Optimizing Protein Separations with Agilent Weak Cation-Exchange Columns

Application Note

Biopharmaceuticals

Author

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Abstract

Columns containing weak cation-exchange stationary phases offer a degree of versatility when used for separation of positively charged proteins. Of particular interest, is the difference in selectivity that can be obtained by adjusting the pH of the mobile phase eluent. We explore the parameters of interest and how these can be changed to optimize the resolution of the compounds of interest using Agilent Bio WCX columns on an Agilent 1260 Infinity Bio-inert Quaternary LC system and Agilent Buffer Advisor software.

Introduction

Complex biomolecules such as proteins contain a large number of amino acids with acidic and basic side chain functionalities, including their N- and C-termini. These residues are most commonly weak in strength and so the net charge of the molecule is dependent on the pH of the surrounding environment. Unlike more common strong cation-exchange sorbents (typically possessing a sulfopropyl functionality which remains negatively charged across the normal operating range pH 2 to 12), weak cation-exchange columns possess carboxyl functionality. This means that at moderate pH (pH 4 to 6) the level of ionization may well change and can also be affected by the ionic strength (Figure 1).

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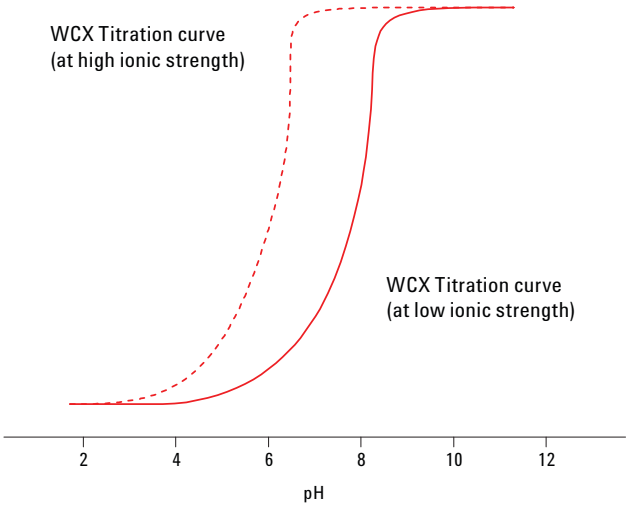

Figure 1. Typical titration curve for weak cation-exchanger.

This is particularly true of the rigid, highly crosslinked, nonporous polystyrene beads with grafted polymeric functional layer that make up the Agilent Bio WCX and Bio MAb product range.

For the solute molecules to be retained, they must possess a net positive charge and the sorbent must possess a net negative charge. Consequently, the normal operating range for a weak cation-exchange column is much narrower than for a strong cation-exchange column, typically pH 5.5 to 8.5 (but can be higher for more basic analytes).

We have demonstrated here the use of hardware and software tools available to quickly and easily maximize the performance and selectivity of your Agilent Bio WCX columns to improve protein separations.

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 (Figure 2).

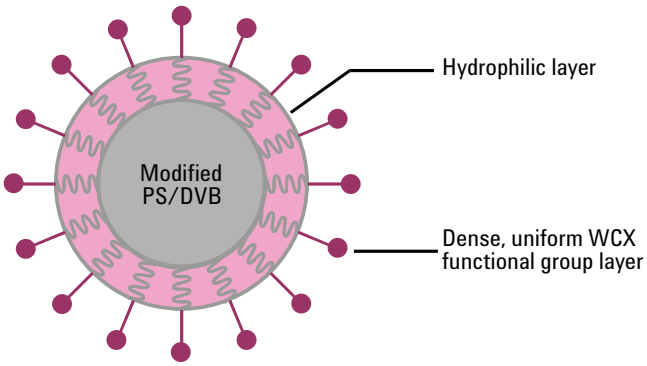

Figure 2. Composition of the Agilent Bio IEX particle.

Buffer compositions of known ionic strength and pH were prepared by using the quaternary channel capabilities of the Agilent 1260 Infinity Bio-inert Quaternary LC pump, by combining different proportions of sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate solutions. This method is similar to the common laboratory technique used for the preparation of Sörensen buffers ranging from pH 5.8 to 8.0 using stock solutions of NaH_2PO_4 and Na_2HPO_4 (as described in Dawson *et al*., 1969).

To facilitate this process, a prototype software program Buffer Advisor from Agilent was used (Figure 3). 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 table for use with a quaternary pump.

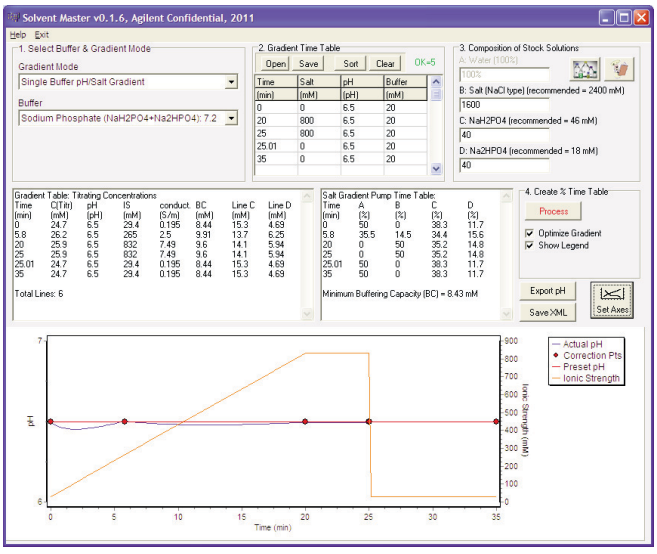

Figure 3. Screenshot from prototype Agilent Buffer Advisor software.

Conditions

Columns	Agilent Bio WCX, 10 µm, 4.6 × 250 mm Agilent Bio WCX, 5 µm, 4.6 × 250 mm
Mobile phase	A: water B: 1.6 M NaCl C: 40.0 mM NaH_2PO_4 D: 40.0 mM Na_2HPO_4 By combining predetermined proportions of C and D, 20 mM buffer solutions at the desired pH range were produced.
Gradient	0 to 50% B, 0 to 20 min 50% B, 20 to 25 min 0% B, 25 to 35 min
Flow rate	1.0 mL/min
Temperature	ambient
Injection volume	10 µL
Sample	Ovalbumin, Ribonuclease A, Cytochrome c, Lysozyme
Sample concentration	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)
Detection	UV, 220 nm
Instrument	Agilent Infinity 1260 Bio-inert Quaternary LC system

Results and Discussion

The retention times for four standard proteins were determined using a standard salt gradient (0 to 800 mM, 0 to 20 minutes) at a range of pH values (5.5, 6.0, 6.5, 7.0 and 7.5). The results are summarized in Figure 4 and are representative of weak cation-exchange columns; the elution order of the proteins is determined by their isoelectric point (pI). Ovalbumin, with a rather low pI (pI 4.5), was chosen as it is not retained on a weak cation-exchange column.

Ribonuclease A (pI 9.6; Tanford & Hauenstein, 1956), cytochrome c (pI 10.0 to 10.1; Barlow & Margoliash, 1966) and lysozyme (pI 11.0; MacRitchie & Alexander, 1963) were the other proteins chosen serving as standards.

The effect of pH on both the retention time and resolution of four protein standards is more clearly shown in the chromatograms in Figures 5a and 5b. However, to optimize the performance using weak cation-exchange columns it is necessary to consider both the pH and ionic strength of the mobile phase.

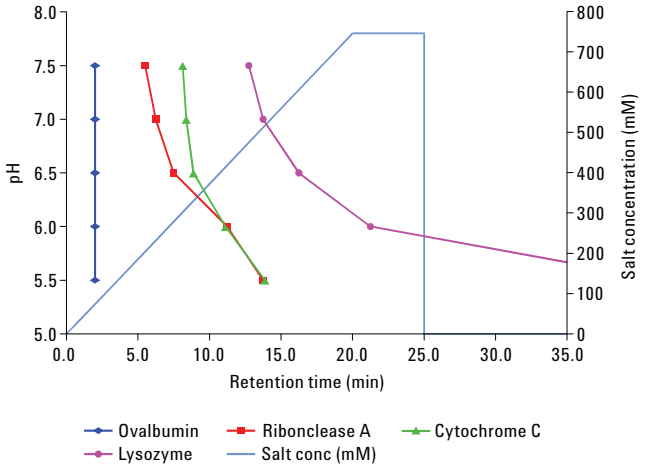

Figure 4. Effect of pH on retention time of protein standards using an Agilent Bio WCX 4.6 × 250 mm, 10 µm column.

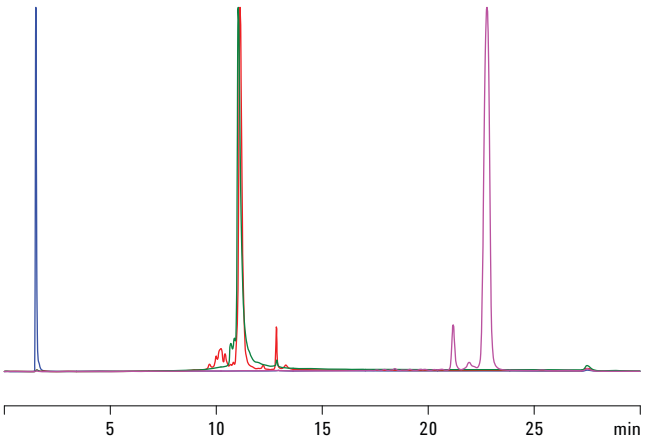

Figure 5a. Separation of protein standards at pH 6.0 using an Agilent Bio WCX, 10 µm, 4.6 × 250 mm column.

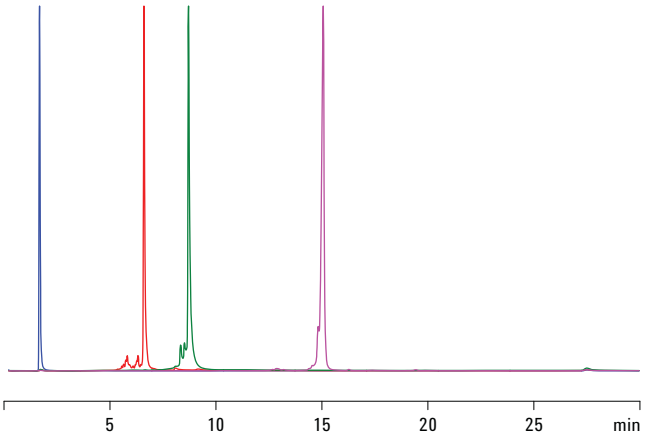

Figure 5b. Separation of protein standards at pH 7.0 using an Agilent Bio WCX, 10 µm, 4.6 × 250 mm column.

Figures 6a, 6b and 6c show the subtle differences between particles of 10 µm and 5 µm in diameter. Unlike totally porous particles, diffusion limited band broadening is restricted to the thickness of the surface coating on Agilent Bio IEX particles. The means that the retention time differences between 10 µm and 5 µm particles is minimal.

The difference in particle size does play a part in column efficiency and back pressure however, so improved resolution is possible as illustrated in Figure 6c where peak response is doubled on the 5 µm packed column compared to the 10 µm packed column.

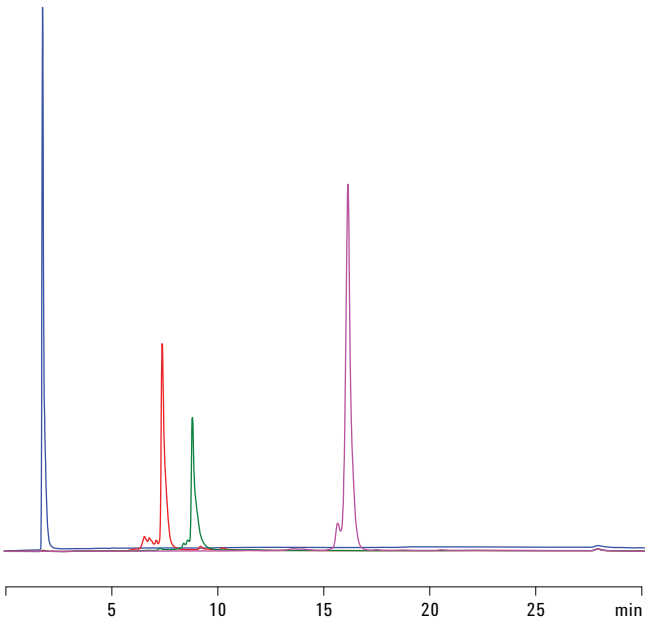

Figure 6a. Separation of protein standards at pH 6.5 using an Agilent Bio WCX, 10 µm, 4.6 × 250 mm column.

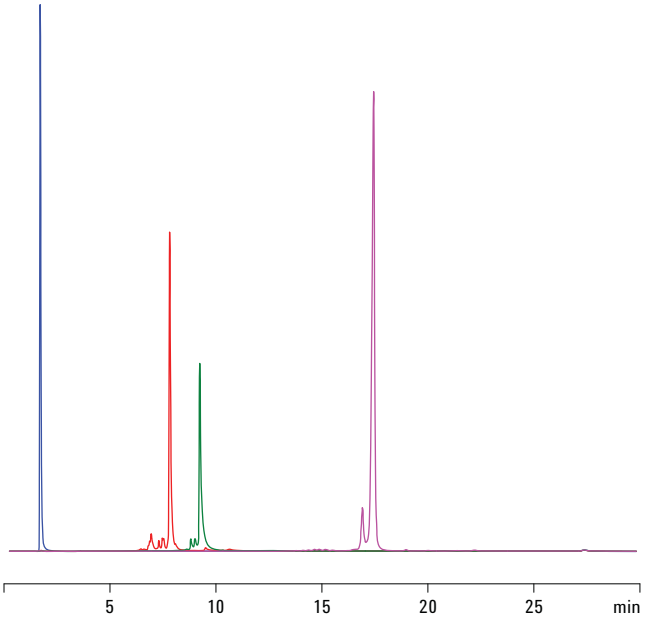

Figure 6b. Separation of protein standards at pH 6.5 using an Agilent Bio WCX, 5 µm*, 4.6 × 250 mm column.*

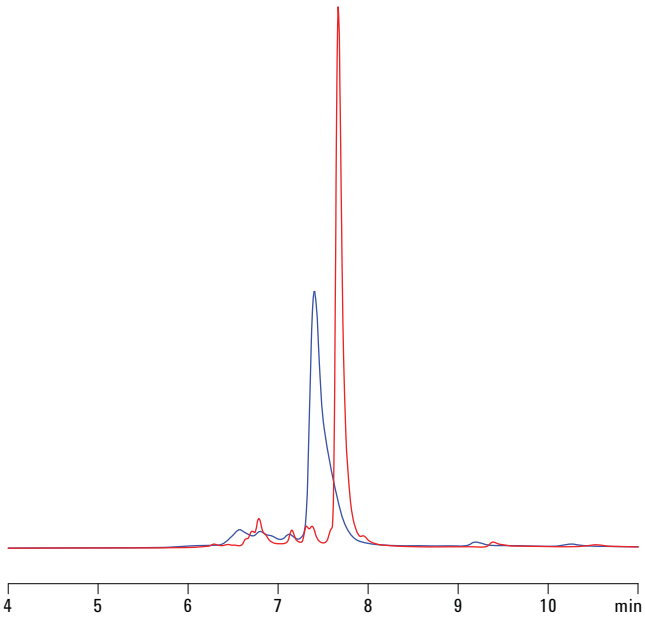

Figure 6c. Overlay of ribonuclease A on an Agilent Bio WCX, 10 µm, 4.6 × 250 mm column, pH 6.5 (blue trace) and an Agilent Bio WCX, 5 µm*, 4.6 × 250 mm column, pH 6.5 (red trace).*

Conclusions

The impact of changes in pH and buffer strength on retention times and selectivity of standard proteins with Agilent Bio WCX columns was revealed. The non-porous particle technology ensured that high performance separations were possible. In future work we will show how these insights can be used to optimize the separation of complex biomolecules such as antibodies, including the use of pH gradient separations. We will also demonstrate the ability to greatly reduce analysis times by using smaller particle sized sorbents packed in shorter columns.

References

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