Optimization of the Agilent 1100 HPLC System for Superior Results with ZORBAX Poroshell Columns

Technical Overview

Biochemical

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Abstract

ZORBAX Poroshell superficially porous particle technology allows the fastest, most rugged HPLC separation of proteins to date. A 0.25-µm porous silica crust formed around a nonporous 4.5-µm diameter silica core results in extremely hard, spherical, 5-µm ZORBAX Poroshell particles. Molecules diffusing the short distance into and out of the thin porous crust are rapidly separated.

While the inherent properties of ZORBAX Poroshell columns make them usable with most modern HPLC instruments, the use of superficially porous particle columns of small diameter (2.1 mm, 1.0 mm, and 0.5 mm i.d.) for the separation of proteins and peptides places certain demands on the instrument configuration.

This technical overview briefly addresses temperature flow rate and gradient adjustment and then details several of the more important parameters that can influence separation performance. These include: detector peak-width setting, extra-column volume, and flow-cell volume.

ZORBAX Poroshell Column Features

ZORBAX Poroshell 300SB columns come in a variety of internal diameters and phases, all being extremely useful for the fast separation of proteins and peptides. Poroshell particles are extremely hard, 5-µm silica spheres, each consisting of a 0.25-µm porous crust formed around a nonporous 4.5-µm core. See Figure 1.

With ZORBAX Poroshell packed columns, protein and peptide separations can be optimized, simplified, and enhanced by choosing appropriate detector settings, tubing diameter, and flow-cell volume.

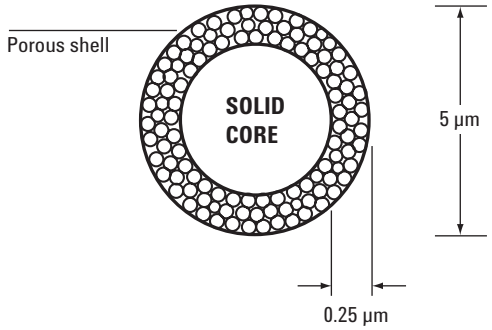

Figure 1. Schematic of a 5-µm superficially porous (ZORBAX Poroshell) HPLC particle.

Introduction

It has long been known that high linear velocity (high relative flow rate) is effective in achieving ultrafast separation of small molecules [1, 2]. This use of high relative flow rates to achieve an extremely fast separation (high-velocity chromatography [3]) is limited when using large molecules (for example, proteins) because of their smaller diffusion constant. As flow rate is increased, a point is reached where mobile phase moves past a column particle faster than a large molecule can get into it and back out; peak broadening is the result. ZORBAX Poroshell technology was designed to circumvent this limitation on flow rate. The reduced diffusion distance required for molecules separated on ZORBAX Poroshell means that a large molecule moves a much shorter distance into and out of the particle and can do this before the mobile phase has moved a significant distance down the column [4, 5]. This allows higher flow rates to be achieved before peak broadening occurs.

Figure 2 allows a visual comparison of separations resulting from reverse-phase HPLC (RP-HPLC) on totally porous and superficially porous (Poroshell) particles at various flow rates. The chromatograms have been aligned along the time axis (for example, the chromatogram obtained at a flow rate of 3 mL/min has been stretched 6-fold relative to the chromatogram obtained at 0.5 mL/min) so that relative peak widths and band spacing are more apparent. The sample mixture used throughout this technical overview consisted of neurotensin (peak 1), RNase A (peak 2), lysozyme (peak 3), and myoglobin (peak 4), each at approximately 0.25 mg/mL in mobile phase A.

The upper set of chromatograms (Figure 2A) were obtained using a 300SB-C18 totally porous packing in a 2.1 × 75-mm column. As flow rate is increased from 0.5 to 3 mL/min, the relative width of peaks 1 through 4 increases significantly. The first two sample components broaden to the extent that they merge into a single peak. The relative elution times stay constant because of the gradient adjustments; but resolution is lost because of peak broadening.

The lower set of chromatograms (Figure 2B) were obtained using ZORBAX Poroshell 300SB-C18 packing in a 2.1 × 75-mm column format. The relative peak width remains almost constant as flow rate is increased from 0.5 to 3 mL/min. The relative elution times stay constant (as in Figure 2A) because of the gradient adjustments. Resolution remains high, especially at flow rates of 2 mL/min or less. A flow rate of 2 mL/min on these 2.1-mm i.d. columns corresponds to a flow of 10 mL/min on a 4.6-mm i.d. column.

Table 1 allows a quantitative comparison of the results from Figure 2. Using the Agilent ChemStation, peak width at half height was determined for peaks 3 and 4 separated at all flow rates. (Peaks 1 and 2 were excluded for simplicity and because they were insufficiently resolved at higher flow rates on the column having totally porous particles.) Values in the table represent absolute peak width rather than the relative peak width depicted in the figure. Therefore, as flow is increased one expects peak width to drop proportionately (for example, increasing flow from 1 to 2 mL/min should cut the peak width 50%). Any band broadening is apparent as peak widths that decrease at less than the expected rate. The value for "Mean Increase in pw1/2" is meant to show this more clearly. First, peak widths were normalized by multiplying their value times the flow rate. Then, the percent increase in normalized peak widths relative to their value at a flow rate of 0.5 mL/min was reported. Mean relative peak width increases quickly with flow rate when using superficially porous particles—reaching 74% at 2 mL/min and 106% at 3 mL/min. In contrast, when using the Poroshell column, mean relative peak width increases by only 14% at 2 mL/min and by 39% at 3 mL/min. These results confirm observations made for the aligned chromatograms of Figure 2.

Table 1. Effect of Flow Rate on Peak Width and Resolution when Using Totally Porous and Superficially Porous Particles

Totally Porous Particle

Flow rate (mL/min)	Peak width (1/2 height)		Mean increase in pw1/2	Resolution
	Peak 3	**Peak 4**	**Peak 3 and Peak 4**	
0.5	0.0158	0.0188	0%	9.9
1	0.0100	0.0121	28%	7.5
2	0.0070	0.0080	74%	5.7
3	0.0057	0.0061	106%	4.1

Superficially Porous Particle - Poroshell

Flow rate (mL/min)	Peak width (1/2 height)		Mean increase in pw1/2	Resolution
	Peak 3	**Peak 4**	**Peak 3 and Peak 4**	
0.5	0.0129	0.0171	0%	10.0
1	0.0075	0.0083	7%	10.1
2	0.0037	0.0048	14%	8.2
3	0.0031	0.0038	39%	7.0

Virtually all modern HPLC instruments can take advantage of Poroshell separation technology; however, it is important to check the different aspects of instrument configuration discussed in this document before starting a separation. Understanding the mechanism of extremely fast separations using Poroshell (Figures 1-2) helps one to see the need for instrument settings that yield optimal performance. As previously mentioned, three main issues will be addressed here.

- The response time (peak-width setting) of the detector
- The effect of tubing diameter (volume)
- The effect of flow-cell volume

The choice of temperature, flow rate, and adaptation of gradient parameters are also very important in achieving optimal performance for Poroshell. A very brief discussion of these parameters follows.

Temperature

Temperature has a number of benefits in rapid separations using Poroshell. First, change of temperature can be used to achieve more-desirable selectivity. Operation at elevated temperature results in reduced retention times as well as reduced peak width of large molecules such as proteins. Finally, operating at elevated temperature dramatically reduces back pressure, facilitating use of higher flow rates. The sterically protected C18 groups of Poroshell 300SB-C18 make it extremely stable at low pH and temperatures up to 80 °C.

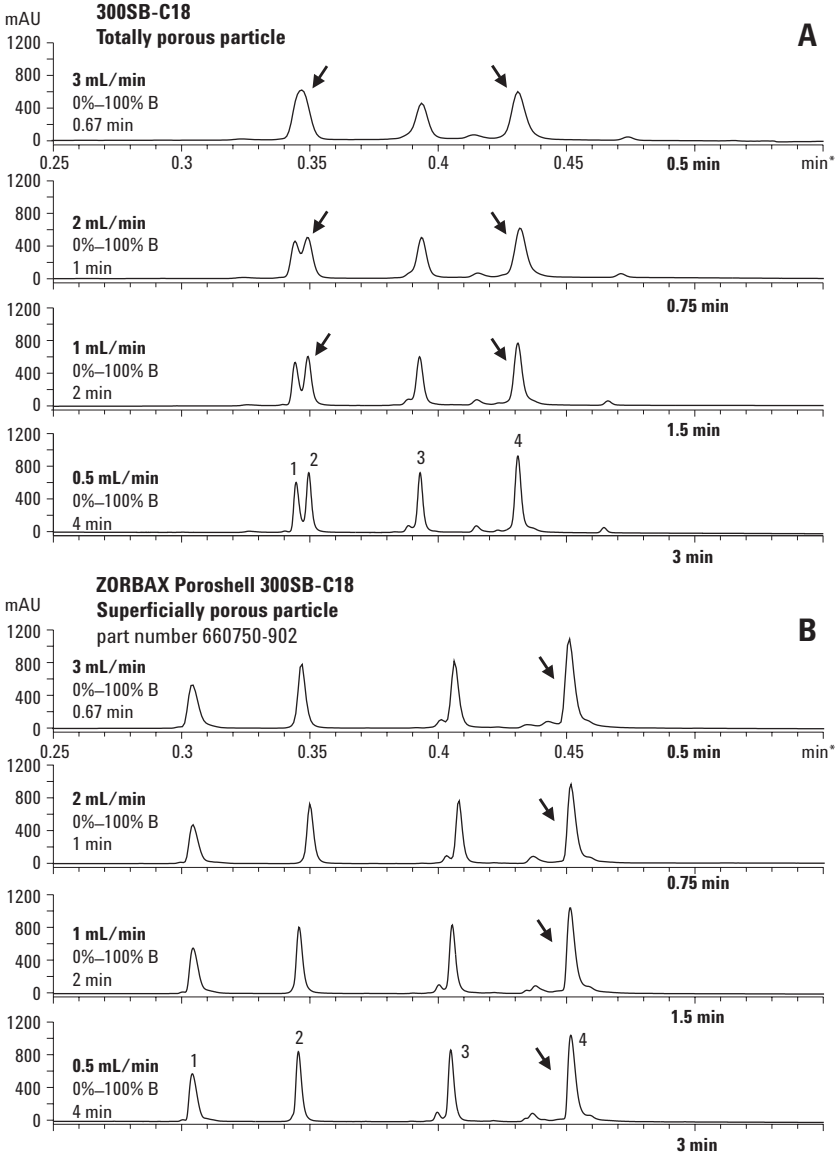

Figure 2. Effect of linear velocity (relative flow rate) on peak widths of a peptide-protein mixture, on wide-pore SB-C18 columns. In Figure 2A, the column contains totally porous particles, while in Figure 2B the column contains superficially porous particles (ZORBAX Poroshell).

Flow Rate

If one considers the optimal flow rate for obtaining the best combination of throughput and resolution with Poroshell, the data suggest a flow rate of 1 or 2 mL/min for the 2.1 × 75-mm column. When desiring the same separation on a column of a different diameter, flow rate should be adjusted relative to the cross-sectional area of the column. This maintains the same relative flow rate (linear velocity). For instance, to keep the same separation when switching from a 2.1- to a 1.0-mm i.d. column, the flow rate should be reduced 5-fold.

The equation below is helpful in these conversions.

$$Flow_2 = Flow_1 \times \frac{(Diameter_2)^2}{(Diameter_1)^2}$$

Gradient Adjustment

Flow rate and gradient time are linked by the following gradient relationship:

$$\text{Gradient retention (k)} = \frac{(tG \times F)}{(C \times Vm)}$$

Where: k is the relative retention within a gradient; tG is the gradient time in minutes; F is flow rate in mL/min.; Vm is the volume in mL of mobile phase in the packed column; and C is a constant (provided that the sample and the change in organic concentration during the gradient are unchanged from run to run). Combining this formula with the understanding that superficially porous (Poroshell) particles may be used at high linear velocities, one can choose gradient and flow rate conditions that optimize use of Poroshell technology.

The formula was used in Figure 2 to keep the relative elution patterns constant while changing flow rate. For instance, k is kept constant by using a 2-min gradient at 1 mL/min and a 1-min gradient 2 mL/min.

The equation also allows one to increase resolution through increased k, and to adjust for different column lengths.

Peak-Width Setting

Because ZORBAX Poroshell separations occur very quickly, peaks are not present in the detector for long periods of time. In the examples shown in Figure 2, measured peak widths vary from 0.01 min (0.6 s) to 0.06 min (3.6 s). The rate of detector response makes a very serious contribution to observed peak width under these conditions, but it also contributes to band broadening under less-demanding conditions. A more systematic demonstration of this is shown in Figure 3.

Peak widths in the 9-min separation shown are approximately 0.1 min (6 s). Use of a large diode array detector (DAD) peak-width setting causes data to be averaged for too long a time before it is reported to the data system for calculations and storage. This can result in another peak or some baseline absorbance being averaged in with the current peak. This gives diminished apparent resolution and peak heights that are easily restored by use of a narrower peak-width setting. The peak widths of the four sample components shown in Figure 3 become smaller as the peak-width setting of the detector is reduced from 0.2 min to <0.01 min. Note the improvement in resolution between peak 3 and the shoulder to its left, as the detector peak-width setting is reduced.

For the experiment shown in Figure 3, a ZORBAX Poroshell 300 SB-C18 column (1.0 mm × 75 mm containing 5-µm particles) was used. The Agilent 1100 HPLC system is configured essentially as it was used for Figure 2, with the exception that a 500-nL DAD flow cell was used and detection was at 212, 4 nm with the reference at 320, 20 nm. Mobile phases consisted of: A = H_2O with 0.1% TFA; B = ACN with 0.07% TFA. Gradients were 5% to 60% B in 8.8 min at a flow rate of 113 µL/min. All tubing was of 0.12 mm i.d.

Table 2 shows percent increases in peak width (determined at half height of the peak) observed as the peak-width setting for detection is increased from <0.01 to 0.2 min (comparing top and bottom chromatograms). The observed peak width increased by about 35%. It is clear that a proper peak-width setting is essential for achieving optimal ZORBAX Poroshell performance.

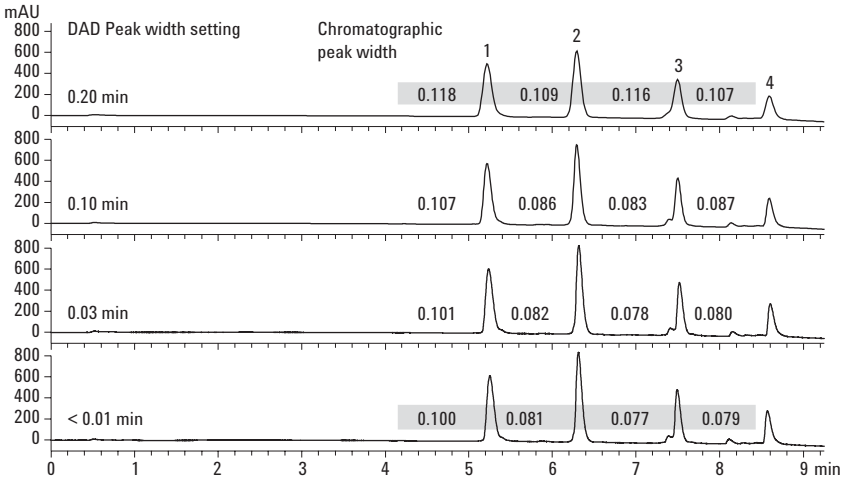

Figure 3. Effect of increasing detector response-time settings on peak widths of test sample peaks.

Table 2. Effect of Detector Response-Time Setting on the Peak Widths of a Peptide-Protein Sample Mixture Analyzed on ZORBAX Poroshell

Peak	% Increase in peak width
1	+18
2	+35
3	+51
4	+35

Values for percent increase in peak width are calculated using peak width at half height for peaks in the top (0.20 min) and bottom (<0.01 min) chromatograms of Figure 3.

The next two figures will focus on the effects of extra-column volume (ECV) relative to peak volume and how this may broaden peaks.

Extra-Column Volume

ECV the volume of all HPLC system components between the injector and the detector (excluding the column). This typically includes the sample loop, connecting tubing, fittings, and flow cell. Tubing diameter and flow-cell volume will be the focus of the next two experiments.

Sample peaks in HPLC elute in a system-dependent volume. Peak volume is reduced when peaks elute from a higher efficiency column (narrow bands, defined by a large theoretical plate number/meter). Peak volume is also reduced when using a smaller-diameter column; a peak analyzed on a 2.1-mm i.d. column has one-fifth the volume of the same peak eluting from a 4.6-mm i.d. column. Peaks eluting from a 1-mm i.d. column (used in many of these ZORBAX Poroshell experiments) elute in a volume that is one-twentieth that of peaks eluting from a 4.6-mm i.d. column. As peak volume decreases, peak width becomes more and more sensitive to ECV effects. Therefore, when using a 1-mm i.d. ZORBAX Poroshell column, additional attention should be paid to the ECV.

Effect of Tubing Diameter

Figure 4 illustrates peak broadening that results from use of column connecting tubing having too large an i.d. The upper chromatogram represents a separation of a peptide-protein mixture on a 1 × 75-mm, ZORBAX Poroshell 300SB-C18 column with a connecting tube of 0.12-mm (0.005-in) i.d. The lower chromatogram was obtained under identical conditions, but the connecting tube was replaced with a 0.17-mm i.d. (0.007 inch) tube of the same length. Peak broadening and asymmetry effects from use of the nonoptimized tubing are clearly observed.

Table 3 shows the percent change in both peak width and tailing (symmetry) for peaks in the peptide-protein sample analyzed under the two conditions in Figure 4. The increase in peak width, when using the larger diameter tubing, is consistent for all peaks and averages around 29%, indicating a significant effect of the larger tubing i.d. Peak symmetry also changes significantly in the direction of more peak tailing. The first peak appears to have improved in symmetry when the wider tubing was used, but this may be a result of the narrower peak in the upper chromatogram unmasking a shoulder peak at its trailing base. This would give the peak observed with smaller i.d. tubing an unusually large tail and correspondingly small symmetry value. For the remaining three peaks (2 through 4), increase in symmetry averages almost 47%.

The beneficial effects of using a fast detector peak-width setting and small-volume connecting tubing were discussed. In the

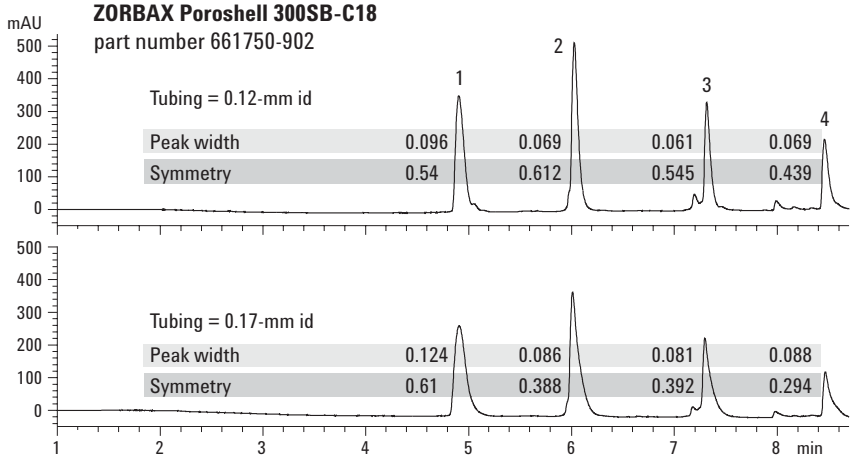

Figure 4. Effect of extra-column tubing volume on peak widths of a peptide-protein mixture separated on ZORBAX Poroshell. Peptide-protein mixture separated using a ZORBAX Poroshell 300 SB-C18 column (1.0 mm × 75 mm containing 5-µm particles). Chromatograms were aligned in the data system by peak elution time, for comparison. The Agilent 1100 HPLC system configured as in Figure 3, with the exception that the tubing diameter connecting the column to the instrument was variable, 0.12- or 0.17-mm i.d., and a 1.7-µL flow cell was used in the DAD. The DAD peak-width setting was <0.01 min.

Table 3. Effect of Column-Connector Tubing on the Peak Shape of a Peptide-Protein Sample Mixture Analyzed on ZORBAX Poroshell

Peak	% Change in peak width	% Change in symmetry value
1	+29	-12
2	+25	+58
3	+33	+33
4	+28	+49

Values for percent change in peak width are calculated using peak width at half height for peaks in the top (0.12-mm i.d. tubing) and bottom 0.17-mm id tubing) chromatograms of Figure 4. Percent change in symmetry values was derived in a similar way using pseudomoment analysis as reported by the Agilent ChemStation.

next section, the effect of different flow cells on peak shape will be shown for a separation of peptide and protein standards run on a ZORBAX Poroshell 300SB-C18 column. As was described in the previous section, sample peaks in HPLC elute in a system-dependent volume. For identical injection volume, peaks eluting from 1-mm i.d. ZORBAX Poroshell columns are one-twentieth the volume of those eluting from a 4.6-mm i.d. column. These small-volume peaks are very sensitive to ECV. In the following experiments, ECV was changed by use of different sized flow cells.

Effect of Flow-Cell Volume

When the flow cell is much larger than the peak volumes, it is more likely that subsequent peaks or baseline absorbance will enter the cell before the current peak has left. The detector "sees" the total absorbance in the flow cell so that peaks may appear overlapped, broadened, and reduced in height.

In Figure 5, these effects are evident for peak 3 and the small shoulder to its left. With a smaller-volume flow cell (1.7 µL, second chromatogram), the peak and shoulder are quite well separated. In contrast, the peak and shoulder merge together when the 13-µL flow cell (bottom chromatogram) is used. Relatively high flow rates may create abnormal turbulence in flow cells of certain dimensions. This may explain why, for 1.0-mm i.d. columns, the 1.7-µL flow cell, not the 500-nL flow cell, provides optimal peak widths. The 1.7-µL flow cell is also the best choice for 2.1-mm i.d. columns. However, when using 0.5-mm i.d. columns, the 500-nL flow cell provides the best performance (data not shown).

Some confusion may be avoided by pointing out the effect that the flow cell's path length has on peak area in Figure 5. The flow cells used in the upper and lower chromatograms (500 nL and 13 µL, respectively) have path lengths of 1 cm. The other two flow cells (1.7 µL and 4.5 µL) have path lengths of only 0.6 mm. The shorter path length proportionately reduces observed absorbance, hence the smaller peaks for the two middle chromatograms.

Table 4 lists the percent increase in peak width as a function of flow-cell volume for a 1.0-mm i.d. ZORBAX Poroshell column. Calculations were made using the peak widths at half height of sample peaks detected using the 1.7-µL and 13-µL flow cells. With peak widths up to twice as large, it is clear that flow-cell configuration can make a significant difference in chromatographic performance.

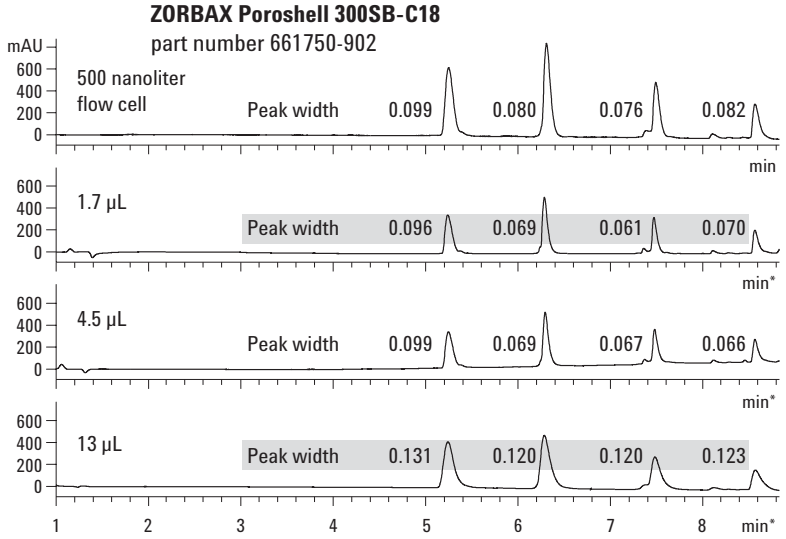

Figure 5. Effect of flow-cell volume on peak widths of a peptide-protein mixture separated on ZORBAX Poroshell. Peptide-protein mixture separated using a ZORBAX Poroshell 300 SBC18 column (1.0 mm × 75 mm containing 5-µm particles). *Chromatograms were aligned in the data system by peak-elution time, for comparison.

The Agilent 1100 HPLC system configured as in Figure 3, with the exception that the peak-width setting was <0.01 min. The flow cell volume was variable, as indicated, and included appropriate Agilent flow cells. The DAD peak-width setting was <0.01 min. Tubing was 0.12 mm i.d.

Table 4. Effect of Detector Cell Volume on the Peak Shape of a Peptide-Protein Sample Mixture Analyzed on ZORBAX Poroshell

Peak	% Change in peak width
1	+36
2	+74
3	+96
4	+75

Values for percent change in peak width are calculated using peak width at half height for peaks in the second and fourth chromatograms of Figure 4 (1.7 µL and 13-µL flow cells, respectively).

Combined Configuration Effect

In an attempt to summarize the findings discussed in this work, the peptide-protein separation on ZORBAX Poroshell was used to demonstrate the combined effect of using a non-optimal system configuration – slow detector peak-width setting, larger tubing, and a larger flow cell.

In Figure 6, the lower chromatogram shows the peptide-protein separation obtained using non-optimized settings: a detector peak width of 0.20 min, a 0.17-mm i.d. tubing, and a 13-µL flow cell. The upper chromatogram in Figure 6 shows the separation achieved when using the optimized system configuration of a <0.01 min detector peak-width setting, a 0.12-mm i.d. tubing, and a 1.7-µL flow cell.

The improvement in chromatographic performance is striking. In addition to much narrower peaks, a variety of smaller peaks are unmasked. Notice the shoulders to the right of peak 1 and to the left of peak 2. The new peak appearing to the left of peak 3 is almost baseline resolved. One more peak appears between peaks 3 and 4; this may or may not have been unmasked. The percent improvements in peak width for peaks observed in the optimized chromatogram compared to those in the deoptimized chromatogram are shown in Table 5.

By optimizing system configuration, peak widths improved by 80% to 180% – up to a 3-fold improvement. The result is a greatly improved resolving power that can be used to provide enhanced qualitative and quantitative data.

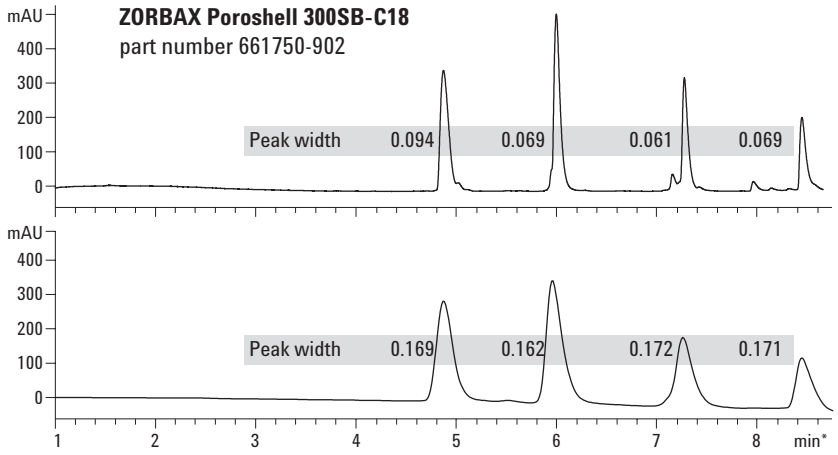

Table 5. Combined Effect of Several Deoptimized System Parameters on the Peak Shape of a Peptide-Protein Sample Mixture Analyzed on ZORBAX Poroshell

Peak	% Change in peak width
1	+79
2	+135
3	+182
4	+148

Values for percent change in peak width are calculated using peak width at half height for peaks in the top and bottom chromatograms of Figure 6.

Figure 6. Effect of deoptimized instrument configuration on chromatographic results. Peptide-protein mixture separated using a ZORBAX Poroshell 300 SB-C18 column (1.0 mm × 75 mm containing 5-µm particles). *Chromatograms were aligned in the data system by peak elution time, for comparison. The Agilent 1100 HPLC system was used in the fully optimized configuration (upper chromatogram) and with all three non-optimized parameters (lower chromatogram). The system configuration was essentially the same as that for Figure 3, with the following exceptions: For the upper chromatogram, the detector peak-width setting was <0.01 min; the tubing diameter connecting the column to the instrument was 0.12-mm id; and the 1.7-µL flow cell was used. For the lower chromatogram, the detector peak-width setting was 0.20 min; the tubing diameter connecting the column to the instrument was 0.17-mm id; and a 13-µL flow cell was used.

Summary

ZORBAX Poroshell technology provides exceptionally rapid HPLC separation of peptides and proteins. The resulting sample peaks are both narrow peak width and small in volume. To ensure that this separation technology is fully used, one needs to consider instrument configuration in the experimental setup. The configurations shown to make a critical difference to chromatographic performance in these experiments are readily available for most modern HPLC systems. Configuration items to evaluate when beginning a separation include:

- The peak-width setting of the detector. The detector must sample fast enough to adequately capture sample peaks present in the flow cell for very short times (obtain >8 points per peak).
- Connecting tubing should be small enough in i.d. so as not to contribute to peak broadening. Tubing of 0.12-mm i.d. or less is required.
- Detector flow cells should be chosen with volumes small enough that they do not contribute significantly to peak broadening. For the Agilent 1100 DAD detector and 2.1-mm or 1.0-mm i.d. ZORBAX Poroshell columns, the 1.7-µL flow cell was shown to provide optimal peak widths. For a 0.5-mm i.d. ZORBAX Poroshell column, the 500-nL flow cell is recommended. Note that a flow-cell with too small of an i.d. can cause peak broadening at high relative flow rates. A few minutes spent checking the instrument setup will ensure outstanding ZORBAX Poroshell analyses.

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Printed in the USA
February 5, 2004
5988-9998EN