Charge Variant Analysis of Monoclonal Antibodies by pH Gradient Separation on Cation-exchange Columns

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Introduction

Proteins are complex biomolecules containing numerous amino acids many of which possess acidic and basic side chains. 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.

Methods of manufacture of recombinant proteins such as monoclonal antibodies may result in formation of charge variants caused by a variety of side reactions (Figure 1), but the overall effect of these differences may be subtle in a large biomolecule such as IgG which has a molecular weight of around 150-160 kDa, equating to around 1330 individual amino acids (approximately 450 amino acids per heavy chain and 215 amino acid per light chain). Consequently analysis by liquid chromatography can prove challenging.

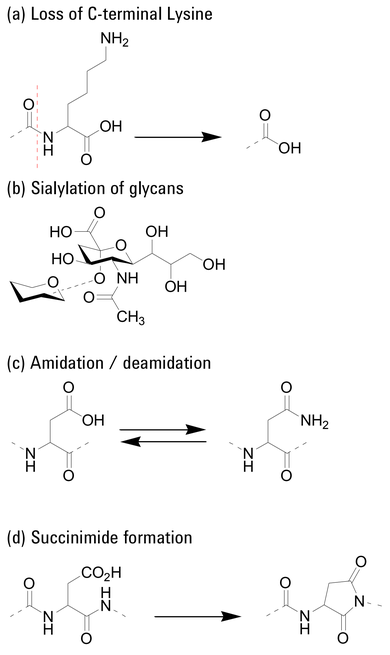

Figure 1. Side reactions leading to charge variants

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.

Normally pH gradient elution is used infrequently, 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. pH gradients may therefore be used so that, with cation-exchange chromatography, an increase in pH will cause the protein to become neutral or negatively charged and will result in elution from the column. Consequently, this approach has begun to receive greater attention particularly for analysis of more complex proteins, such as monoclonal antibodies.

Materials and Method

The range of Agilent Bio IEX columns are packed with rigid polymeric, non-porous 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 – 8.0).

To facilitate this process, a prototype software program, Buffer Advisor, from Agilent was used (Figures 2a and 2b). By entering the appropriate buffer conditions and the composition of the stock solutions (or relying on the software to recommend the best choice of stock solution and the formulation for creating the required concentration), the software calculates the necessary gradient time table for use with a quaternary pump.

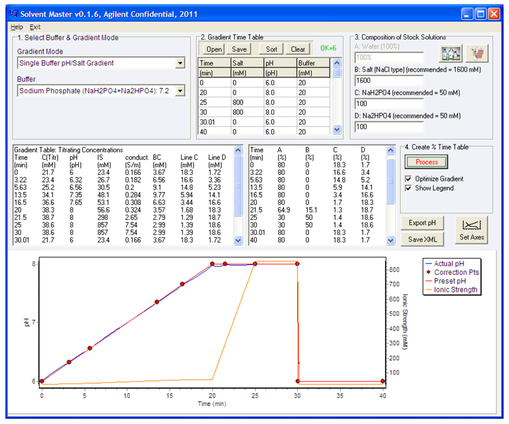

Figure 2a. Screenshot from prototype Buffer Advisor software

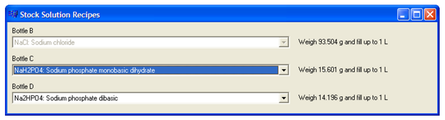

Figure 2b. Screenshot from prototype Buffer Advisor software showing recipe for preparing buffer stock solutions

Conditions

Columns	Agilent Bio MAb NP5, 4.6 x 250 mm SS Agilent Bio MAb NP1.7, 4.6 x 50 mm SS
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 & D as determined by the Buffer Advisor software, buffer solutions at the desired pH range and strength were created.
Gradient	pH 6.0 – 8.0, 0 – 20 minutes 0 – 800 mM NaCl, 20 – 25 minutes 800 mM NaCl, 25 – 30 minutes
Flow rate	1.0 mL/min
Temperature	Ambient
Injection volume	10 µL
Sample	IgG monoclonal antibody
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

Results and Discussion

We have previously found 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. 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. It is therefore unsurprising that ionic strength also plays an important part in pH gradient experiments.

Figure 3 shows a series of chromatograms of an IgG monoclonal antibody. Each was run from pH 6.0 – 8.0 (0 – 20 minutes), followed by a conventional salt gradient clean-up (20 – 25 minutes) and re-equilibration (25.01 – 35 minutes). Each chromatogram was obtained at different buffer concentration and resulting ionic strengths (20 – 50 mM). For this task the Buffer Advisor software was used to create the necessary gradient time table from the same stock solutions.

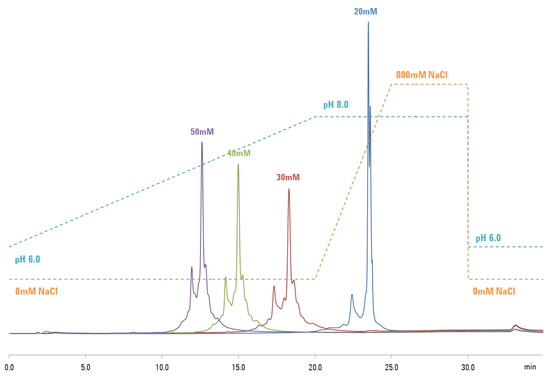

Figure 3. Chromatogram of IgG monoclonal antibody at different ionic strengths

Results and Discussion

From Figure 3 it can be seen that, 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. With 30 mM buffer strength elution conditions, a greater degree of resolution is observed. It was therefore decided to explore the separation of this antibody at 30 mM buffer strength using a smaller column packed with particles of 1.7 µm in diameter.

In order to maximize the benefits of the smaller, higher performance column the gradient was modified to reduce the length of time per analysis. The conditions used were pH 7.0 – 8.0 (0 – 4 minutes), followed by a conventional salt gradient clean-up (4 – 7 minutes) and re-equilibration (7 – 9 minutes).

Figure 4 shows the resulting chromatogram with the main IgG peak eluting at 11 minutes (with acidic variants eluting earlier and basic variants eluting later).

Further optimization could be performed to try and improve resolution, perhaps by shortening the gradient or by reducing the pH range still further.

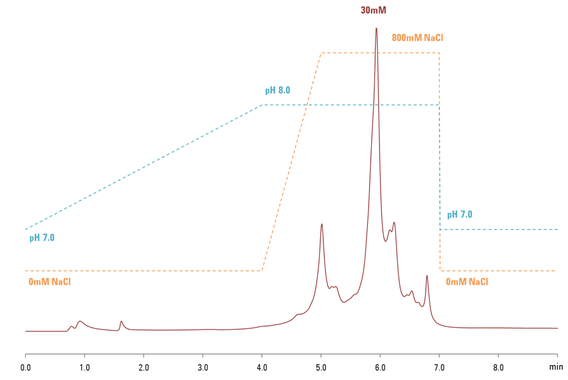

Figure 4. Chromatogram of IgG monoclonal antibody at different ionic strengths

Conclusion

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.

It is also possible to utilize smaller column sizes packed with high efficiency particles to significantly reduce runs times allowing greater throughput.

For more information visit

www.agilent.com/chem/bioHPLC

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