# Fast Protein Separations Using Agilent Poroshell 300

## Technical Note

The number of proteins and antibodies in drug development and in clinical phase trials is growing rapidly. For investigators working with these complex bio-molecules, looking for faster, more efficient separations of peptides, proteins and antibodies, Agilent offers the Poroshell 300 reversed-phase HPLC columns.

### Poroshell Offers More Resolution at High Flow Rates

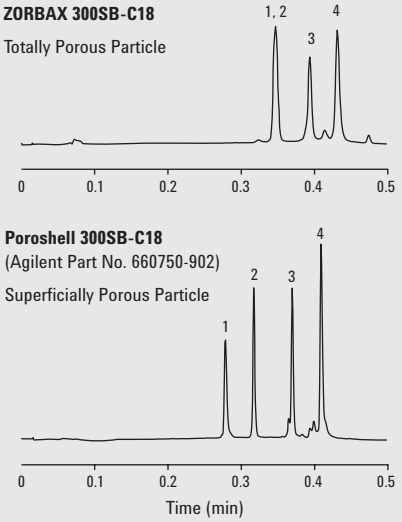
With this technology, higher resolving power for proteins at high flow rates is driven by particle design. The particle design allows large molecules to quickly diffuse in and out of the particle pore structure, resulting in improved efficiency and decreased peak width with more chromatographic detail at higher flow rates. This is clearly illustrated in Figure 1, where the separation of four proteins is run on a totally porous Agilent ZORBAX 300SB-C18 column and on the superficially porous Poroshell 300SB-C18 column of the same dimension and particle size.

The Poroshell 300SB-C18 column easily separates the first two proteins of this mixture – neurotensin and ribonuclease A – while these same proteins co-elute on the 300SB-C18 totally porous product at this high flow rate.

Figure 1

**Comparison of Superficially and Totally Porous Particles in Proteins Separations at High Flow**

| | |
|---|---|
| Column: | 2.1 x 75 mm, 5 µm |
| Mobile Phase: | |
| Gradient: | 5-100% B in 0.67 min.<br>A: 95% $H_2O$, 5% ACN with 0.1% TFA<br>B: 5% $H_2O$, 95% ACN with 0.07% TFA |
| Flow: | 3.0 mL/min |
| Temperature: | 70° C |
| Detector: | UV 215 nm |
| Sample: | 200 µg each<br>1. Neurotensin<br>2. RNaseA<br>3. Lysozyme<br>4. Myoglobin |




More notably, the peak width of each of these proteins eluted at 3.0 mL/min is considerably narrower on the Poroshell 300SB-C18 product. The peak widths average 70% narrower on the Poroshell 300SB-C18 column when compared to the standard totally porous 300SB-C18 product. Additionally, the chromatographic detail in the baseline of the Poroshell separation is more crisply defined. This leads to the finer resolution of trace components on the Poroshell 300SB-C18 column.

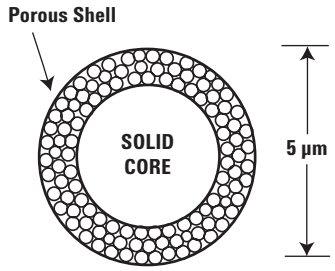
## The Poroshell Design

**The Particle**

The unique, patented Poroshell particle design, illustrated in Figure 2, consists of a solid core of high purity silica surrounded by a thin layer of bonded, porous, high purity silica. Pores in the thin-porous layer measure 300Å in diameter and are maximally bonded with diisobutyloctadecylsilane for SB-C18 for maximum column life with low pH mobile phases (i.e. TFA-containing). In contrast, most wide-pore reversed-phase packings are comprised of a bonded, totally porous, silica particle.

Figure 2

**Poroshell Superficially Porous Particle**




During a separation on the Poroshell column, proteins diffuse in and out of the thin, porous bonded layer – the "Poroshell layer." Because larger molecules diffuse slower than small molecules, the reduced diffusion path length of a Poroshell particle provides:

- More rapid diffusion of proteins between the particle and the mobile phase.
- More efficient peak widths at high flow rates.

## The Configuration

A reduced column diameter and column length, i.e., 2.1 x 75 mm, operated at flow rates between 1-3 mL/min, are key to providing the very short, highly improved gradient peptide and protein separations illustrated here. (See Guidelines for more details.)

## The Bonding

The rugged StableBond bonding of Poroshell enables methods run at low pH and at temperatures up to 90° C. This capability allows you to take advantage of a wide pH range and higher temperature to further improve peak widths and also decrease column backpressure and analysis time.

## Unleash the Power of Poroshell 300 in Your Laboratory

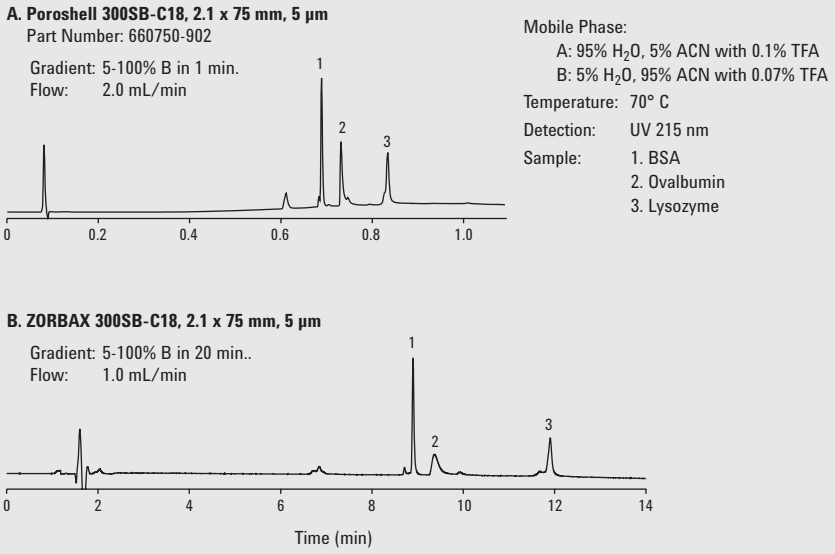
The power of Poroshell to enhance chromatographic details in your separations can be achieved while decreasing analysis time. In fact, you can reduce analysis time up to an impressive 90%, when adapting your wide-pore reversed-phase method on a 4.6 mm I.D. column to a 2.1 x 75 mm, 300SB-C18 Poroshell product run under similar conditions.

Figure 3A shows the outstanding peak shape and resolution of a three-component mixture, which includes the difficult to analyze BSA and Ovalbumin, separated on the Poroshell 300SB-C18 column in less than one minute. Compared to a similar separation on a 4.6 x 150 mm, 300SB-C18 totally porous material (Figure 3B), the Poroshell separation is concluded in less than one-tenth the time, with overall improved performance. The high temperature of 70° C for this assay, a practical choice due to 300SB-C18 bonding, assists in providing the narrow peak widths and high resolution observed here.

Note that in this case, the selectivity (relative retention) of these two column types is not the same. The selectivity differs because the volume of the stationary phase of these two products is quite different relative to the volume of the mobile phase, so differences in relative peak position are expected when comparing the Poroshell product with a totally porous product. Such selectivity differences, coupled with the high resolving power of Poroshell, can however, lead to very favorable improvements in your separation.

Figure 3

**Poroshell Separates Proteins in Less Than One Minute**



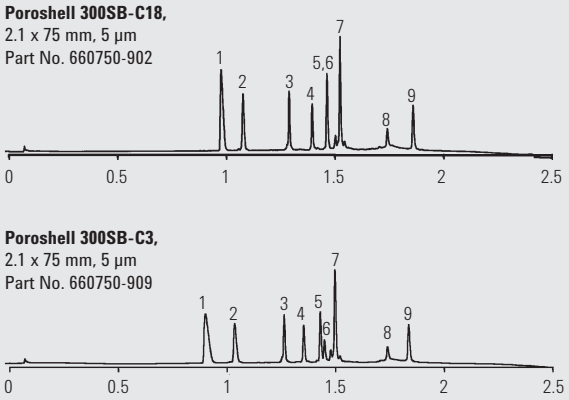
## Bonded phase choices offer more resolving power and fewer carryover concerns

The Poroshell 300 HPLC column line is offered in three bonded phases or selectivities – the 300SB-C18, the 300SB-C8 and the 300SB-C3. Since reducing bonded-phase chain length makes the polar silica support more accessible, the 300SB-C8 and 300SB-C3 are less hydrophobic bonded phases, and offer more separation options for moderately polar biomolecules. For example, Insulin and Cytochrome C are baseline resolved on the Poroshell 300SB-C3, while these solutes co-elute on the Poroshell 300SB-C18 column under the conditions described in Figure 4.

For some complex mixtures, like fermentation broth and milk-derived samples, protein carryover into a blank gradient can be problematic. The use of the less hydrophobic Poroshell 300SB-C8 and C3 columns has been shown to eliminate these "memory effects." Figure 5 shows the traces resulting from the injection of 100 µL of fermentation broth on a Poroshell 300SB-C3 column, followed by the clean trace resulting from the immediately following blank injection of 100 µL of water.

Figure 4

**Poroshell 300SB-C3 provides complete resolution of Insulin and Cytochrome C.**

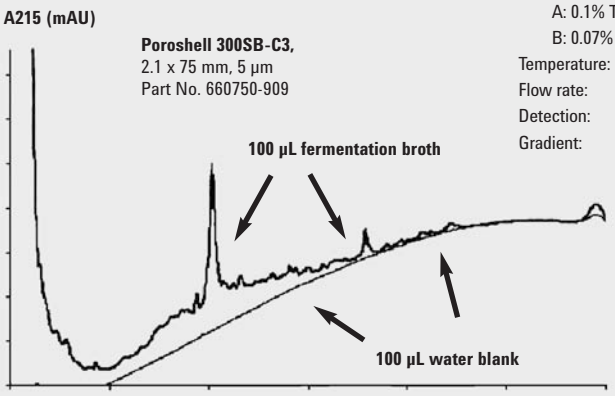



Mobile Phase:
A: 0.1% TFA/$H_2O$,
B: 0.07% TFA/ACN
Temperature: 70° C;
Flow rate: 0.5 mL/min;
Detection: UV 215 nm;
Gradient: 5-100% B in 3.0 min

Samples:
1. Angiotensin II
2. Neurotensin
3. RNase A
4. Insulin B Chain
5. Insulin
6. Cytochrome C
7. Lysozyme
8. Myoglobin
9. Carbonic Anhydrase

Figure 5

**Essentially no carryover is observed using the Poroshell 300SB-C3.**

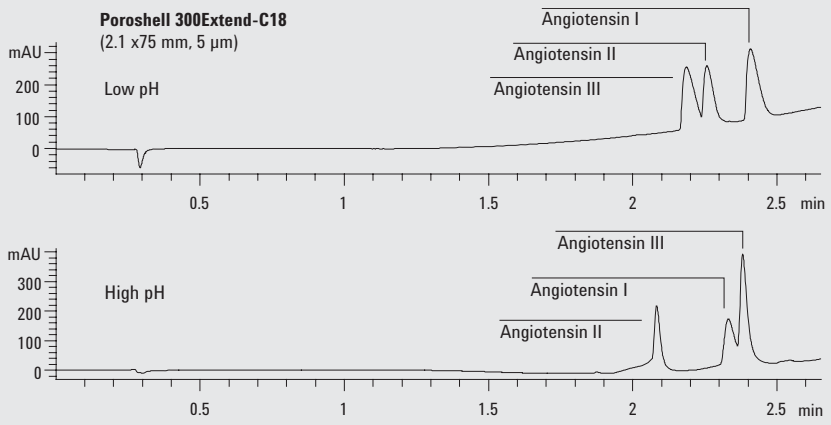



Mobile Phase:
A: 0.1% TFA in $H_2O$
B: 0.07% TFA in ACN
Temperature: 50° C
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Gradient: 10-60% B in 10.5 min, to 100% B in 1 min. Water injection (blank) immediately follows an injection of 100 µL of clarified fermentation broth.

## Achieve unique selectivity from pH 2 to pH 11.5

Poroshell 300Extend-C18 columns incorporate a unique patented bidentate silane, combined with a double-endcapping process that protects the silica from dissolution at high pH. At high pH, the retention and selectivity of peptides can change dramatically as a result of changes in amino acid charge. Excellent recoveries of hydrophobic polypeptides have been achieved at high pH. In addition, LC/MS sensitivity of peptides can also be improved at high pH using a simple ammonium hydroxide mobile phase. Angiotensins I, II and III differ only slightly in polarity and length. In Figure 6 the three related forms of angiotnesin were separated on a Poroshell 300Extend-C18 column at high pH (pH 10, lower chromatogram) and low pH (pH 2.4, upper chromatogram). At high pH angiotensin II is well resolved and at lower pH angiotensin I is well resolved. The Poroshell 300Extend-C18 column rapidly resolves similar peptides with very minor charge differences, while maintaining a robust column lifetime.

Figure 6




**Conditions**

| | |
|---|---|
| Column | **Poroshell 300Extend-C18** (2.1 x 75 mm, 5 µm) (p/n 670750-902) |
| Sample | 330 ng each peptide |
| Gradient | 0% – 100% B in 2 min (see mobile phase at right). |
| Detection | UV (215 nm) |
| Temperature | Ambient |
| Injection | 1 µL |
| Flow rate | 0.5 mL/min |

**Low pH mobile phase (pH 2.4)**

A: 0.1% Formic acid in $H_2O$
B: 0.1% Formic acid in ACN

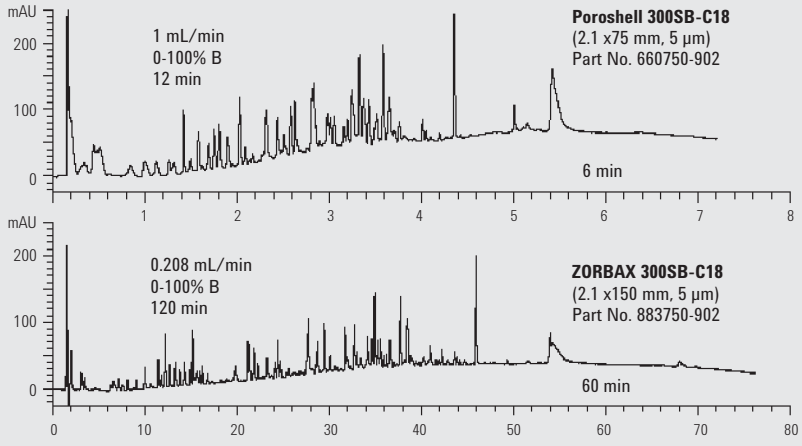
**High pH mobile phase (pH 10)**

A: 10 mM $NH_4OH$ in $H_2O$
B: 10 mM $NH_4OH$ in ACN

## Resolve complex peptide maps in minutes

Poroshell 300SB can offer tremendous time savings for complex bioseparations, in many cases reducing analysis time up to 90 percent. For example, a single chromatographic run of a protein tryptic digest using a totally porous ZORBAX 300SB-C18 column can require an hour or more to complete, as shown in Figure 7. Using the Poroshell 300SB-C18, this same complex separation is concluded in just six minutes. The combined ten-fold difference in flow rate and column length used for the Poroshell separation allows for a ten-fold reduction in gradient time from 120 to 12 minutes, while maintaining the same resolution and peak elution profile.

Figure 7
**Reduce peptide map analysis time by 90% with Poroshell 300SB.**




**Conditions**

| | |
|---|---|
| Instrument | Agilent 1100 binary system |
| Mobile phase | A: 95% $H_2O$, 5% ACN, 0.1% TFA<br>A: 95% $H_2O$, 5% ACN, 0.1% TFA |
| Flow | As above |
| Piston stroke | 20 µL |
| Detection | UV (215 nm) |
| Temperature | 70° C |
| Agilent 1100 well-plate autosampler with delay volume reduction | |
| Injection volume | 20 µL (0.22 Mg/1 µL) |
| Sample | BSA Tryptic digest (15 hours, 70 pmol) |

# Guidelines for Using Poroshell 300SB-C18

In order to take advantage of the Poroshell 300SB-C18 technology for your peptide and protein separations, you may want to consider the following guidelines when starting method development with Poroshell or when adapting a method from a totally porous HPLC column.

## Suggestions for Developing Fast Separations on Poroshell

1. Prepare the following mobile phase:
   A: 95% water, 5% acetonitrile with 0.1% TFA
   B: 5% water, 95% acetonitrile with 0.07% TFA
2. Keep injection volume below 5 µL.
3. Using the 2.1 x 75 mm, 5 µm Poroshell column, start with a flow rate of 1.0 mL/min and a gradient of 0-100% B/4 min at 35° C. If more resolution is needed for your sample, increase the gradient time by a factor of 2, 3, 4 or more, but retain the same flow rate.
4. Consider increasing column temperature at this point. Column temperatures up to 90° C will significantly decrease peak width and column backpressure.
5. To decrease analysis time significantly, increase the flow rate to 2.0 mL/min and cut the final gradient time in Step 3 in half. To decrease analysis time further, increase flow rate up to 3.0 mL/min and decrease gradient time proportionately to retain the same peak elution pattern. Flow rate increases may be limited by column backpressure.

## Suggestions for Adapting Existing Separations to Poroshell – Reduce Analysis Time up to 90%

1. At first, maintain the same "change in organic" during the gradient run.

   For example, if you are running 5-100% B on a 4.6 I.D. column, use the same change in organic, 5-100% B on the 2.1 x 75 mm, 5 µm Poroshell column; where B is the organic mobile phase component.
2. Use the same column temperature.
3. Reduce the injection volume five-fold.
4. When adapting the Poroshell method from a 4.6 x 150 mm column, use a flow rate and gradient time for the Poroshell separation such that the product of flow rate and gradient time is one-tenth that on the 4.6 mm I.D. column.

   For example, if the flow rate (F) is 1.0 mL/min. and gradient time (tG) is 40 minutes on the 4.6 x 150 mm column, the following combinations can be used with the 2.1 x 75 mm Poroshell product.

| Flow Rate (F) | Gradient Time (tG) |
|---|---|
| 3.0 mL/min | 1.3 min |
| 2 mL/min | 2 min |
| 1 mL/min | 4 min |
| 0.5 mL/min | 8 min |

*High flow rates coupled with shorter gradient times result in shorter analysis times with 1.0 to 3.0 mL/min being the most advantageous flow rate range.*

**NOTE:** In this example, column volume and length are reduced by a factor of five and two, respectively. So to retain similar gradient retention – relative peak position may differ on Poroshell – the product of gradient time and flow rate is reduced by a corresponding factor of ten.

5. Increase temperature up to 90° C to decrease peak width, increase resolution and reduce column backpressure.

**If you have any additional questions on how to use Poroshell HPLC columns, contact Agilent, or your Agilent Authorized Distributor for HPLC column support.**

**www.agilent.com/chem/contactus for a complete listing by country.**


Printed in the USA November 25, 2008
5989-9899EN

Agilent Technologies