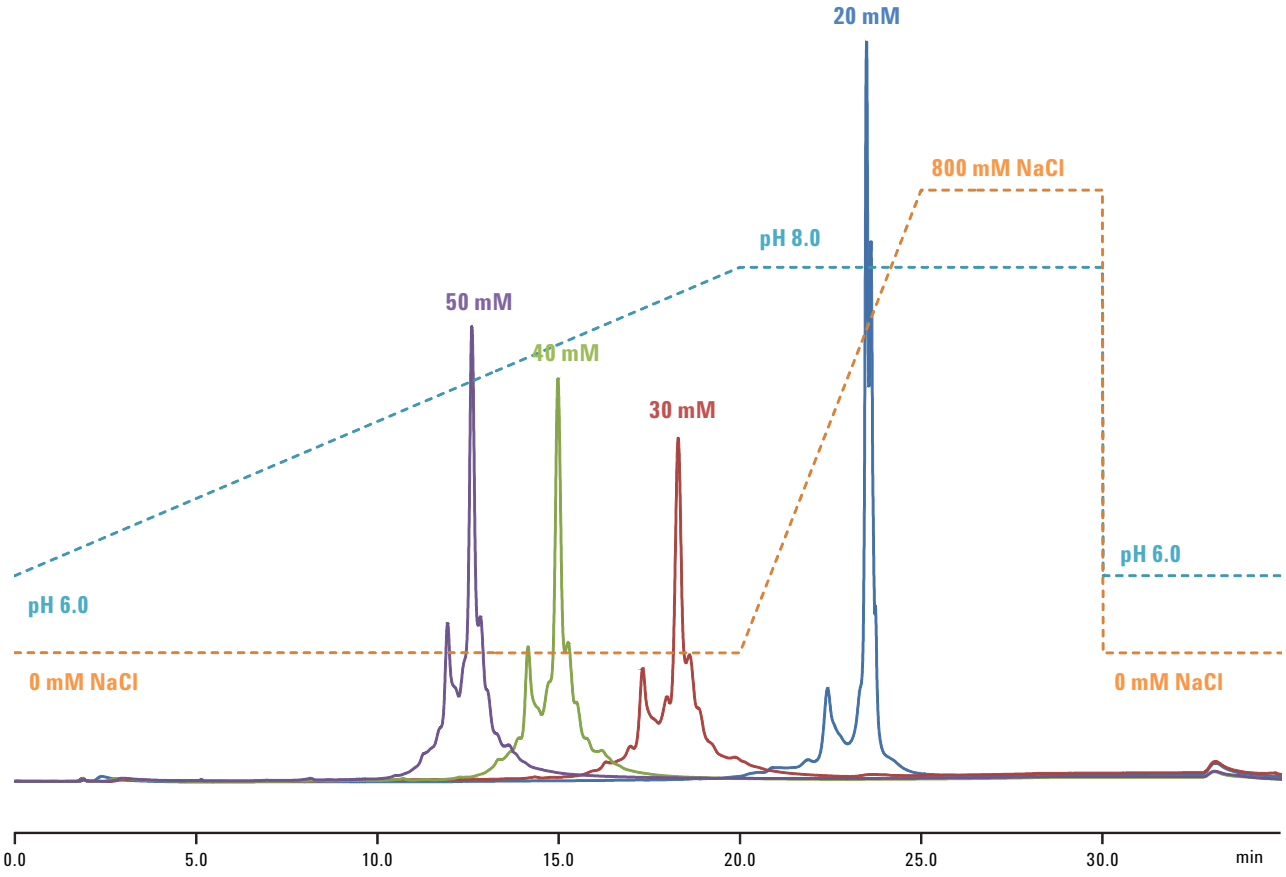


Figure 4. Chromatograms of IgG monoclonal antibody at different ionic strengths.

Figures 5a and 5b show the resulting chromatograms with the main IgG peak eluting at 11 minutes (with acidic variants eluting earlier and basic variants eluting later). The two chromatograms are in fact remarkably similar, although slightly better peak definition for the charge variants is evident at 30 mM buffer strength.

Further optimization could be performed to try and improve resolution, perhaps by extending the gradient over a longer time period or by reducing the pH range still further.

Conclusions

The use of pH gradient ion-exchange chromatography for separating complex proteins, such as monoclonal antibodies, is a valuable tool in the chromatographer's armory. However, simply preparing two different buffer solutions at different pH values and running a linear gradient does not result in a linear change in pH. Computer software is necessary to calculate the refinements needed to ensure the desired gradient outcome. The technique is sensitive to the analysis conditions and ionic strength as well as starting and ending pH play a part in selectivity.

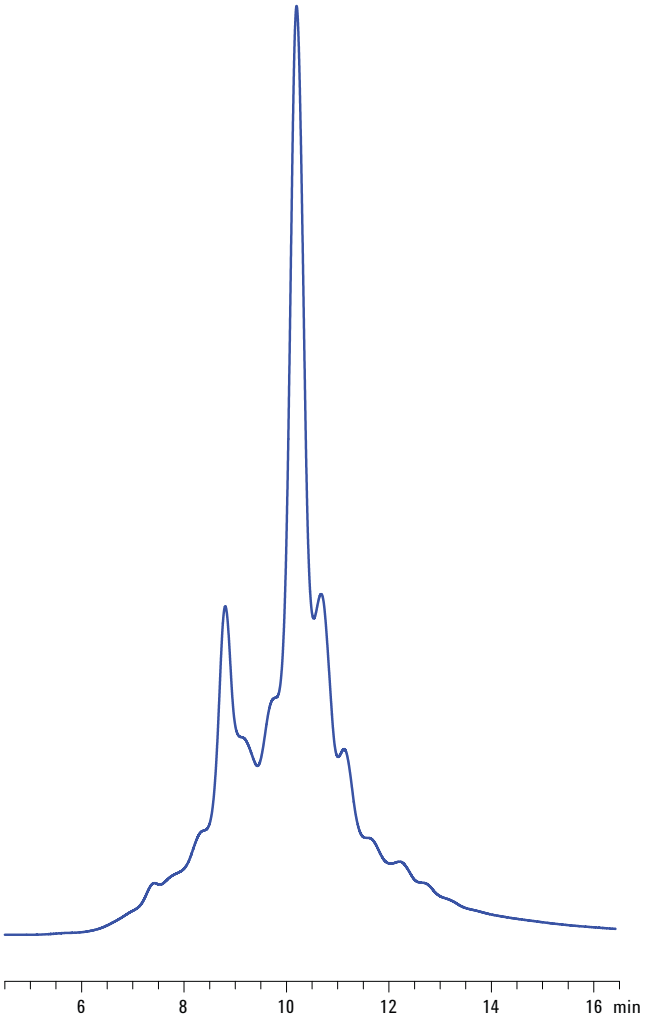


Figure 5a. Chromatogram of IgG monoclonal antibody pH 7.0 to 8.0 (0 to 20 minutes), 30 mM.

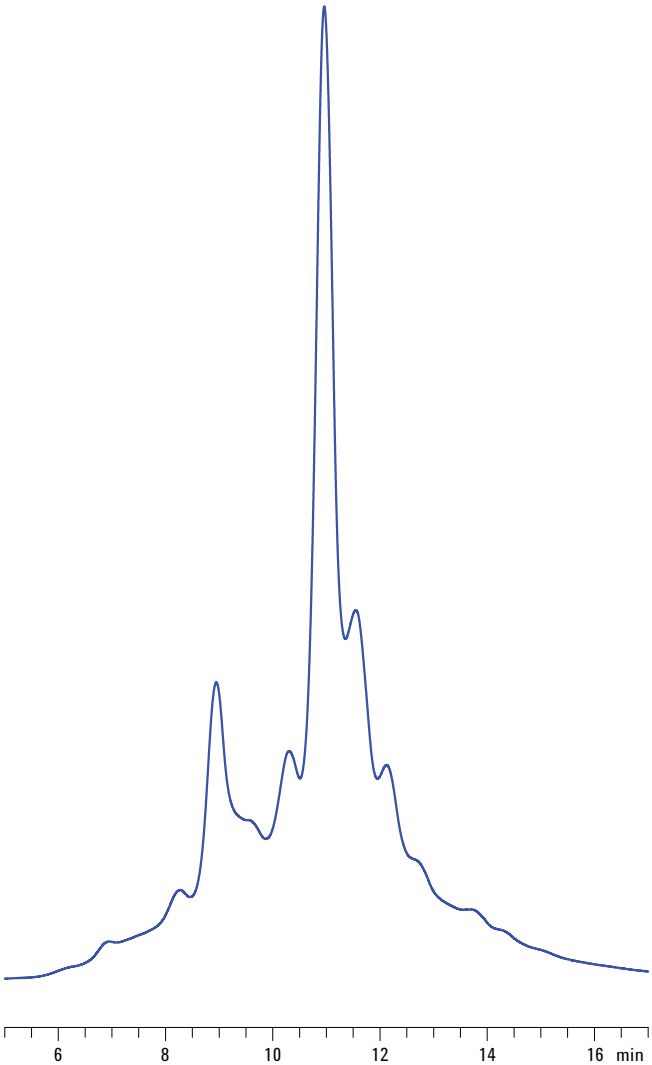


Figure 5b. Chromatogram of IgG monoclonal antibody pH 6.5 to 7.5 (0 to 20 minutes), 50 mM.

For More Information

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