Fast Separation of Large and Heterogeneous Proteins Using ZORBAX Poroshell C18, C8, and C3 Phases

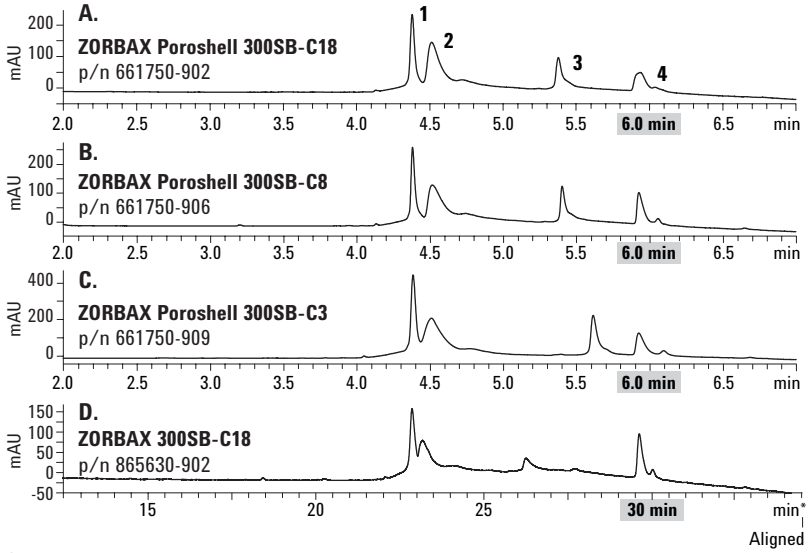
Application

Biochemical

Cliff Woodward and Robert D. Ricker

The use of C18-bonded totally porous particle columns is the traditional way to separate proteins by reversed phase liquid chromatography. This mode of analysis becomes somewhat problematic when the proteins are very large and/or very heterogeneous. These types of proteins have more complex interactions with the surface of the column packing and show improved peak shapes and resolution on superficially porous particles bonded with shorter phases such as C3 and C8. The simplified interaction of protein with the surface of the packing is the reason for improved chromatography.

The chromatograms below demonstrate differences in chromatography that result when analyzing very large, complex proteins using different ZORBAX Poroshell bonded phases (SB-C3, SB-C8, and SB-C18). The ZORBAX 300SB-C18 column is also shown for comparison of chromatographic results on a column containing totally porous particles. The sample is a mixture of four very large proteins – a monoclonal rabbit IgG, human IgM, α2-macroglobulin, and glycophorin. Molecular weights (MW) span the range of 50 to 950 kDa. (See legend for protein description and MW.)



Conditions

Column	**A–C: ZORBAX Poroshell 300SB-C18, C8, or C3** (1.0 × 75 mm, 5 µm) (see p/n above) **D: ZORBAX 300SB-C18** (1.0 × 50 mm, 3.5 µm) (see p/n above)
Temperature	70 °C, automatic delay volume reduction, binary pump, no mixer, 0.12-mm id tubing throughout
Flow rate	**A–C:** 0.454 mL/min, **D:** 0.071 mL/min
Detection	UV (212 nm), 1.7 µL flow cell, "<0.01 min" peak width setting

Highlights

- ZORBAX Poroshell 300SB columns come in many internal diameters and bonded phases. This gives a wide variety of choices for the optimal fast separation of proteins and peptides.
- The choice of which ZORBAX Poroshell column to use will depend on the molecular weight (MW) and heterogeneity of the protein sample.
- For very large, heterogeneous proteins, ZORBAX Poroshell C3 or C8 columns give the best peak shape in a separation.

Peak	Protein type	MW (kDa)*
1	IgG, rabbit monoclonal	150 kDa
2	IgM, human	950 kDa
3	α2-Macroglobulin, human	720 kDa
4	Glycophorin, human 60% carbohydrate	~50 kDa

*Approximate MW in kilodaltons

Mobile phase

A = 0.1% TFA in H_2O

B = 0.07% TFA in ACN

Gradient timetable

Column	Time (min)	% Solvent B
A–C	0.00	5.0
	10.00	100.0
D	0.00	5.0
	50.00	100.0

Chromatograms in the figure reveal that IgG and IgM are better separated on the ZORBAX Poroshell phases than on the totally porous material. This is also true for α2-macroglobulin, which shows better peak shape and recovery on the three ZORBAX Poroshell phases. Glycophorin shows better peak shape on ZORBAX Poroshell C8 and C3 than C18; but, it is best on the ZORBAX 300SB-C18. This difference may be attributable to the unusual post translational modification of this protein, (for example, 60% glycosylation). While protein elution order is the same between the different column types, there are significant changes in selectivity. This is most apparent for the third peak, which elutes somewhat later on the ZORBAX Poroshell 300SB-C3, and earlier on the ZORBAX 300SB-C18. This type of selectivity difference, combined with narrower peaks, can be significant tools in achieving a desired separation.

Each of the four columns was run at an optimum flow rate and gradient time for its configuration. This choice of parameters takes into consideration the particle type and diameter, as well as the column id and length. It is beyond the scope of this note to discuss gradient selection and modification, but a very simple explanation can be found in Agilent publication number 5989-0095EN. (Agilent publication number 5988-9998EN shows how to optimize your 1100 for best results with ZORBAX Poroshell columns.) ZORBAX Poroshell particles are designed for high linear velocity (high relative flow rates). The short diffusion distance into and out of the superficially porous outer layer allows higher flow rates without a deterioration of peak widths. Hence, the ZORBAX Poroshell columns were run at 454 µL per min and the ZORBAX columns at 71 µL per min. Shortening the gradient time in proportion to flow and column length results in similar elution patterns on all the columns but ZORBAX Poroshell analyses. These analyses are only 6 min long, in contrast to 30 min analyses using the totally porous material. Speed is a welcome benefit to nearly every analysis.

In summary, use of different bonded phases (see table below) can be crucial to successful separation of a particular protein sample, especially if these proteins are very large and heterogeneous. Bonded-phase choice can improve peak shape and recovery, as well as achieve desired selectivity. ZORBAX Poroshell often enhances peak shape and recovery through its simplified interaction with large proteins. The structure of ZORBAX Poroshell particles allows very short analysis times that can facilitate rapid method development as well as high-throughput analysis.

Column Types and Part Numbers for ZORBAX Columns Used in this Study

Column type	Part number
ZORBAX Poroshell 300SB-C3	661750-909
300SB-C8	661750-906
300SB-C18	661750-902
ZORBAX 300SB-C18	865630-902

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.
Search "Poroshell".

The authors, Cliff Woodward and Robert Ricker, are application biochemists based at Agilent Technologies, Wilmington, Delaware.

Printed in the USA
October 17, 2003
5989-0015EN

Agilent Technologies