Agilent BioHPLC Columns

PROTEIN IDENTIFICATION AND IMPURITY PROFILING USING REVERSED-PHASE HPLC/UHPLC

The Measure of Confidence

Agilent Technologies

REVERSED-PHASE HPLC/UHPLC

Agilent can boost your accuracy and productivity

Reversed-phase is used to confirm protein identity, impurity profiling, and quantify post-translational modifications. The technique separates on the basis of differences in hydrophobicity, and uses denaturing conditions. This provides information about the molecule's primary amino acid sequence, as well as variations and modifications to the sequence.

Agilent offers the most comprehensive range of wide-pore, 300Å, 450Å, and larger, reversed-phase BioHPLC columns, all backed by technical support experts and application chemists around the globe. The family includes 1.8, 3.5, and 5 µm totally porous particles for pressures from 400 to 1200 bar, Poroshell (superficially porous) particles for UHPLC separations at lower pressure, and polymeric columns for analysis under the most extreme conditions.

Agilent AdvanceBio RP-mAb columns: based on Poroshell technology with unique engineering for pore size and bonded phase, these columns deliver higher resolution and faster run times to provide accurate, reproducible results when analyzing intact monoclonal antibodies and mAb fragments.

Agilent AdvanceBio Peptide Mapping columns: quickly resolve and identify amino acid modifications in primary structure. With their 2.7 µm particles and C18 functionality, AdvanceBio Peptide Mapping columns deliver excellent retention, resolution, and peak shape for basic hydrophobic peptides.

Agilent Poroshell 300 columns: the industry's first superficially porous small particle columns for fast polypeptide and protein separations.

Agilent ZORBAX RRHD 300Å 1.8 µm columns: deliver UHPLC performance for reversed-phase separations of intact proteins, protein fragments, and digests with 1200 bar stability.

Agilent ZORBAX 300Å 3.5 & 5 µm columns: fully porous materials for HPLC and prep separations; many of the bonded phases are scalable from the 1.8 µm particle.

Agilent PLRP-S columns: macroporous polymer particles deliver HPLC separations over the widest pH range. With 3 wide-pore sizes and 8 particle sizes, the PLRP-S columns provide optimum solutions for analytical prep separations of peptides, proteins, and protein complexes.

INSIDE

To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

REVERSED-PHASE HPLC

Which Fast LC column is best for your reversed-phase separation?

Agilent offers the widest range of fast HPLC/UHPLC wide-pore columns. So you have the flexibility to create methods with maximum resolution, whether you have a 400, 600, or 1200 bar instrument. Wide-pore, 300Å columns are necessary for efficient separation of proteins and peptides because they allow these analytes to completely access the bonded phase.

Reversed-Phase Column Selection

Application	Agilent Columns	Notes
Intact monoclonal antibodies and mAb fragments	AdvanceBio RP-mAb, 450Å, 3.5 µm • SB-C8 • C4 • Diphenyl	Particles with a wide pore diameter are necessary for an efficient separation of large biomolecules such as intact mAbs, as they allow the analytes to completely access the bonded phase. The use of Poroshell technology, with reduced diffusion distances, enhances this efficiency even further. C4 chemistry is well suited to mAb separations, providing stability at low pH and compatibility with methods that require USP L26 columns. StableBond C8 gives scalability and method transfer. The Diphenyl phase, unique to Agilent, offers alternative selectivity.
Intact proteins, monoclonal antibodies, mAb fragments and polypeptides	ZORBAX 300Å, 1.8 µm • RRHD 300SB-C18 • RRHD 300SB-C8 • RRHD 300SB-C3 • RRHD 300-Diphenyl	Optimized packing processes achieve stability up to 1,200 bar for use with the Agilent 1290 Infinity LC. RRHD 1.8 µm columns are available in 50 and 100 mm lengths for fast or high resolution – truly high definition – separations of the most complex samples. StableBond C18 is ideal for complex protein and protein digest separations.
	ZORBAX 300Å, 3.5 and 5 µm • 300SB-C18 • 300SB-C8 • 300SB-C3 • 300SB-CN	Ideal for use with HPLC systems. StableBond C3 and CN are useful for larger, more hydrophobic compounds.
	ZORBAX 300Å, Extend-C18	Incorporate a unique bidentate silane, combined with a double-endcapping process that protects the silica from dissolution at high pH – up to pH 11.5.
Large intact proteins, monoclonal antibodies	Poroshell 300 • 300SB-C18 • 300SB-C8 • 300SB-C3 • 300Extend-C18	Poroshell columns use a unique particle made with a layer of porous silica on a solid core of silica. This reduces the diffusion distance for proteins, making possible practical, rapid HPLC separations of peptides and proteins.
Peptides	AdvanceBio Peptide Mapping	An ideal 120Å pore size for identifying a wide molecular weight range of peptides. Tested with a challenging peptide mix to ensure performance. The unique Agilent Poroshell technology enables higher flow rates and better resolution of the full peptide sequence.
Peptides to DNA	PLRP-S • 100Å • 300Å • 1000Å • 4000Å	Particles are inherently hydrophobic and so an alkyl ligand bonded phase is not required for reversed-phase separations. This gives a highly reproducible material 100Å that is free from silanols and heavy metal ions.
Small molecules/synthesis	PLRP-S 100Å	
Recombinant peptides/ proteins	PLRP-S 300Å	
Large proteins	PLRP-S 1000Å	
DNA/high speed separation	PLRP-S 4000Å	
Amino acids	ZORBAX Amino Acid Analysis (AAA)	Tested for amino acid analysis using well-known OPA and FMOC precolumn derivatization chemistry. Options for HPLC and UHPLC available.

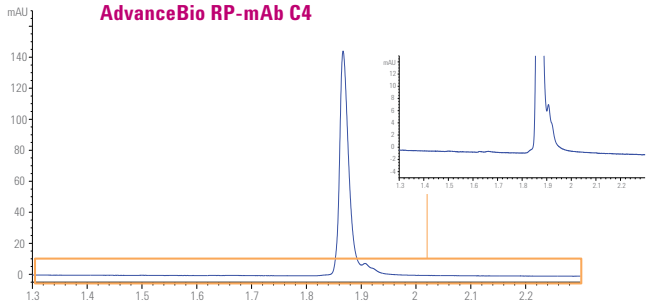
ADVANCEBIO RP-mAb COLUMNS RESOLVE mAbs FASTER AND BETTER

The Agilent AdvanceBio RP-mAb column delivers higher resolution and faster run times to provide accurate, reproducible results when analyzing monoclonal antibodies for biopharma discovery, development, and QA/QC applications.

Exclusive Agilent Poroshell technology, built into every AdvanceBio RP-mAb column, gives you the advantages of:

- **Improved accuracy:** Superficially porous particles (3.5 µm) with wide pores (450Å) increase mAb resolution while maintaining compatibility with all LC instruments
- **Speed:** Shorter analysis times compared to columns packed with fully porous particles of the same size **(Figure 1)**
- **Lower costs:** The robust Poroshell packed bed and 2 µm inlet frit extend column lifetime by helping prevent inlet blockage
- **Flexible method development:** Range of chemistries – SB-C8, C4, and diphenyl

Sharp peaks with fine detail for short runs – Characterization in less than 2 minutes

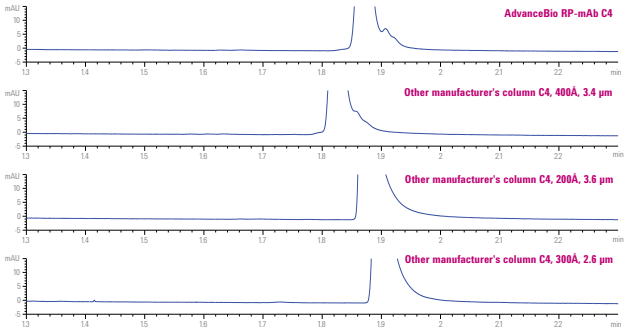


Method Parameters

Column dimensions:	2.1 x 100 mm, 3.5 µm
Mobile phase A:	0.1% TFA in water:IPA (98:2)
Mobile phase B:	IPA:ACN:Mobile phase A (70:20:10)
Flow rate:	1.0 mL/min
Gradient:	10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Sample:	5 µL injection of humanized recombinant Herceptin Variant IgG1 intact from Creative Biolabs (1 mg/mL)
Temperature:	80 °C
Detection:	UV @ 254 nm

Figure 1. Here, an AdvanceBio RP-mAb C4 column delivered excellent peak shape and detailed resolution of intact humanized recombinant Herceptin IgG1 in less than 2 minutes.

Agilent AdvanceBio vs. the competition – Superior to other protein columns



Method Parameters

Column dimensions:	2.1 x 100 mm, 3.5 µm
Mobile phase A:	0.1% TFA in water:IPA (98:2)
Mobile phase B:	IPA:ACN:Mobile phase A (70:20:10)
Flow rate:	1.0 mL/min
Gradient:	10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Sample:	5 µL injection of humanized recombinant Herceptin Variant IgG1 intact from Creative Biolabs (1 mg/mL)
Temperature:	80 °C
Detection:	UV @ 254 nm

Figure 2. Specifically designed for mAb separatioins, AdvanceBio RP-mAb provides superior peak shape and resolution than other columns used for intact protein separations.

To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

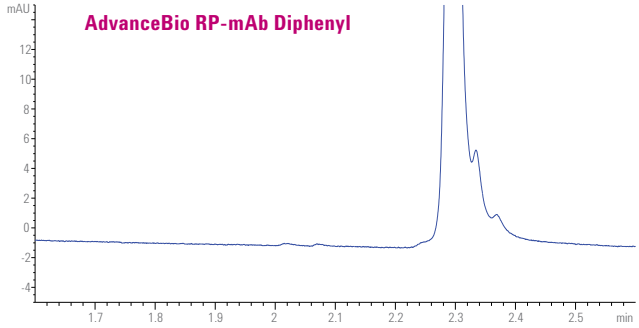
Exceptional speed and confidence for mAb separations

As with *all* columns manufactured by Agilent, AdvanceBio RP-mAb columns undergo rigorous end-to-end QC testing to ensure reproducibility and performance.

In this example **(Figure 3)**, intact humanized recombinant Herceptin IgG1 was characterized using an AdvanceBio RP-mAb Diphenyl column. The unique diphenyl phase resolves even more fine detail.

Figure 4 demonstrates how the wide-pore Poroshell technology of the AdvanceBio RP-mAb column delivers high efficiency, a short analysis time, and low pressure, at temperatures below 80 °C – the typical temperature of many reversed-phase methods.

Selective diphenyl phase – More fine details resolved

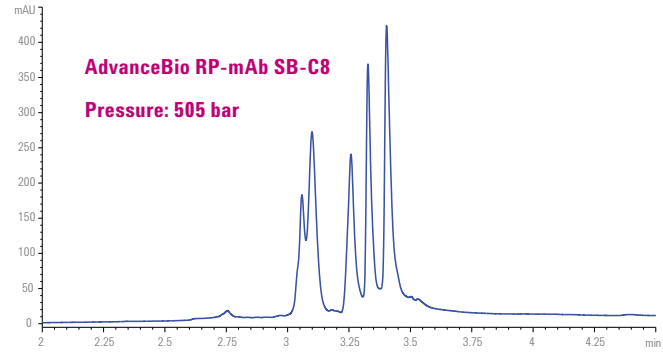


Method Parameters

Column dimensions:	2.1 x 100 mm, 3.5 µm
Mobile phase A:	0.1% TFA in water:IPA (98:2)
Mobile phase B:	IPA:ACN:Mobile phase A (70:20:10)
Flow rate:	1.0 mL/min
Gradient:	10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Sample:	5 µL injection of humanized recombinant Herceptin Variant IgG1 intact from Creative Biolabs (1 mg/mL)
Temperature:	80 °C
Detection:	UV @ 254 nm

Figure 3. The unique selectivity of AdvanceBio RP-mAb Diphenyl resolves even more fine detail.

The Poroshell advantage – High accuracy, low backpressure



Method Parameters

Column dimensions:	2.1 x 100 mm, 3.5 µm
Mobile phase A:	0.1% TFA in water
Mobile phase B:	n-Propanol:ACN:Mobile phase A (80:10:10)
Flow rate:	0.8 mL/min
Gradient:	5-40% B in 5 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Sample:	1 µL injection of Fc/Fab, papain-digested humanized recombinant Herceptin Variant IgG1 from Creative Biolabs (2 mg/mL)
Temperature:	60 °C
Detection:	UV @ 220 nm

Figure 4. AdvanceBio RP-mAb columns perform well at temperatures below 80 °C.

ADVANCEBIO PEPTIDE MAPPING COLUMNS

Reduce peptide mapping time without losing resolution

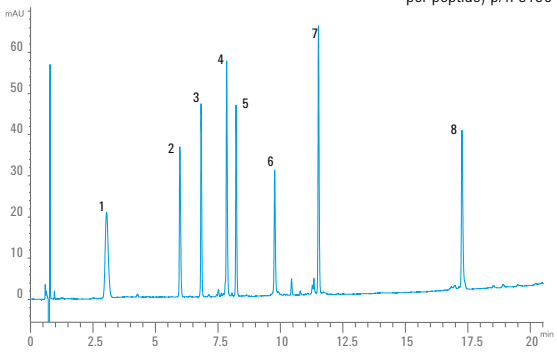
Agilent AdvanceBio Peptide Mapping columns let you quickly resolve and identify amino acid modifications in primary structure, unlike fully porous columns which can take 60 minutes.

These advanced biocolumns feature a 120Å pore size with superficially porous 2.7 µm particles, and are designed to deliver:

- **Greater analytical confidence:** Each batch of AdvanceBio Peptide Mapping media is tested with a rigorous peptide mix to ensure suitability and reproducibility, and to enable the identification of key peptides in complex peptide maps.
- **Time savings:** Perform high-resolution separations 2 to 3 times faster than with fully porous HPLC columns.
- **Every instrument works harder:** 4.6, 3.0, and 2.1 mm id columns are stable to 600 bar, enabling you to get the most from your UHPLC instruments. They can also deliver excellent performance for your legacy 400 bar instruments, too.
- **More flexibility:** Increase MS sensitivity with formic acid mobile phases on any HPLC.

Quality assurance testing with peptide mix

Column: **AdvanceBio Peptide Mapping**, 2.1 x 150 mm, 2.7 µm, p/n 653750-902
Flow rate: 0.5 mL/min
Injection: 5 µL
Temp: 55 °C
Detection: 220 nm
Gradient: A, water (0.1% TFA), B, ACN (0.08% TFA), 0-25 min,15-65% B; 25-26 min, 65-95% B
Sample: Agilent Peptide Mapping Standards Mix (0.5-1.0 µg/µL per peptide) p/n 5190-0583



Pk no.	MW	Component
1	757	Bradykinin fragment 1-7
2	1060	Bradykinin
3	1046	Angiotensin II
4	1673	Neurotensin
5	1295	Angiotensin I
6	2465	ACTH fragment 18-39
7	1759	Renin
8	2845	Melittin

Figure 5. Test mix used for every batch of AdvanceBio Peptide Mapping media. The mixture contains 8 hydrophilic, hydrophobic, and basic peptides, ranging in molecular weight from 757 Da to 2845 Da. Every column is also tested with a small-molecule probe to ensure efficiency.

Peptide map of a biosimilar EPO

Column: **AdvanceBio Peptide Mapping**, 2.1 x 250 mm, 2.7 µm, p/n 651750-902
Flow rate: 0.5 mL/min
Injection: 5 µL (2.0 mg/mL)
Temp: 55 °C
Detection: 220 nm
Gradient: A, w n, 60-95% B

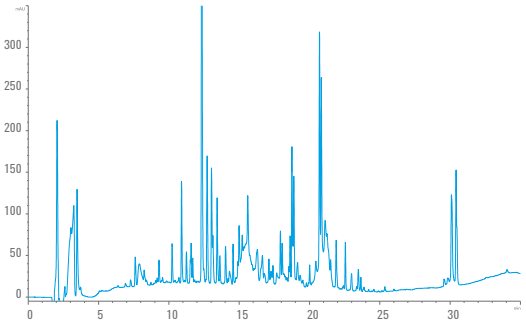


Figure 6. AdvanceBio Peptide Mapping column easily confirms protein identity and identifies all post-translational modifications.

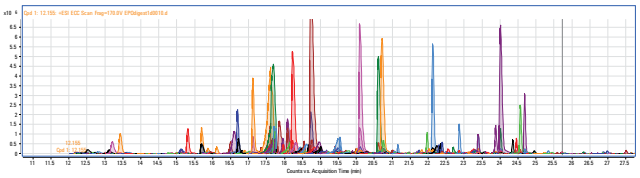


Figure 6a. EPO digest, LC/MS TOF 95% sequence coverage achieved using MassHunter Workstation software.

To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

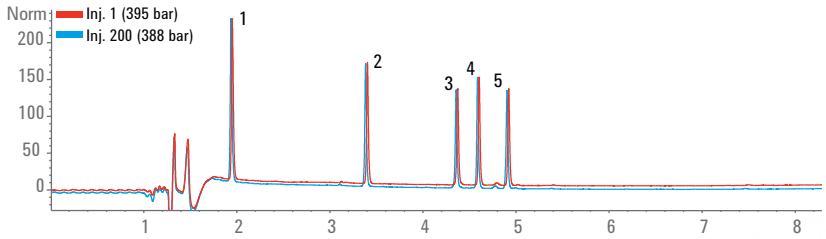
Excellent reproducibility

The science behind AdvanceBio columns helps increase accuracy and productivity to support faster biopharmaceutical analysis and efficiency. In addition, AdvanceBio columns are rigorously tested by Agilent to ensure reproducibility, giving you greater confidence in your results.

Figure 7 demonstrates the superior lot-to-lot and run-to-run reproducibility that can be achieved using AdvanceBio Peptide Mapping columns.

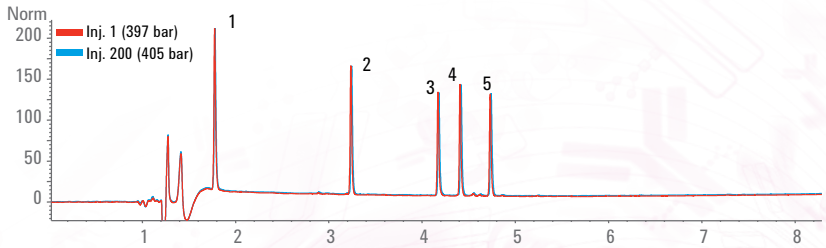
Lot-to-lot reproducibility after 200 injections

Silica lot PEP1227229



Injection	RT2 (min)	RT3 (min)	RT4 (min)	RT5 (min)
1	3.39	4.36	4.59	4.90
200	3.52	4.48	4.70	5.02
Injection	**PW2**	**PW3**	**PW4**	**PW5**
1	0.020	0.021	0.020	0.022
200	0.020	0.021	0.019	0.021

Silica lot B12169



Injection	RT2 (min)	RT3 (min)	RT4 (min)	RT5 (min)
1	3.36	4.29	4.52	4.85
200	3.24	4.18	4.41	4.74
Injection	**PW2**	**PW3**	**PW4**	**PW5**
1	0.019	0.020	0.019	0.020
200	0.019	0.020	0.019	0.020

Column:	**AdvanceBio Peptide Mapping**, 2.1 x 250 mm, 2.7 µm, p/n 651750-902	Detection:	220 nm
Flow rate:	0.50 mL/min.	Gradient:	A, water (0.1% TFA), B, ACN (0.08% TFA), 0-8 min, 10-60% B; 8.1-9 min, hold 95% B
Injection:	1 µL	Sample:	Sigma HPLC peptide standards: 1-Gly-Tyr, 2-Val-Tyr-Val, 3-Met Enk, 4- Angio II, 5- Leu Enk
Temp:	55 °C		

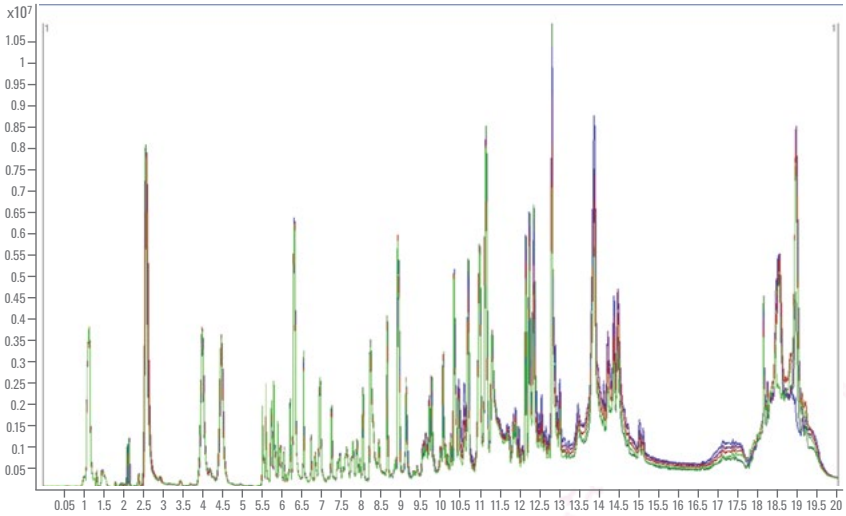
Figure 7. A 2.1 x 250 mm AdvanceBio Peptide Mapping column was used for maximum resolution.

Ideal for fast or high resolution peptide separation

Agilent AdvanceBio Peptide Mapping columns are made with 2.7 µm ultra-high purity (>99.995% SiO_2) superficially porous silica, and are densely bonded with C18 to provide the high selectivity needed for peptide separations. This type of particle delivers high efficiency at lower pressures when compared to small, totally porous particles.

In **Figure 8**, you can see how AdvanceBio Peptide Mapping columns ensure reproducibility of peak heights and retention times for more accurate target peptide identification.

LC/MS reproducibility



Column:	**AdvanceBio Peptide Mapping**, 3.0 x 150 mm, 2.7 µm, p/n 653950-302
LC/MS (Agilent 6520 Q-TOF) Parameters	
Dry gas:	10 L/min, Vcap: 4000 V,
Fragmentor:	150 V
Flow rate:	0.3 mL/min
Injection:	1 µL
Temp:	40 °C
Gradient:	A, water (0.1% FA), B, ACN (0.10% FA), 0-3 min, 2% B; 3-13 min, 2-45% B; 13-15 min, 45-65% B; 15.1-17 min., hold 90% B
Sample:	Stratagene mAb, in-house tryptic digestion

Figure 8. This entire IgG1 tryptic peptide map was completed in just 20 minutes (n=5).

To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

POROSHELL 300 COLUMNS

Quick, confident separation of intact proteins and protein fragments

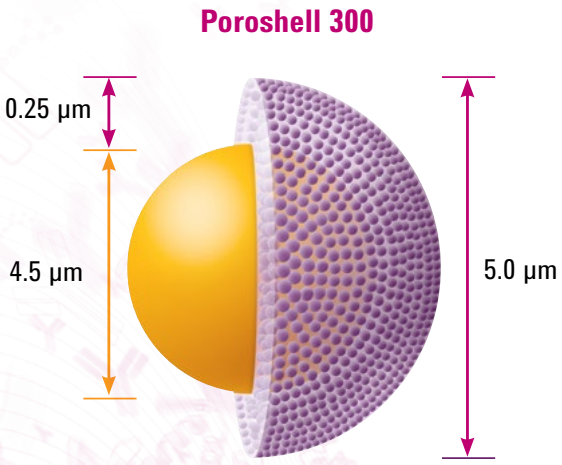
Agilent Poroshell columns are the ideal choice for separating and characterizing complex bio-molecules, including intact and protein fragments at pressures up to 400 bar.

For fast analysis of intact proteins, we recommend Agilent Poroshell 300 columns. The Poroshell 300 superficially porous particle is a revolutionary chromatography material that produces very fast, high-resolution, RP-HPLC separations of proteins and other macromolecules.

Poroshell columns work so well for fast separations of macromolecules because of their rapid mass transfer into and out of their thin 300Å porous shell, providing sharper peaks for higher resolution, for improved accuracy of impurity profiling and post-translational modifications.

Shorter analysis time, higher resolution, with lower column pressures

Feature	Advantage
0.25 µm, 300Å porous shell on solid particle	• Shorter diffusion distances for reduced analysis times
5 µm particle	• Lower operating pressure • Long column lifetime due to reduced sample trapping • UHPLC resolution and efficiency at lower pressures, for faster separations
StableBond chemistry	• Proven stability at low pH • Long column lifetime with TFA and formic acid



Use Poroshell 300 columns for analyzing intact proteins and large peptide fragments.

High flow rates with 2.1 mm id

Poroshell 300 columns, with their larger 300Å pore size and thin shell, are a reliable choice for fast separations of intact proteins. The separation shown in **Figure 9** was completed in less than 1.5 minutes.

With the rapid mass transfer capability of the superficially porous particle, Poroshell 300 columns are the best columns for high efficiency at higher flow rates for extremely rapid protein separations.

Separation of peptides and proteins

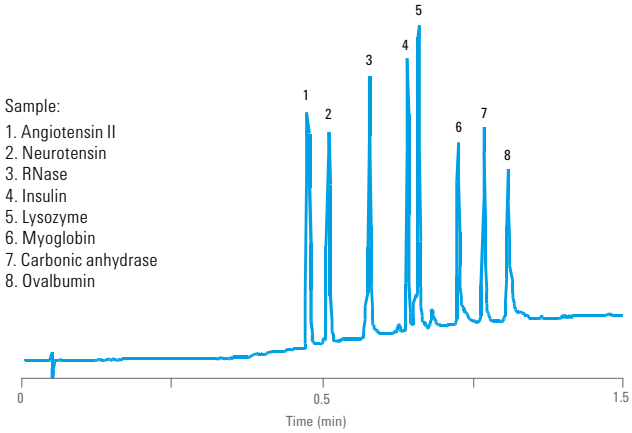


Figure 9. Separation of 8 peptides and proteins in under 1.5 minutes – good peak capacity for rapidly separating complex samples.

Column:	**Poroshell 300SB-C18**, 2.1 x 75 mm, 5 µm
Mobile phase:	A: 0.1% TFA
	B: 0.07% TFA in ACN
Flow rate:	3.0 mL/min
Temperature:	70 °C
Detection:	UV 215 nm
Gradient:	5 to 100% B in 1.0 min
Pressure:	250 bar

Separation of monoclonal antibody heavy and light chains

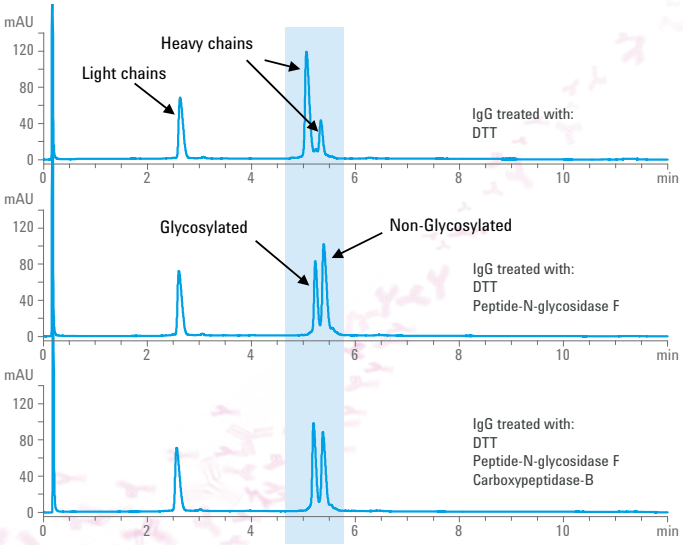


Figure 10. Chromatographic comparison of antiboday IgG after reduction and enzymatic cleavage.

Gradient:

Time (min)	% Solvent B
0.00	25
10.00	40
10.10	25
12.00	25

Column:	**Poroshell 300SB-C8**, 2.1 x 75 mm, 5 µm
Mobile phase:	A: H_2O-ACN (90:10)
	B: H_2O-ACN (10:90)
	A and B contain 0.1% TFA and 3 mL/L of PEG 300
Flow rate:	1.0 mL/min
Temperature:	70 °C
Detection:	UV 210 nm

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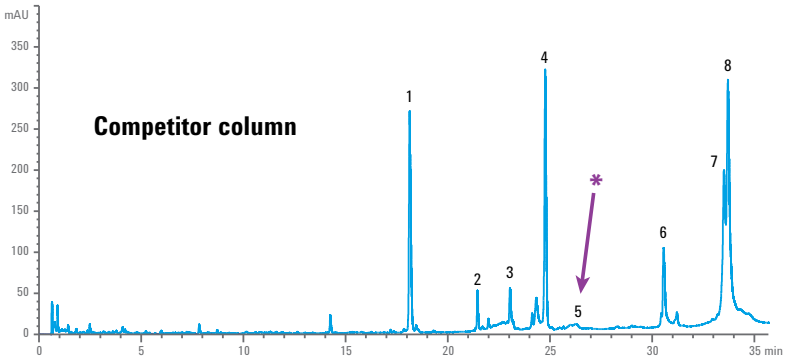
Ultra fast separation advantage

5 µm Poroshell 300 columns can deliver compelling fast-separation advantages versus a competitor's superficially porous 3.7 µm, 150 mm (low flow) column.

In **Figure 11**, the Poroshell 300 column maintained critical resolution at ultra-fast separation speeds with ballistic gradients – while maintaining pressure drops for HPLC less than 400 bar. The Agilent Poroshell 300 column resolves the 8 proteins 12x faster than the alternative superficially porous column.

Poroshell 300 vs. Competitor

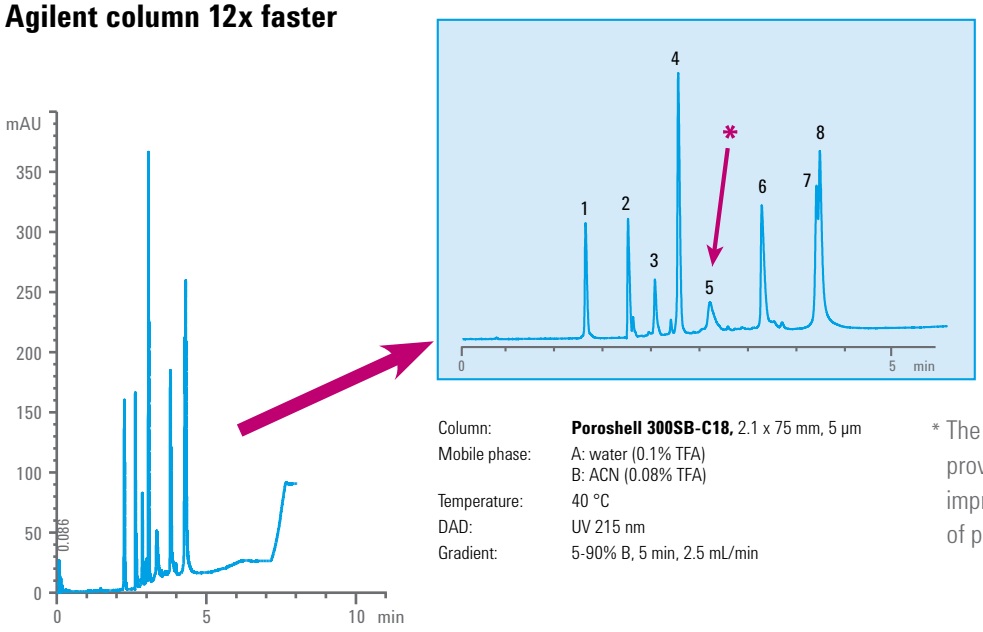


Column:	**Competitor column: C18**, 2.1 x 150 mm, 3.7 µm
Sample:	Protein Standard Mix (13 kDa- 660 kDa)
Mobile phase:	A: water (0.1% TFA) B: ACN (0.08% TFA)
Temperature:	40 °C
DAD:	UV 215 nm
Gradient:	5-90% B, 60 min, 0.3 mL/min

Sample:

1. Ribonuclease A
2. Lysozyme
3. Cytochrome c
4. Insulin
5. Transferrin
6. Myoglobin
7. B-amylase
8. Thyroglobulin

Agilent column 12x faster



Column:	**Poroshell 300SB-C18,** 2.1 x 75 mm, 5 µm
Mobile phase:	A: water (0.1% TFA) B: ACN (0.08% TFA)
Temperature:	40 °C
DAD:	UV 215 nm
Gradient:	5-90% B, 5 min, 2.5 mL/min

* The Agilent Poroshell 300 column provides better peak shape for improved accuracy in the analysis of peak 5, transferrin.

Figure 11. Ultra-fast separation advantage with Poroshell 300 SB-C18 columns versus a competitor.

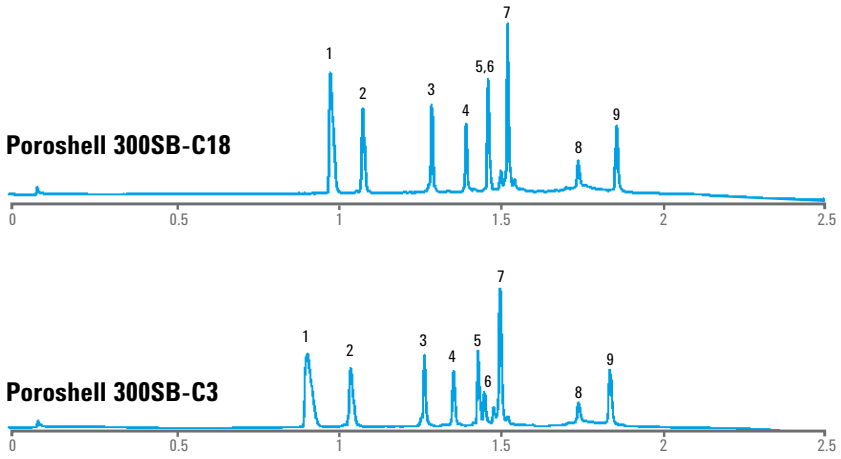
Bonded phase choices offer more resolving power and improved recovery

Poroshell 300 HPLC columns are available in four bonded phases 300SB-C18, C8, C3, and 300Extend-C18.

Reducing the bonded phase chain reduces the hydrophobicity of the 300SB-C8 and 300SB-C3 bonded phases. For example, insulin and cytochrome c are baseline resolved on the Poroshell 300SB-C3 column, while these same analytes co-elute on the Poroshell 300SB-C18 column under the conditions described in **Figure 12**.

For some complex samples, protein recovery can be an issue. Using the less hydrophobic Poroshell 300SB-C8 and C3 columns has been shown to improve recovery.

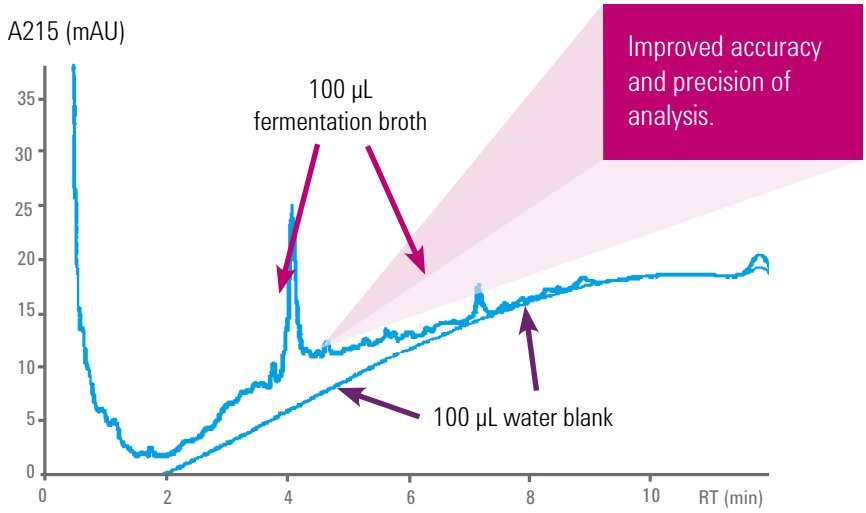
Figure 13 shows the traces that result from injecting 100 µL of fermentation broth on a Poroshell 300SB-C3 column, followed by the clean trace immediately after a blank injection of 100 µL of water. Improved sample recovery and resolution of critical pairs of peaks improves the accuracy of protein analytics.



Columns: **Poroshell 300SB-C18**, 2.1 x 75 mm, 5 µm
Poroshell 300SB-C3, 2.1 x 75 mm, 5 µm
Mobile phase: A: 0.1% TFA/H_2O
B: 0.07% TFA/ACN
Flow rate: 0.5 mL/min
Temperature: 70 °C
Gradient: 5 to 100% B in 3.0 min
Detection: UV 215 nm

Sample:
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 12. Poroshell 300SB-C3 resolves peaks 5 and 6, insulin and cytochrome c, which co-elute with the more hydrophobic C18 phase.



Column: **Poroshell 300SB-C3**, 2.1 x 75 mm, 5 µm
Mobile phase: A: 0.1% TFA /H_2O
B: 0.07% TFA/ACN
Flow rate: 1.0 mL/min
Temperature: 50 °C
Gradient: 10 to 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
Detection: UV 215 nm

Figure 13. No carryover when using the Agilent Poroshell 300SB-C3 column.

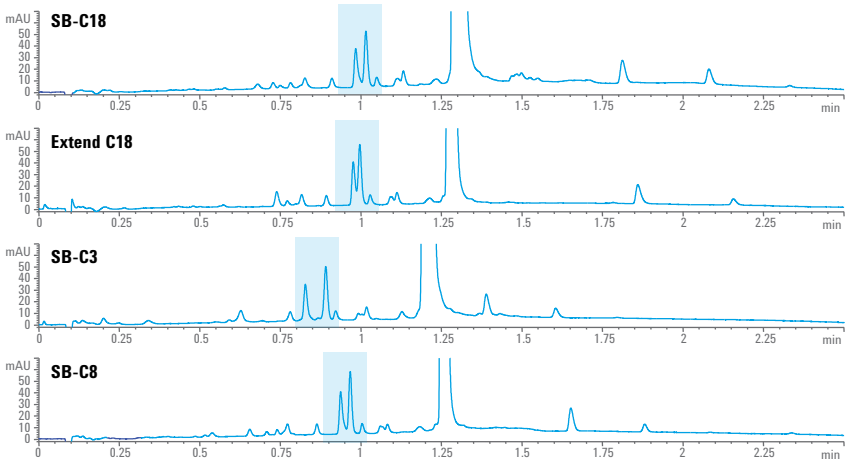
To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

Achieve unique selectivity from pH 2-11.5

Figure 14 shows that selectivity differences, coupled with the high resolving power of Poroshell 300 columns, can help you achieve very favorable improvements in your separation.

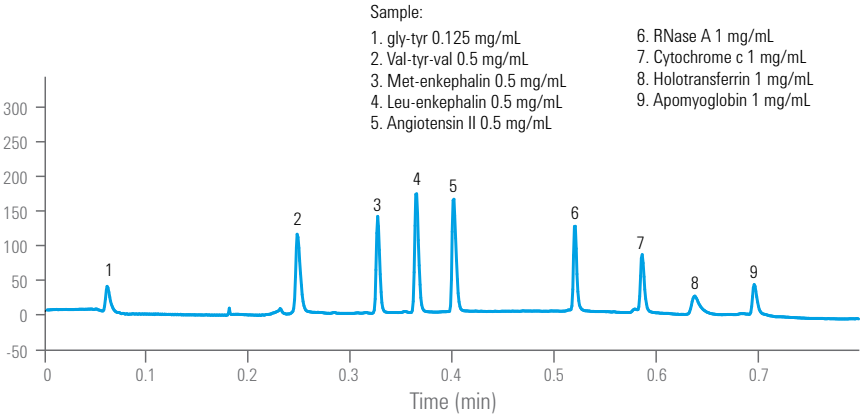
Poroshell 300Extend-C18 columns combine bidentate silane with a double-endcapping process that protects the silica from dissolution at high pH, to provide longer column lifetime and improve baseline at higher pH.

Figure 15 shows fast separation of small proteins and polypeptides in less than one minute, using the most hydrophobic phase, C18.



Column:	**Poroshell 300,** 2.1 x 75 mm, 5 µm
Sample:	Degraded insulin
Mobile phase:	A: water (0.1% TFA) B: ACN (0.08% TFA)
Flow rate:	1.75 mL/min
Temperature:	45 °C
Gradient:	5% B hold 0.3 min, 5-65% B, 2.7 min.

Figure 14. Changing the bonded phase improves resolution of the critical pair of peaks to improve accuracy of analysis.



Column:	**Poroshell 300SB-C18**, 2.1 x 75 mm, 5 µm
Sample:	peptides/proteins, 0.5 µL
	Mixer bypassed with P/N G1312-67301; Loop-bypass program
Mobile phase:	A: 0.1% TFA, H2O
	B: 0.07% TFA, ACN
Flow rate:	3 mL/min.
Gradient:	0-100% B in 1.33 min
Temperature:	70 °C
Detector:	DAD 215/16 nm, ref = 310/10 nm

Figure 15. Fast separation of small proteins and polypeptides in less than one minute.

ZORBAX RRHD COLUMNS

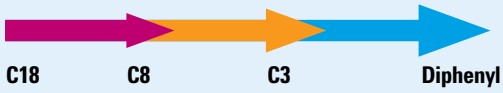
300Å 1.8 µm particles ensure stability at 1200 bar

Wide-pore ZORBAX RRHD 300SB-C18, C8, C3, and 300-Diphenyl 1.8 µm columns deliver UHPLC performance for separations of intact proteins and peptide digests. Together with UHPLC instruments, such as the Agilent 1290 Infinity LC, these versatile columns enable higher order characterization and shorter analysis times.

The Diphenyl phase provides unique selectivity, and ZORBAX StableBond technology (C18, C8, and C3) gives you the advantages of:

- **Low pH stability,** which lets you confidently perform protein and peptide separations down to pH 1 using trifluoroacetic acid (TFA) and formic acid eluents.
- **Temperature stability,** up to 80 °C, allows you to run separations at higher temperatures without compromising column lifetime. So you can improve efficiency and reduce eluent viscosity.

Increasing protein size and hydrophobicity



With four different ligand types; C18, C8, and C3 alkyl chains; and diphenyl for additional selectivity based on pi-pi aromatic amino acids, Agilent has the widest range of reversed-phase columns for peptide and protein UHPLC separations.

Reproducibility and recovery of monoclonal antibodies

For larger proteins, including monoclonal antibodies, a shorter, less hydrophobic C8 functionality is used. This provides improved resolution and high recovery.

Column: **ZORBAX RRHD 300SB-C8**, 2.1 x 50 mm, 1.8 µm
Sample: mAb
Mobile phase: A: H_2O:IPA (98:2), 0.1% TFA
B: PA:ACN:H_2O (70:20:10), 0.1% TFA
Flow rate: 1.0 mL/min
Temperature: 80 °C
Detection: 1290 Infinity LC at 225 nm

Gradient:

Time (min)	% B
0.00	25
3.00	35
4.00	90
5.00	25

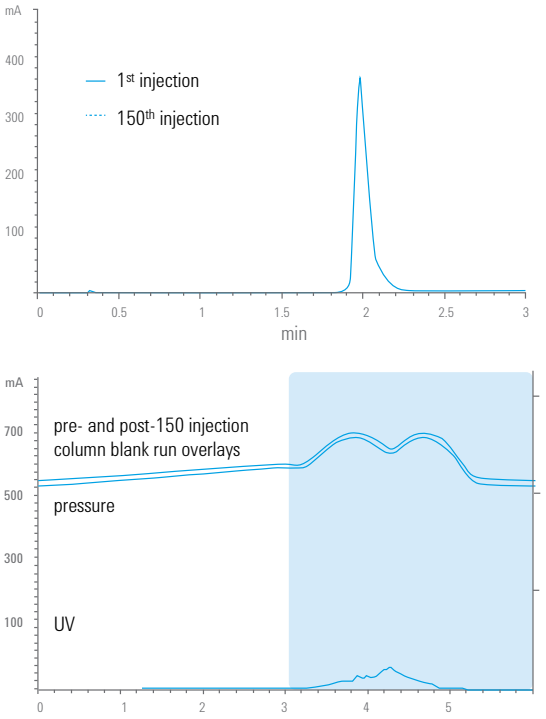


Figure 16. This example demonstrates the reproducibility and lifetime of an Agilent ZORBAX RRHD 300SB-C8 column over 150 injections, with no retention time shifts or peak shape abnormalities. The bottom chromatogram shows the pre- and post-150 injection blank runs and gradient pressure curves, proving that there was no ghosting or pressure increase after 150 injections – therefore, **no column failure or sample losses which will improve accuracy of quantitation.**

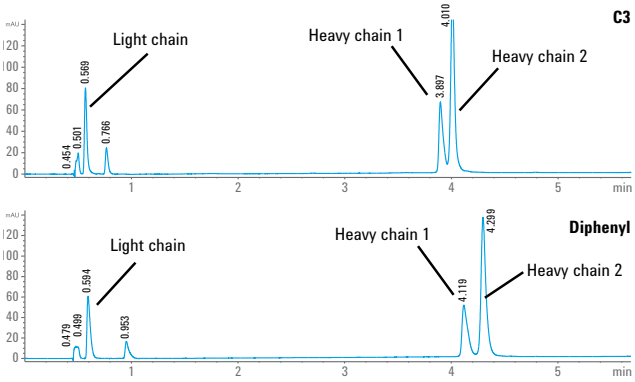
To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

More speed, more resolution

The **unique diphenyl phase** was previously available only on smaller pore 100Å Pursuit XRs and 200Å Pursuit columns. Now, by applying this proven bonding chemistry to ZORBAX 300Å 1.8 µm columns, this unique selectivity can be used for larger protein separations.

1.8 µm RRHD columns can deliver fast separation advantages over competitive 3.7 µm columns (low-flow model), showing appreciable gains in analysis speed while maintaining comparable resolution. In **Figure 18**, 1.8 µm columns maintained (and surpassed) critical resolution at **ultra-fast** separation speeds with ballistic gradients – a demonstrable UHPLC advantage.

Fast separation of reduced monoclonal antibody

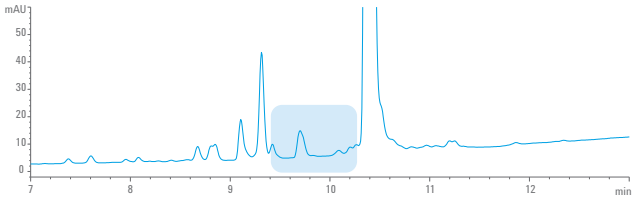


Columns:	**ZORBAX RRHD 300SB-C3** and **300-Diphenyl**, 2.1 x 100 mm, 1.8 µm
Sample:	Reduced monoclonal antibody (IgG1) (1.0 mg/mL)
Sample injection:	2 µL
Mobile phase:	A: 0.1% TFA in water
	B: 80% n-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Gradient:	0 min-1% B, 2 min-20% B, 5 min-50% B
Flow rate:	0.5 mL/min
Temperature:	74 °C
Detection:	UV 280

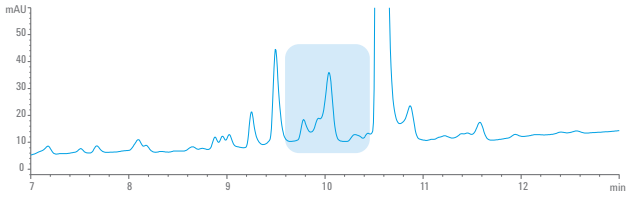
Figure 17. Comparison of fast separation of reduced monoclonal antibody using Agilent ZORBAX RRHD 300SB-C3 and 300-Diphenyl, 2.1 x 100 mm, 1.8 µm – better resolution of the two heavy chains is obtained with the diphenyl column.

ZORBAX RRHD vs. Competitor column

Competitor column: C18, 2.1 x 150 mm
164 bar



ZORBAX RRHD 300SB-C18, 2.1 x 100 mm, 1.8 µm
359 bar



Sample:	Degraded insulin
Mobile phase:	A: water (0.1% TFA) B: ACN (0.08% TFA)
Gradient:	3% B hold 3.0 min 3-65% B, 15 min
Flow rate:	0.3 mL/min
Temperature:	40 °C
DAD:	225 nm

Figure 18. Degraded insulin separation. Agilent Rapid Resolution High Definition 300Å 1.8 µm columns achieve superior bandwidths and peak shapes as the competition (improved resolution of degradation products).

ZORBAX 300Å 3.5 AND 5 µm COLUMNS

Extraordinary chemical and temperature stability in the pH 1-6 range

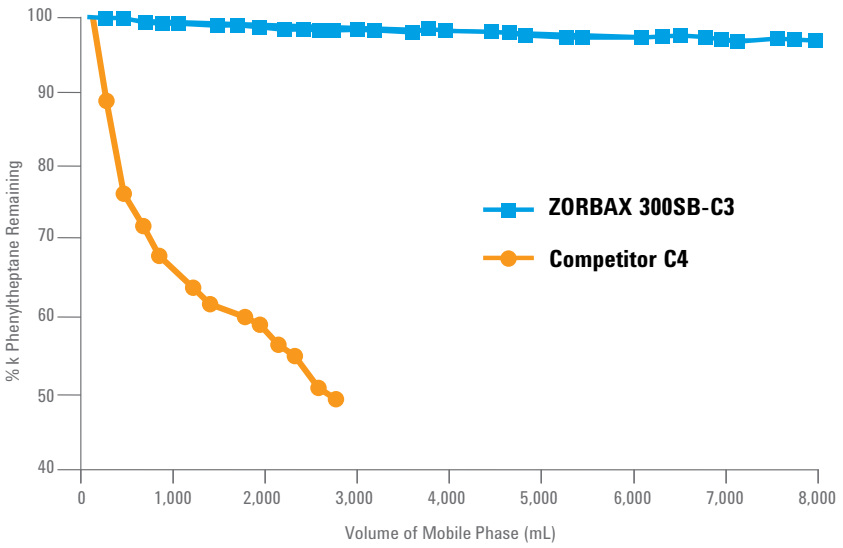
Agilent ZORBAX 300StableBond columns are ideal for the reproducible separation of proteins and peptides for two reasons:

- Wide pore, 300Å columns allow proteins, peptides, and other large molecules to completely access the bonded phase.
- ZORBAX 300StableBond columns have unmatched durability with the low-pH mobile phases (including TFA) that are typically used for protein and peptide separations.

For LC/MS separations at low pH, ZORBAX 300StableBond columns can also be used with formic acid and acetic acid mobile phase modifiers.

These columns are available in four different bonded phases, **StableBond C18, C8, C3, and Extend-C18** for selectivity and optimized recovery of proteins and polypeptides. To further increase sample recovery and improve efficiency for difficult proteins, 300StableBond columns can be used up to 80-90 °C.

Short-chain ZORBAX 300SB-C3 is stable at low pH and high temperature for reproducible separations and longer column lifetime



Column:	**ZORBAX 300SB-C3**, 4.6 x 150 mm, 5 µm
Mobile phase:	Gradients 0-100% B in 80 min A: 0.5% TFA in Water B: 0.5% TFA in Acetonitrile Isocratic Retention Test Conditions: 1-phenylheptane 50% A, 50% B
Flow rate:	1.0 mL/min
Temperature:	60 °C

Figure 19. Typical mobile phases for protein and peptide separations combine a very low pH with TFA (or other acids) with high temperature to denature and solubilize proteins. Agilent StableBond columns have extremely long lifetimes under these conditions.

To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

PLRP-S HPLC COLUMNS

Reproducible separations under extreme conditions

The Agilent PLRP-S column family includes a range of pore and particle sizes, all with identical chemistries and fundamental chromatographic characteristics. They feature:

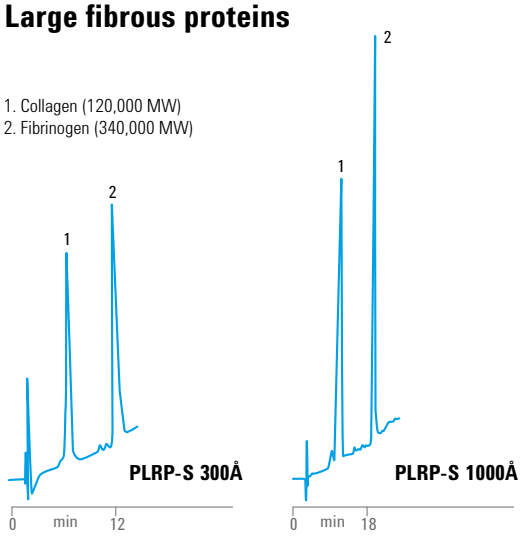
- Durable, resilient polymer particles that deliver reproducible results for longer column lifetimes
- Thermal and chemical stability for separations at extremes of pH and high temperature
- 100-4000Å pore sizes that provide high efficiency separations over the full range of protein and peptide sizes

PLRP-S particles are inherently hydrophobic; and so no bonded phase, alkyl ligand, is required. This ensures a highly reproducible material that is free from silanols and heavy metal ions.

In addition, PLRP-S provides scalability from analytical separations through purification, prep columns, and bulk media.

When purifying proteins it may be necessary to sanitize the column with the PLRP-S material. It is possible to use extremely aggressive cleaning in place, including 1M NaOH, as demonstrated in **Figure 21**. Media can be cleaned in a packed column (CIP), or in bulk, using a range of solubilizing agents such as sodium hydroxide to ensure unsurpassed column/media lifetimes.

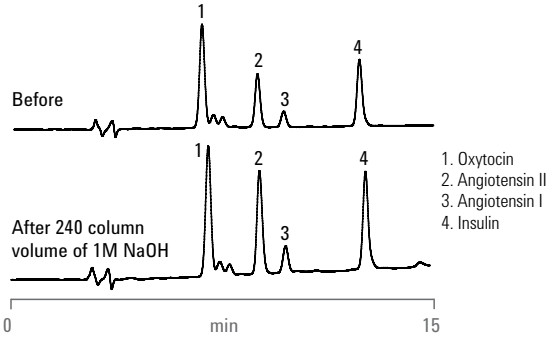
Large fibrous proteins



Column:	**PLRP-S 300Å** 4.6 x 150 mm, 8 µm	Mobile phase:	A: 0.25% TFA in wate B: 0.25% TFA in 5% water: 95% ACN
Column:	**PLRP-S 1000Å** 4.6 x 150 mm, 8 µm	Flow rate:	1.0 mL/min
		Gradient:	20-60% B in 15 min
		Detector:	UV, 220 nm

Figure 20. Agilent PLRP-S 300Å and PLRP-S 1000Å material separate large fibrous proteins, as shown here. However, improved peak shape and increased peak height were obtained from the larger pore size PLRP-S 1000Å column.

Exploiting chemical stability – NaOH concentration



Column:	**PLRP-S 300Å** 4.6 x 250 mm, 10-15 µm	Gradient:	20-50% B in 15 mins
		Flow rate:	1.0 mL/min
Mobile phase:	A: 0.1% TFA in Water B: 0.1% TFA in ACN	Detector:	UV 220 nm

Figure 21. The Agilent PLRP-S media is chemically robust and can withstand extremely aggressive sanitizing/cleaning protocols, to ensure unsurpassed column/media lifetimes.

INSTRUMENTS FOR PROTEIN IDENTIFICATION AND IMPURITY PROFILING

Use for protein identification – for best results use with Poroshell 300

Agilent 1260 Infinity Bio-inert Quaternary LC System: your best choice for protein separations

The only UHPLC that provides a metal-free sample flow path. Other advantages include:

- 100% Bio-inertness
  - No stainless steel: sample does not touch metal surfaces
  - pH 1 to pH 13 (pH 14 short-term)
  - Handles 2 M salt and 8 M urea
  - New capillary technology
- UHPLC capability: 600 bar
- Robust and easy to use with low surface activity, corrosion resistance, active seal wash, and quaternary buffer mixing

Use for impurity profiling, peptide mapping or ultra-fast gradients – for best results use with ZORBAX RRHD 300Å 1.8 µm

Agilent 1290 Infinity Binary LC System: most adaptive UHPLC system with the widest application range

Best-in-class performance in terms of resolution per time, dispersion, sensitivity, accuracy and precision in LC/UV and LC/MS. By combining innovative active damping, microfluidic mixing and optofluidic waveguides detection technology:

- UHPLC power range with up to 1200 bar and 5 mL/min
- The fastest and easiest method transfer using ISET, Agilent's unique Intelligent System Emulation Technology
- UHPLC productivity, HPLC-like costs of ownership

Use for any kind of standard UHPLC application

Agilent 1260 Infinity Binary LC System: raising the standard in analytical HPLC – 600 bar,high-speed 80 Hz detector, up to 10x higher sensitivity

100% HPLC compatibility, UHPLC capability:

- UHPLC performance, HPLC-like costs of ownership
- Support of LC and LC/MS applications, with any narrow and standard bore analytical column (2.1 - 4.6 mm id)
- Superior gradient accuracy by high-pressure mixing

Use for method development or walk-up systems with accurate buffer blending

Agilent 1290 Infinity Quaternary LC System: combine performance with flexibility

The only quaternary UHPLC system with binary-like accuracy and precision. Further advantages include:

- UHPLC power range with up to 1200 bar and 5 mL/min
- BlendAssist, the easiest tool for accurate buffer and additive blending
- UHPLC productivity, HPLC-like costs of ownership

To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

Ordering Information and Specifications

AdvanceBio RP-mAb columns

Bonded phase	Pore size	Temp. limits	pH range	Endcapped
C4	450Å	90 °C	1.0 to 8.0	Yes
SB-C8	450Å	90 °C	1.0 to 8.0	No
Diphenyl	450Å	90 °C	1.0 to 8.0	Yes

Description	Size (mm)	Particle Size (µm)	Part Number
C4	2.1 x 50	3.5	799775-904
C4	2.1 x 75	3.5	797775-904
C4	2.1 x 100	3.5	795775-904
C4	2.1 x 150	3.5	793775-904
C4	4.6 x 50	3.5	799975-904
C4	4.6 x 100	3.5	795975-904
C4	4.6 x 150	3.5	793975-904
SB-C8	2.1 x 50	3.5	789775-906
SB-C8	2.1 x 75	3.5	787775-906
SB-C8	2.1 x 100	3.5	785775-906
SB-C8	2.1 x 150	3.5	783775-906
SB-C8	4.6 x 50	3.5	789975-906
SB-C8	4.6 x 100	3.5	785975-906
SB-C8	4.6 x 150	3.5	783975-906
Diphenyl	2.1 x 50	3.5	799775-944
Diphenyl	2.1 x 75	3.5	797775-944
Diphenyl	2.1 x 100	3.5	795775-944
Diphenyl	2.1 x 150	3.5	793775-944
Diphenyl	4.6 x 50	3.5	799975-944
Diphenyl	4.6 x 100	3.5	795975-944
Diphenyl	4.6 x 150	3.5	793975-944

Poroshell 300 columns for protein analysis

Bonded phase	Pore size	Temp. limits	pH range	Endcapped
300SB-C18, C8, C3	300Å	90 °C	1.0 to 8.0	No
300Extend-C18	300Å	40 °C above pH 8, 60 °C below pH 8	2.0 to 11.0	Yes

Description	Size (mm)	300SB-C18 USP L1	300SB-C8 USP L7	300SB-C3	300Extend-C18 USP L1
Capillary	0.5 x 75		5065-4468		
Capillary	0.5 x 75		5065-4468		
MicroBore	1.0 x 75	661750-902	661750-906	661750-909	971750-902
Narrow Bore	2.1 x 75	660750-902	660750-906	660750-909	970750-902
Guard cartridge, 4/pk	2.1 x 12.5	821075-920	821075-918	821075-924	
Guard hardware kit		820888-901	820888-901	820888-901	
MicroBore guard, 3/pk	1.0 x 17	5185-5968	5185-5968	5185-5968	5185-5968

ZORBAX 300Å columns for HPLC and UHPLC protein separations

Bonded phase	Pore size	Temp. limits	pH range	Endcapped
300SB-C18	300Å	90 °C	1.0 to 8.0	No
300SB-C8	300Å	80 °C	1.0 to 8.0	No
300SB-C3	300Å	80 °C	1.0 to 8.0	No
300SB-CN	300Å	80 °C	1.0 to 8.0	No
300Extend-C18	300Å	60 °C	2.0 to 11.5	Double
300-Diphenyl	300Å	80 °C	1.0 to 8.0	Yes

Description	Size (mm)	Particle Size (µm)	300SB-C18 USP L1	300SB-C8 USP L7	300SB-CN USP L10	300SB-C3 USP L56	300Extend-C18 USP L1	300-Diphenyl USP L11
MicroBore	1.0 x 250	5	861630-902					
MicroBore RR	1.0 x 150	3.5	863630-902	863630-906				
MicroBore RR	1.0 x 50	3.5	865630-902	865630-906				
Narrow Bore	2.1 x 250	5	881750-902					
Narrow Bore	2.1 x 150	5	883750-902	883750-906	883750-905	883750-909		
Narrow Bore	2.1 x 100	1.8	858750-902	858750-906		858750-909		858750-944
Narrow Bore	2.1 x 50	1.8	857750-902	857750-906		857750-909		857750-944
Narrow Bore RR	2.1 x 150	3.5		863750-906			763750-902	
Narrow Bore RR	2.1 x 100	3.5	861775-902	861775-906			761775-902	
Narrow Bore RR	2.1 x 50	3.5	865750-902	865750-906			765750-902	
Solvent Saver Plus	3.0 x 150	3.5	863974-302	863974-306		863974-309		
Solvent Saver Plus	3.0 x 100	3.5		861973-306				
Analytical	4.6 x 250	5	880995-902	880995-906	880995-905	880995-909	770995-902	
Analytical	4.6 x 150	5	883995-902	883995-906	883995-905	883995-909	773995-902	
Analytical	4.6 x 50	5	860950-902	860950-906	860950-905	860950-909		
Rapid Resolution	4.6 x 150	3.5	863973-902	863973-906	863973-905	863973-909	763973-902	
Rapid Resolution	4.6 x 100	3.5	861973-902	861973-906			761973-902	
Rapid Resolution	4.6 x 50	3.5	865973-902	865973-906	865973-905	865973-909	765973-902	
Semi-Preparative	9.4 x 250	5	880995-202	880995-206	880995-205	880995-209		
MicroBore Guard, 3/pk	1.0 x 17	5	5185-5920	5185-5920				
Guard Cartridge, 4/pk	4.6 x 12.5	5	820950-921	820950-918	820950-923	820950-924	820950-932	
Guard Cartridge, 4/pk	2.1 x 12.5	5	821125-918	821125-918	821125-924	821125-924	821125-932	
PrepHT Cartridge	21.2 x 250	7	897250-102	897250-106	897250-105	897250-109		
PrepHT Cartridge	21.2 x 150	7	897150-102	897150-106		897150-109		
PrepHT Cartridge	21.2 x 150	5	895150-902	895150-906		895150-909		
PrepHT Cartridge	21.2 x 100	5	895100-902	895100-906		895100-909		
PrepHT Cartridge	21.2 x 50	5	895050-902	895050-906		895050-909		
PrepHT Endfittings, 2/pk			820400-901	820400-901	820400-901	820400-901		
PrepHT Guard Cartridge, 2/pk	17 x 7.5	5	820212-921	820212-918	820212-924	820212-924		
Guard Cartridge Hardware			820444-901	820444-901	820444-901	820444-901		

To learn more about performing high-resolution protein separations with Agilent reversed-phase columns, visit **agilent.com/chem/AdvanceBio**

PLRP-S HPLC columns for widest pH range

Description	Size (mm)	Particle Size (µm)	PLRP-S 100Å USP L21	PLRP-S 300Å USP L21	PLRP-S 1000Å USP L21	PLRP-S 4000Å USP L21
MicroBore	1.0 x 50	3	PL1312-1300	PL1312-1301		
MicroBore	1.0 x 50	5	PL1312-1500		PL1312-1502	
MicroBore	1.0 x 150	3	PL1312-3300			
Analytical	4.6 x 50	8		PL1512-1801	PL1512-1802	PL1512-1803
Analytical	4.6 x 250	5	PL1512-5500	PL1512-5501		
Analytical	4.6 x 150	5	PL1111-3500	PL1512-3501		
Analytical	4.6 x 50	5	PL1512-1500	PL1512-1501	PL1512-1502	PL1512-1503
Analytical	4.6 x 150	3	PL1512-3300	PL1512-3301		
Analytical	4.6 x 50	3	PL1512-1300	PL1512-1301		
Analytical	2.1 x 250	8		PL1912-5801		
Analytical	2.1 x 150	8		PL1912-3801	PL1912-3802	PL1912-3803
Analytical	2.1 x 50	8		PL1912-1801	PL1912-1802	PL1912-1803
Analytical	2.1 x 250	5	PL1912-5500	PL1912-5501		
Analytical	2.1 x 150	5	PL1912-3500	PL1912-3501		
Analytical	2.1 x 50	5	PL1912-1500	PL1912-1501	PL1912-1502	PL1912-1503
Analytical	2.1 x 150	3	PL1912-3300	PL1912-3301		
Analytical	2.1 x 50	3	PL1912-1300	PL1912-1301		
Method development	4.6 x 250	30		PL1512-5702	PL1512-5703	821125-918
Method development	4.6 x 250	15-20	PL1512-5200	PL1512-5201		
Method development	4.6 x 250	10-15	PL1512-5400	PL1512-5401		
Method development	4.6 x 250	10	PL1512-5100	PL1512-5101	PL1512-5102	PL1512-5103
Method development	4.6 x 250	8	PL1512-5800	PL1512-5801	PL1512-5802	
Method development	4.6 x 150	30			PL1512-3702	PL1512-3703
Method development	4.6 x 150	15-20	PL1512-3200	PL1512-3201		
Method development	4.6 x 150	10-15		PL1512-3401		
Method development	4.6 x 150	10	PL1512-3100	PL1512-3101	PL1512-3102	PL1512-3103
Method development	4.6 x 150	8	PL1512-3800	PL1512-3801	PL1512-3802	PL1512-3803
Prep to Process	100 x 300	30			PL1812-3102	PL1812-3103
Prep to Process	100 x 300	15-20	PL1812-6200	PL1812-6201	880995-902	880995-906
Prep to Process	100 x 300	10-15	PL1812-6400	PL1812-6401	883995-902	883995-906
Prep to Process	100 x 300	10	PL1812-6100	PL1812-6101	860950-902	860950-906
Prep to Process	100 x 300	8	PL1812-6800	PL1812-6801	863973-902	863973-906
Prep to Process	50 x 300	8	PL1712-6800	PL1712-6801	861973-902	861973-906
Prep to Process	50 x 150	30			PL1712-3702	PL1712-3703
Prep to Process	50 x 150	15-20	PL1712-3200	PL1712-3201	863974-302	863974-306
Prep to Process	50 x 150	10-15	PL1712-3400	PL1712-3401		861973-306
Prep to Process	50 x 150	10	PL1712-3100	PL1712-3101	PL1712-3102	PL1712-3103
Prep to Process	50 x 150	8	PL1712-3800	PL1712-3801	883750-902	883750-906
Prep to Process	25 x 300	15-20	PL1212-6200	PL1212-6201		863750-906
Prep to Process	25 x 300	10-15	PL1212-6400	PL1212-6401	861775-902	861775-906
Prep to Process	25 x 300	10	PL1212-6100	PL1212-6101	865750-902	865750-906
Prep to Process	25 x 300	8	PL1212-6800	PL1212-6801	861630-902	
Prep to Process	25 x 150	30			PL1212-3702	PL1212-3703
Prep to Process	25 x 150	10	PL1212-3100	PL1212-3101	PL1712-3102	PL1712-3103
Prep to Process	25 x 150	8	PL1212-3800	PL1212-3801	5185-5920	5185-5920
Prep to Process	25 x 50	10			PL1212-1102	PL1212-1103
PLRP-S Guard Cartridges	for 5 x 3 mm, 2/pk		PL1612-1801	PL1612-1801	PL1612-1801	PL1612-1801
Guard Cartridge holder	for 3.0 x 5.0 mm cartridges		PL1310-0016	PL1310-0016	PL1310-0016	PL1310-0016

Bulk PLRP-S HPLC

Particle Size (µm)	Unit	PLRP-S 100Å USP L21	PLRP-S 300Å USP L21	PLRP-S 1000Å USP L21	PLRP-S 4000Å USP L21
50	1 kg	PL1412-6K00	PL1412-6K01	PL1412-6K02	
	100 g	PL1412-4K00	PL1412-4K01	PL1412-4K02	
30	1 kg			PL1412-6702	PL1412-6703
	100 g			PL1412-4702	PL1412-4703
15-20	1 kg	PL1412-6200	PL1412-6201	861973-906	
	100 g	PL1412-4200	PL1412-4201		
10-15	1 kg	PL1412-6400	PL1412-6401		
	100 g	PL1412-4400	PL1412-4401		
10	1 kg	PL1412-6100	PL1412-6101	PL1412-6102	PL1412-6103
	100 g	PL1412-4100	PL1412-4101	PL1412-4102	PL1412-4103
8	1 kg	PL1412-6800	PL1412-6801		

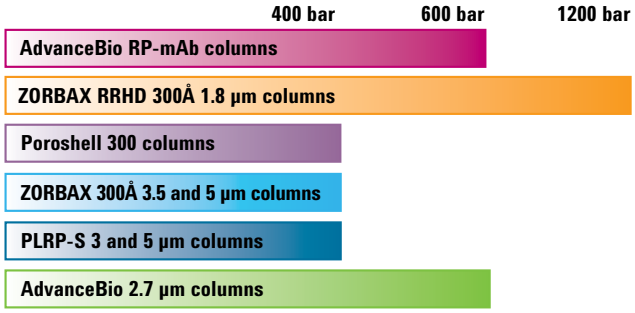
For larger quantities, please contact your local Agilent sales office.

AdvanceBio Peptide Mapping Columns

Description	Part Number
4.6 x 150 mm, 2.7 µm	653950-902
3.0 x 150 mm, 2.7 µm	653950-302
2.1 x 250 mm, 2.7 µm	651750-902
2.1 x 150 mm, 2.7 µm	653750-902
2.1 x 100 mm, 2.7 µm	655750-902
4.6 mm Fast Guard*	850750-911
3.0 mm Fast Guard*	853750-911
2.1 mm Fast Guard*	851725-911

*Fast Guards extend column lifetime without slowing down the separation or affecting resolution.

Maximum Operating Pressure



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