# Faster Separations Using Agilent Weak Cation Exchange Columns

## Application Note

BioPharma


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## Abstract

Ion exchange is a commonly used technique for the separation of complex protein mixtures. Traditionally, such separations are performed using shallow gradients of increasing salt concentration with long column lengths providing the necessary resolution. The columns have often been packed using large diameter particles to minimize backpressure. This application note demonstrates how analysis times can be significantly reduced, increasing throughput without compromising analytical performance, by exploiting the benefits of small particle size, non-porous ion exchange sorbents.


Agilent Technologies

## Introduction

Proteins, polypeptides and oligonucleotides are often analyzed by ion exchange chromatography because they are complex molecules with multiple charges on their surfaces. The technique is ideally suited to the separation of charged biomolecules as it is nondenaturing and can provide good performance and resolution.

Traditionally, this has meant using highly porous particles to enable such large molecules to permeate the particles. In turn, columns of 15 cm or 25 cm in length, packed with 5 µm or 10 µm particles are commonly used.

The advent of non-porous sorbents such as Agilent's Bio IEX range, comprising a rigid polymeric core particle with a grafted hydrophilic layer containing the ion-exchange functionality, can improve resolution. This is because the diffusion-limited band broadening associated with a molecule penetrating the core of a large particle is eliminated. In turn, this means smaller particles and shorter column lengths can be used to significantly improve throughput, greatly reducing analysis times. The benefits for improved productivity for tasks such as fraction analysis are immediately evident.

## Materials and methods

Agilent Bio IEX columns are packed with rigid polymeric, nonporous particles grafted with a functionalized hydrophilic polymer layer. The resultant 1.7, 3, and 5 µm 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 nonspecific binding and results in greater levels of recovery.

### Conditions

| | |
|---|---|
| Columns | Agilent Bio WCX 5 µm, 4.6 × 250 mm SS (p/n 5190-2445)<br>Agilent Bio WCX 3 µm, 4.6 × 50 mm SS (p/n 5190-2443)<br>Agilent Bio WCX 1.7 µm, 4.6 × 50 mm SS (p/n 5190-2441) |
| Mobile phase | A: 20 mM sodium phosphate, pH 6.5<br>B: A + 1.6 M NaCl |
| Gradient | 0 to 50% B |
| Temperature | Ambient |
| Injection volume | 10 µL |
| Sample | Ovalbumin (1), Ribonuclease A (2), Cytochrome c (3), Lysozyme (4) |
| Concentration | 0.5 mg/mL |
| Detection | UV, 220 nm |
| Instrument | Agilent 1260 Infinity Bio-inert Quaternary LC system |

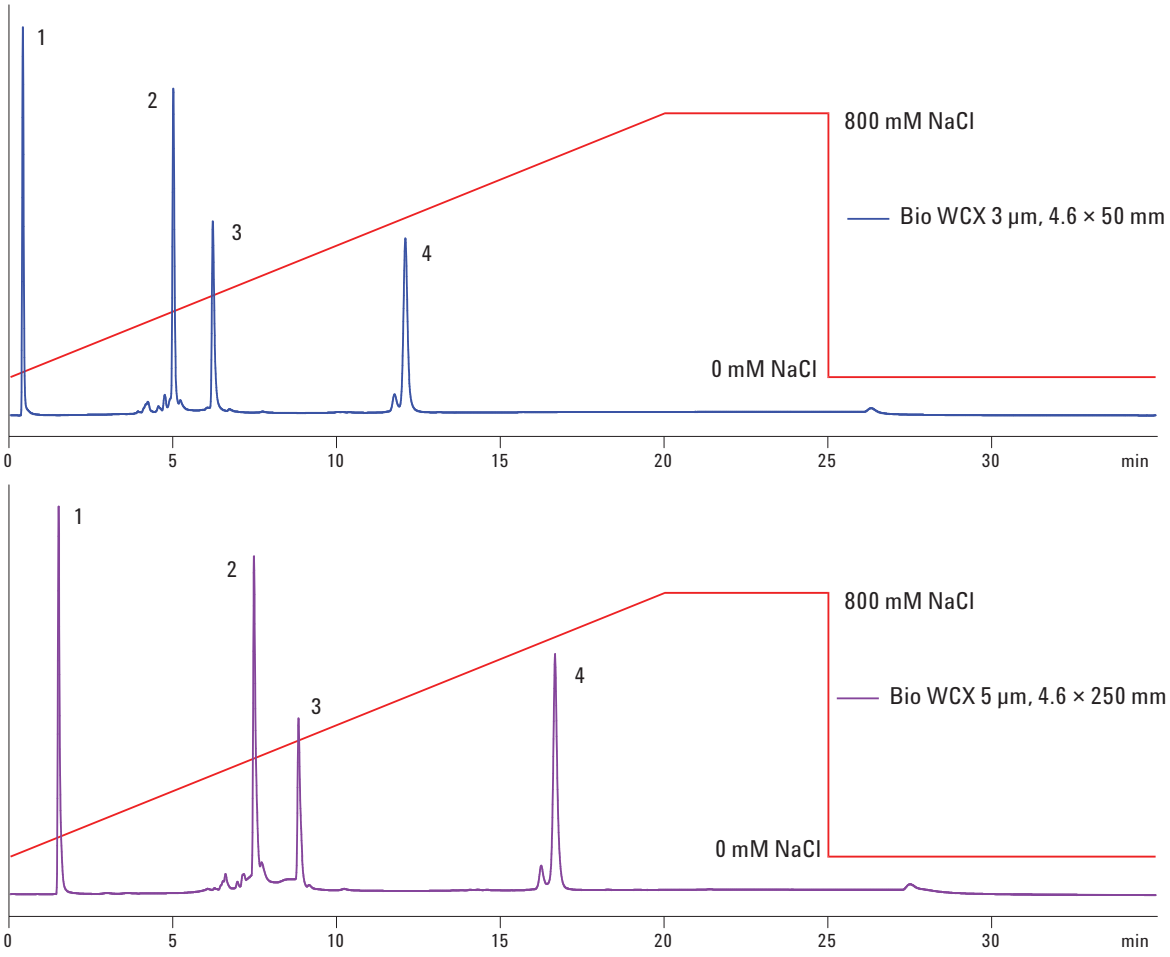


*Figure 1. Protein separation on Agilent Bio WCX 5 µm 4.6 × 250 mm versus Agilent Bio WCX 3 µm, 4.6 × 50 mm (flow rate 1.0 mL/min).*

## Results and Discussion

The performance of a column, as measured by plate count, is dependent on particle size and column length. From this it may be inferred that a shorter column packed with smaller particles can be used to achieve the same level of performance when compared to a longer column packed with larger particles (Figure 1). This is commonly found in practice. However, for gradient elution, further modifications to the method need to be employed to provide the additional benefits of shorter run times and greater productivity.

Converting gradient times into column volumes is a useful way of calculating the shorter gradient program and can provide the desired outcome in terms of higher speed separations (Table 1). However, smaller particle sizes may require higher flow rates to attain maximum performance. This is illustrated by the van Deemter curves shown in Figure 2.

To maximize the separation efficiency using the Agilent Bio WCX 3 µm, 4.6 × 50 mm column, the 4 minute gradient separation was carried out at 1.0, 1.5, 2.0, and 2.5 mL/min (Figure 3). As expected, the higher linear velocity created from higher flow rates improved the peak shape.

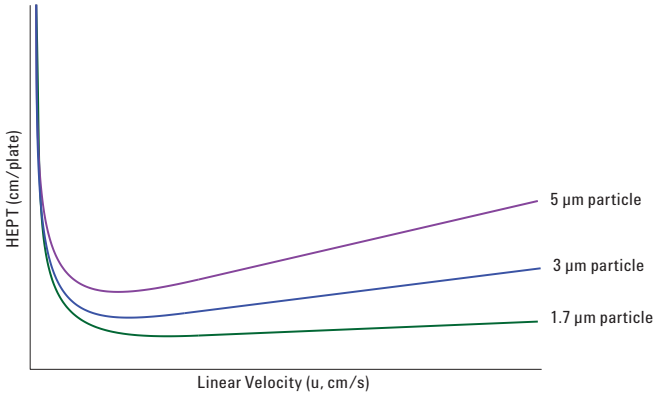


*Figure 2. Typical van Deemter curves.*

*Table 1. Gradient Time to Column Volume Conversion*

| Time (minutes) | mM NaCl | #CV |
|---|---|---|
| 0 | 0 | 0.0 |
| 20 | 800 | 4.8 |
| 25 | 800 | 6.0 |
| 25.01 | 0 | 6.0 |
| 35 | 0 | 8.4 |

#CV = number of column volumes at 1.0 mL/min (4.6 × 250 mm column)

| Time (minutes) | mM NaCl | #CV |
|---|---|---|
| 0 | 0 | 0.0 |
| 4 | 800 | 4.8 |
| 5 | 800 | 6.0 |
| 5.01 | 0 | 6.0 |
| 7 | 0 | 8.4 |

#CV = number of column volumes at 1.0 mL/min (4.6 × 50 mm column)

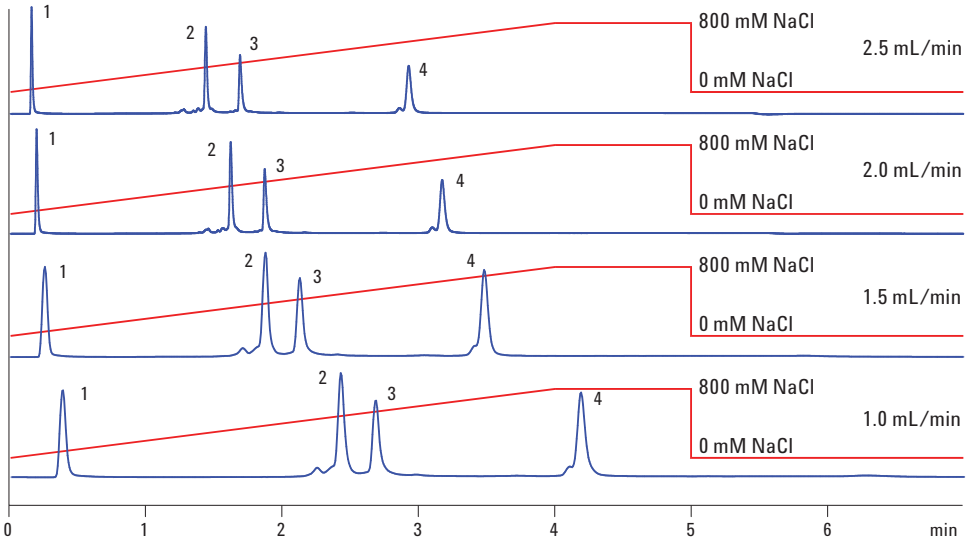


*Figure 3. Effect of flow rate on chromatographic performance (Agilent Bio WCX 3 µm, 4.6 × 50 mm).*

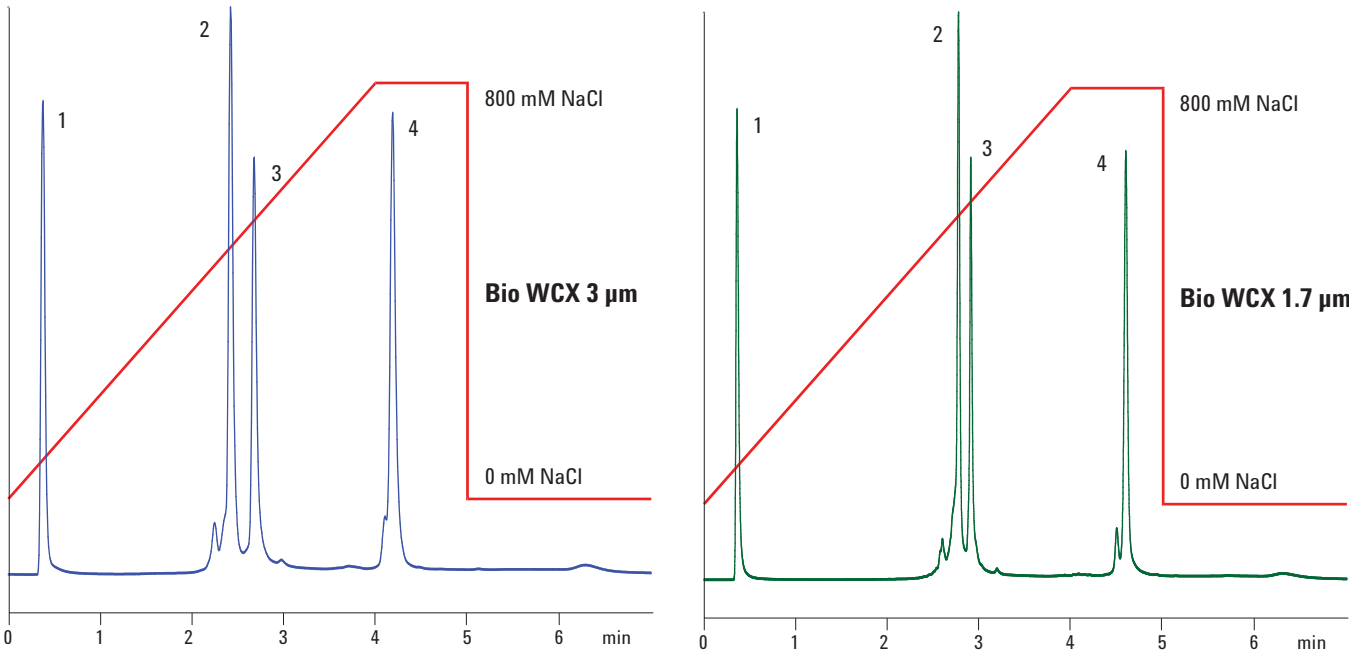


*Figure 4. Comparison of Agilent Bio WCX 3 µm, 4.6 × 50 mm versus Agilent Bio WCX 1.7 µm*

In comparison, the Agilent Bio WCX 1.7 µm, 4.6 × 50 mm column provided sharper peaks under identical conditions (Figure 4).

Increasing the flow rate should mean that it is possible to further reduce the gradient time. This was investigated using the Bio WCX 1.7 µm, 4.6 × 50 mm column. The 0 to 800 mM NaCl gradient was reduced from 4 to 2 minutes.

It was found that at a flow rate of 1.7 mL/min the backpressure remained below 400 bar and still provided exceptional peak shape and resolution (Figure 5).

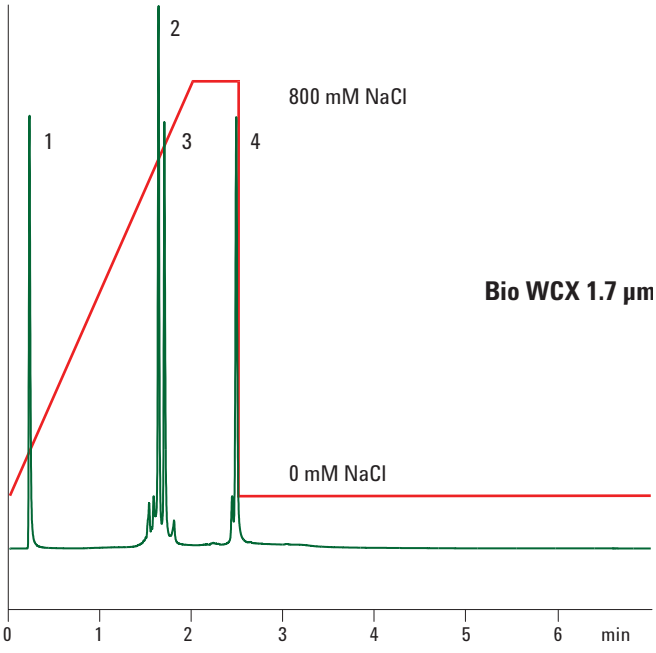


*Figure 5. Agilent Bio WCX 1.7 µm, 4.6 × 50 mm for protein separations under 3 minutes (flow rate 1.7 mL/min).*

## Conclusions

We have shown that by using shorter 5 cm columns packed with smaller particle size (3 µm and 1.7 µm), Agilent Bio WCX products can lead to significant reductions in run times from 20 or 30 minutes down to less than 3 minutes, and still retain excellent peak resolution. This enables much higher throughput in time-critical applications.

The backpressure of 400 bar shows that, by reducing the analysis time dramatically from over 30 minutes to less than four minutes for the entire gradient, a 600 bar system such as the Agilent 1260 Infinity Bio-inert LC is still sufficient.

## For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

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