Technical Overview

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Scale-Up of Protein Separations from Analytical to Semipreparative HPLC

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Introduction

Method development for preparative HPLC is the same as for analytical HPLC – adjusting conditions for optimal chromatographic performance. That is why it is convenient and cost-effective to modify the analytical column method by scaling up the analytical conditions to achieve the preparative separation needed for the collection and isolation of compounds. This application note demonstrates how easy it is to develop a gradient separation of four proteins on a 4.6 × 250 mm, 5 µm, Agilent ZORBAX 300SB-C8 analytical column, then optimize for sample load on a 9.4 mm inner diameter (id) column of identical length and packing material.

Agilent ZORBAX 300SB analytical columns can provide:

- Quick and easy scale-up of protein separations from analytical to preparative HPLC
- Optimization of separations and reduced costs
- Minimal time spent on method development

Conditions	
Column	Agilent ZORBAX 300SB-C8, 4.6 × 250 mm, 5 µm (p/n 880995-906)
Mobile Phase	A) 0.2% TFA in water B) 0.15% TFA in acetonitrile
Injection Volume	5 µm
Flow Rate	1.0 mL/min
Detector	UV (280 nm)
Temperature	40 °C
Gradient	(A) 26 to 55% B for 30 min (B) 26 to 50% B for 30 min

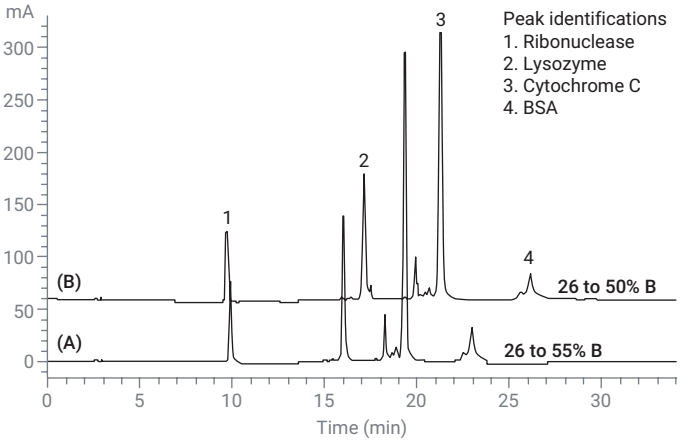

Figure 1. Optimizing gradient conditions for protein separation on an Agilent ZORBAX 300SB-C8, 4.6 × 250 mm, 5 µm column.

Method development

Using an analytical column to develop a preparative method not only streamlines the process of preparative method development, but minimizes the amount of solvent and sample required for method optimization. For this protein scale-up exercise, a wide-pore packing, ZORBAX 300SB-C8 was selected, which maximized diffusion of large molecules into the pore structure of the packing, thereby maximizing protein peak separation efficiency and sample loading. The ZORBAX 300SB-C8 analytical and semipreparative columns chosen have ids of 4.6 and 9.4 mm, respectively, and have identical column lengths, making gradient scale-up, in particular, straightforward.

Using a trifluoroacetic acid/acetonitrile (TFA/ACN) gradient, four proteins were resolved on the selected analytical column, as shown in Figure 1 (A). Gradient conditions were optimized to give a good overall separation. Since the goal was to scale up to a preparative column while achieving maximum resolution between the individual proteins, the actual gradient used was slightly shallower (Figure 1 (B)) with a slight increase in analysis time. In general, gradient conditions accommodate larger injection volumes—up to 100 µL, equivalent to a loading of 1 mg/protein. This separation was performed under these finalized conditions without significant loss in resolution (results are shown in reference 1). Preparative scale-up can now be performed.

Preparative scale-up

As stated previously, since ZORBAX analytical, semipreparative, and PrepHT preparative columns are packed with identical packings and with identical column lengths, scale-up can simply involve the change of columns and flow rate. Flow velocity must be constant to maintain the same time as for the analytical separation. The flow rate used in preparative separations is higher at the same linear velocity because the column diameter, and thus the volume, is larger.

Flow rate scale-up

$$F_{preparative} = (d_{preparative}/d_{analytical})^2 \times F_{analytical}$$

d = Diameter of the column

F = Flow rate

Using the scale-up calculation, Figure 2 shows the final separation achieved using the ZORBAX 300SB-C8 semipreparative (9.4 × 250 mm, 5 µm) column under various loading conditions.

Conditions	
Column	Agilent ZORBAX 300SB-C8, 9.4 × 250 mm, 5 µm (p/n 880995-206)
Mobile Phase	A) 0.2% TFA in water B) 0.15% TFA in acetonitrile
Injection Volume	Varies (100 to 500 µL)
Flow Rate	4.18 mL/min
Detector	UV (280 nm)
Temperature	40 °C
Gradient	26 to 50% B for 30 min

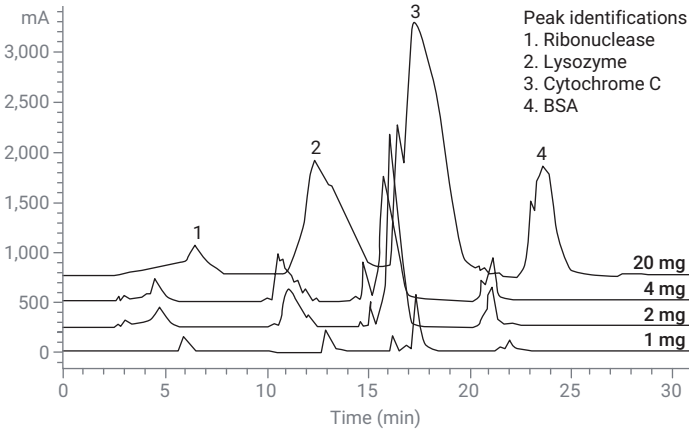

Figure 2. Scaling-up a gradient separation for a mixture of proteins.

Because the lengths of the analytical semipreparative columns used were the same, a semipreparative flow rate of 4.18 mL/min was calculated by the ratio of the column diameters, while leaving all other gradient conditions unchanged. Good resolution was maintained as the concentration load was increased from 1 to 4 mg of protein. There was a slight decrease in retention time as the sample load increased as well as some peak fronting as the column approached an overloaded condition. Note that for the largest mass injection (20 mg/protein), a 0.5 mL loop was required, and this extra volume caused a longer shift in retention. A total of 80 mg of total protein was successfully loaded and separated on this semipreparative column.

Conclusion

This work demonstrates that with scalable columns such as Agilent ZORBAX analytical, semipreparative, and PrepHT columns, carrying out method development work on less expensive analytical columns can save time and money, especially in solvent costs. The process of optimizing the separation on small matched columns, adjusting mobile phase conditions to achieve the necessary separation factor and plate count, is successful because the column surface chemistry is identical, minimizing problems when implementing the methods on larger scale preparative columns.

Instrumentation

All work was performed using an Agilent 1100 LC equipped with a solvent degasser, binary pump, autosampler, heated column compartment, and an ultraviolet detector.

Reference

1. Long, W. Separation Times, *16(2)*, 15–16, *Agilent Technologies*, publication number 5988-9110EN, **2003**.

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