BETTER CHARACTERIZATION OF BIOMOLECULES USING AGILENT ADVANCEBIO REVERSED-PHASE COLUMNS

The Measure of Confidence

White Paper

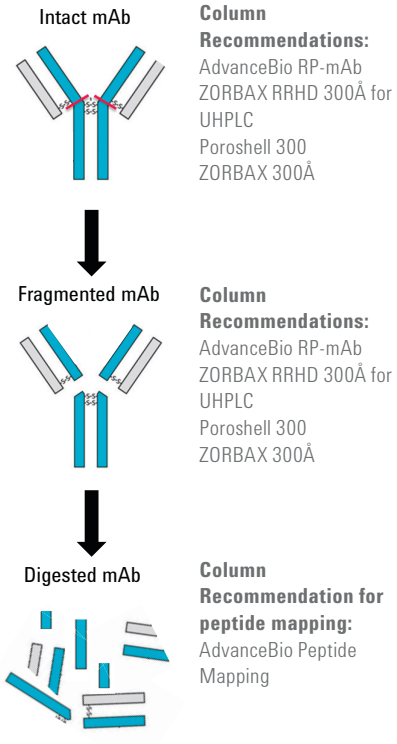


Figure 1. From intact protein to digested protein.

Why Reversed-Phase?

Protein biopharmaceuticals are very heterogeneous, so a number of chromatographic techniques may be required to fully characterize an active pharmaceutical ingredient (API). Methods include size exclusion chromatography for the quantitation of dimers and aggregates, and ion-exchange for the identification of charge variants. Both of these techniques use aqueous eluents and nondenaturing conditions. However, as part of the full characterization of a protein, it is also necessary to look at the primary amino acid sequence and any post-translational modifications to the sequence that may have occurred during the purification or formulation steps of manufacture. To perform this type of analysis, denaturing conditions are required, so reversed-phase HPLC is normally the technique of choice.

Reversed-phase (RP) is one of the three key techniques used in biochromatography, and is particularly valuable because of its compatibility with LC/MS detection. And small particle improvements, such as those found in Agilent ZORBAX RRHD 300Å, 1.8 µm columns, make RP an attractive choice for many biopharmaceutical applications. For intact monoclonal antibodies and large fragment separations, the Agilent Poroshell technology used in AdvanceBio RP-mAb offers unique advantages due to its reduced diffusion distances, which delivers significant speed and resolution advantages. With the introduction of newer phase chemistries, RP separations can provide alternative selectivities, some of which can have greater sensitivity for proteins. For example, the unique Diphenyl phase available for both AdvanceBio RP-mAb and ZORBAX RRHD 300Å 1.8 µm resolves fine detail that is simply not visible with other chemistries.

One of the advantages of reversed-phase chromatography is its versatility in analyzing different protein stages. Figure 1 illustrates the steps, from intact mAb to peptide mAb fragments, that are part of the process for fully characterizing the mAb. The characterization begins with the intact protein followed by reduction, alkylation, and digestion. A larger pore reversed-phase column is appropriate for the first two steps, and a smaller pore size column is best for analyzing the digested protein. Different selectivity can be obtained through the use of different bonded phases, column dimensions, and chromatographic conditions.

Agilent Technologies

Columns with wide pores can be used for the heavy and light chains or Fab and Fc regions, for example. We recommend AdvanceBio RP-mAb for molecules that are very large, 150 kDa. For UHPLC, or applications that require the highest resolution, it may be preferable to use ZORBAX RRHD 300Å, 1.8 µm columns. The final step in protein digestion is enzymatic digestion, which produces small peptide fragments. Peptide mapping is then accomplished with 120Å pore sizes, as are found in Agilent AdvanceBio Peptide Mapping columns.

This brief overview of the protein characterization process is a beginning, but what about sample preparation, the starting point for all protein analysis?

Sample preparation for better biochromatography

Sample preparation is an important step towards achieving accurate, reproducible, and meaningful results from biochromatography. Proteins isolated from biological cells or organisms contain contaminants such as keratin, albumin, serum proteins, nucleic acids, lipids, carbohydrates, and polysaccharides that are naturally present. In addition, various inorganic salts, buffers, reducing agents, surfactants, detergents, and preservatives can be added to a sample to retain enzymatic or biological activity. These materials affect the performance of liquid chromatography techniques such as detection and/or quantitation by mass spectrometry, and must be removed for successful results.

No universal sample preparation procedure exists to isolate all proteins in a mixture because proteins are present in multiple forms, are found within different cell locations (for example, membrane or cytoplasm), and have varying solubilities.

Common protein sample preparation processes include desalting, concentration, centrifugation, affinity, dialysis, filtration and ultrafiltration, precipitation, and lyophilization. Distinctive characteristics of the protein, such as isoelectric point, molecular weight, shape, solubility, and hydrophobicity guide the design of this purification. Since proteins are very fragile, care must be taken in the sample preparation process to avoid the introduction of unwanted modifications that change conformation and biological activity.

The biochromatographic sample preparation method is dictated by the type of sample and the kind of separation required, based on the detector, instrument, and other factors. Table 1 shows the main options when choosing a sample prep method. These are not mutually exclusive, and so it is quite likely that a syringe pre-filter would be used to remove particulates from a dirty sample, followed by a Captiva ND Lipids plate or tube to remove phospholipids and prevent ion suppression.

Table 1. Sample prep options for biochromatography.

Sample	Sample prep device	Notes	Reference
High-abundance proteins	Agilent Multiple Affinity Removal System	The Agilent Multiple Affinity Removal System (MARS) enables the identification and characterization of high-value, low abundant proteins and biomarkers found in serum, plasma, and other biological fluids. MARS reproducibly and specifically removes up to 14 high-abundant proteins found in human biological fluids and three high-abundant proteins from mouse biological fluids, in a variety of LC column dimensions and in spin cartridge format. When combined with Agilent's optimized buffers, convenient spin filters and concentrators, MARS creates an automated, integrated depletion solution compatible with most LC instruments (columns), and bench top centrifuges (spin cartridges). Samples depleted using MARS are ready for downstream analyses such as 2-D gel electrophoresis, LC/MS, and other analytical techniques.	Agilent BioHPLC Column Selection Guide
Desalting	mRP-C18	The Agilent mRP-C18 column provides optimized desalting and concentration for human serum after depletion of the high-abundance proteins using the MARS. This combined workflow enables high recovery for further downstram processing in biomarker research. Additionally, the elimination of a spin concentration step with a molecular weight cutoff (MWCO) allows the safe recovery of peptides and polypeptides less than the typical 5 kDa MWCO. This technology can be coupled, allowing for orthoganol separations without interference from salts or other additives removable by the mRP-C18 column.	Application Note 5989-2506EN
High particle-load samples	Captiva Premium Syringe Filter Glass Fiber/Nylon	Glass fiber functions as a pre-filter catching particulates before they can clog the membrane, leaving PTFE to efficiently filter particulates from the sample	Application note 5991-1308EN
Protein analysis, biomolecules, buffers	Captiva Premium Syringe Filter PES	PES is recommended due to its extremely low protein binding characteristics. It is a hydrophobic membrane and is exceptionally low in extractables. PES is compatible with aqueous and some organic solutions	Application note 5991-1308EN
Micro-volume SPE	OMIX	OMIX pipette tips reliably purify and enrich femtomole and picomole levels of peptides and proteins prior to MALDI-TOF or LC/MS/MS. The unique monolithic sorbent technology used in OMIX consistently delivers uniform flow and strong analyte-to-surface interactions. The high binding capacity of OMIX delivers high productivity. The 10 µL tips bind up to 8 µg of peptide. The OMIX superior flow and exceptional binding capacity ensure reliable recovery of peptides, minimizing peptide loss during multi-aliquot, multi-tip and evaporation steps.	Application note 5990-8885EN
Lysate filtration	Captiva ND plates or tubes	A simple-to-use filtration device designed for high-throughput, automated, in-well protein precipitation. Built with a unique non-drip (ND) membrane, Captiva ND plates allow for solvent-first protein precipitation using methanol or acetonitrile. Captiva's unique dual filter design offers fast uniform flow while avoiding sample loss and filter plugging.	Application note 5990-8388EN
Lysate filtration with lipid removal	Captiva ND Lipids plates or tubes	Specifically designed for LC/MS bioanalysis of plasma, Captiva ND Lipids combine the ease of use and superior flow properties of Captiva ND with a unique chemical filter. The plate efficiently removes ion-suppressing phospholipids, proteins, and surfactant interferences from precipitated plasma samples.	Application note 5991-0445EN

Drug discovery

Discovery applications require the highest levels of sensitivity and resolution making extensive use of mass spectrometry (MS), and are ideal for reversed-phase separations. The increased sensitivity of MS helps with characterizing mAbs and other recombinant proteins, and identifying complex peptide digests. MS provides information on peptide identity and post translational modifications.

Tips for better drug discovery

Select the right pore size for your separation

The pore size of silica is a very important parameter of a reversed-phase column for biochromatography. For molecules with molecular weights less than 5,000 Da, a column with a small pores size such as 90 to 150Å is typically selected (do not use 60Å for bio-applications). A pore size of 200 to 300Å is usually used for low molecular weight proteins (5 to 150 kDa). Monoclonal antibodies are approximately 150 kDa, so 300Å, 450Å and larger pore sizes, such as 1000 to 4000Å, are appropriate for RP. The largest silica pore size is 450Å.

Polymeric PLRP-S is available with 1000 and 4000Å pore sizes for very high molecular weight proteins and vaccines. A small protein, such as insulin with a molecular weight of about 5,800 Da, could be analyzed on columns with small pores, but larger pore sizes should be evaluated. The proper choice of pore size maximizes efficiency. Too small a pore size and the molecule experiences restricted diffusion in and out of the pores. Higher efficiency is obtained from an appropriate pore size. To maximize efficiency, smaller particle sizes with an optimum pore size should be selected.

Columns with smaller pore sizes, such as the 120Å found in AdvanceBio Peptide Mapping column, are often considered optimal for peptides. For polypeptides, protein fragments, and intact proteins, 300Å pores and larger are preferable. Figure 4 shows that the separation efficiency of insulin is more than doubled when comparing the results from columns with <100Å pore sizes to a column with 300Å pore size. The peak shape also improved dramatically between the smallest pore size column (ZORBAX SB-C18) and the largest pore size column (ZORBAX 300SB-C18). These two columns differ only in pore size; the type of bonding and particle size of the columns are the same. This provides a more exact comparison and shows that a pore size larger than 80Å is needed for the most efficient separation of insulin.

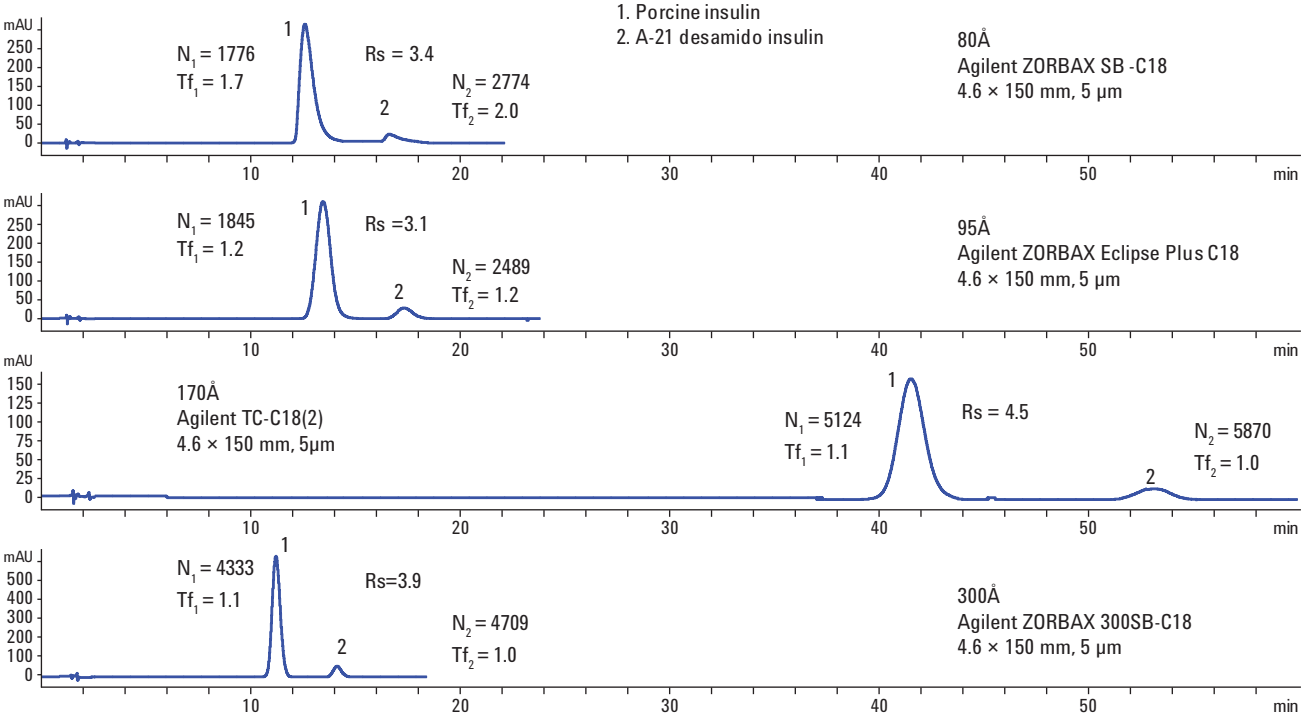


Figure 4. Chromatograms of insulin separations on an Agilent ZORBAX 4.6 × 150 mm, 5 µm LC columns, showing the effect of pore size on separation efficiency.

Select columns with smaller particle size for increased resolution

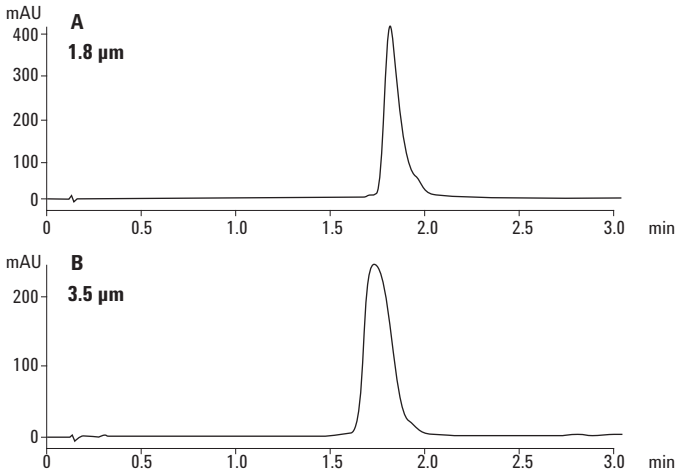
Smaller particles enable sharper peaks and better resolution. In Figure 5, we analyze an intact mAb employing a steep gradient with faster flow rate, the 1.8 µm ZORBAX 300SB-C8 column gives increased resolution, enhanced peak shape and almost twice the sensitivity.

Select columns with narrow internal diameters for more sensitivity

Narrower ID columns elute sharper bands and, when you control extra column effects, you can get greater sensitivity and compatibility with MS. Analysts using MS or UHPLC, or both, will want to use a 2.1 mm id column for best results.

Adjust the ion-pairing agent to improve the separation

Mobile phases used in RPLC for the analysis of proteins and peptides contain an additive which works as an ion-pairing agent. This component increases the hydrophobicity of molecules by forming ionic pairs with their charged groups. As a consequence, interaction of the molecules with the hydrophobic stationary phase is possible and, therefore, so is their separation. The more common additives such as trifluoroacetic acid (TFA), formic acid (FA) and acetic acid (AcOH) can yield very low pHs and promote protein unfolding and denaturation. Thus, molecules elute in sharper and more symmetrical peaks. The ion-pairing agent most widely used for the separation of proteins and peptides is TFA, however, TFA reduces the signal and trace sensitivity of the method when using ESI LC/MS. This can limit the utility of the method, making it more difficult to identify minor peaks. Alternatively, FA and AcOH can be used to replace TFA for



Conditions

Columns:	Agilent ZORBAX RRHD 300SB-C8, 2.1 × 50 mm, 1.8 µm (p/n 857750-906) Agilent ZORBAX RRHD 300SB-C8, 2.1 × 50 mm, 3.5 µm (p/n 865750-906)
Eluent:	A: H_2O:IPA (98:2) + 0.1% TFA (v/v) B: IPA:ACN:H_2O (70:20:10) + 0.1% TFA (v/v)
Injection volume:	1 µL (2 mg/mL)
Flow rate:	1 mL/min
Gradient:	Multi-segmented and linear elution

Time (min)	% B
0	25
3	25
4	90
5	25

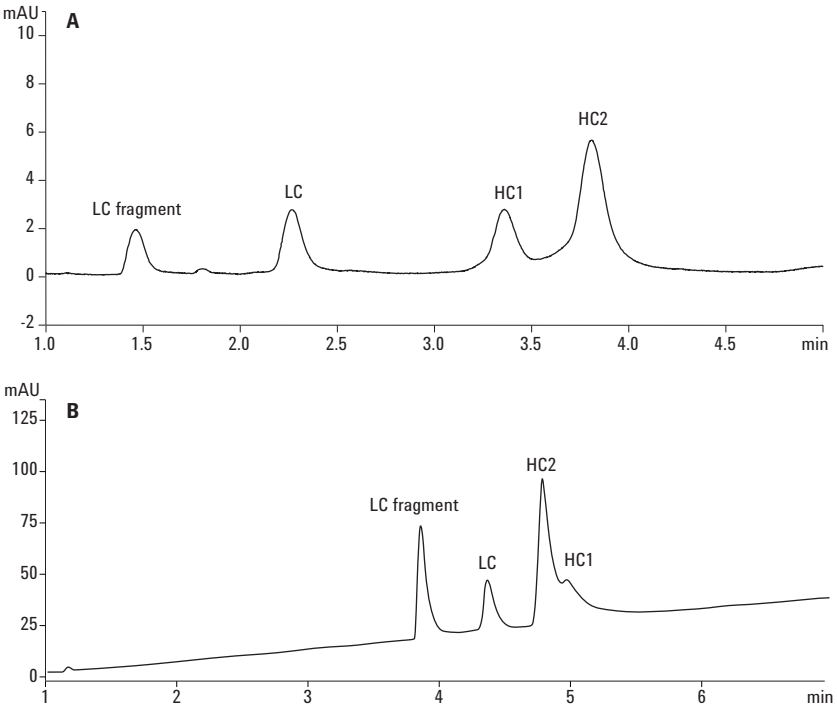
Temperature:	80 °C
Detector:	UV, 225 nm
System:	Agilent 1290 Infinity LC System with auto injector, binary pump, thermostatted column compartment, and diode array detector

Figure 5. Separation of intact mAb, comparing 1.8 µm (A) and 3.5 µm (B) Agilent ZORBAX RRHD 300SB-C8, 2.1 × 50 mm LC columns. The 1.8 µm chromatogram shows improved peak shape (A), resolution, and sensitivity.

ESI LC/MS while using less TFA in the organic can also help to enhance the signal.

We evaluated several combinations of organic mobile phase B with various ion-pair additives to provide higher signal intensity alternatives to conventional ACN/TFA-only systems for LC/MS analysis. The concentration amounts of the ion-pair reagents were adjusted between 0.5% and 5% while the B mobile phases included ACN, 1-propanol, and iso-propanol or combinations thereof.

Figure 6 displays the best separation results from this study. Under the defined gradient conditions, separation of light and two heavy chain variants were fully optimized for fast analysis with each separation completing in under 5 minutes. The top panel separation was optimized using TFA at 0.05%, while 0.5% AcOH was added. The bottom panel shows the separation from a more conventional water/ACN gradient with TFA at 0.05%, and 0.05% FA added. In this separation, the two heavy chain variants show different selectivity compared to the upper panel, where HC1 and HC2 have switched retention positions. We found that using the same gradient slope and mobile phase compositions for Figure 6, but eliminating FA, did not provide sufficient resolution between HC1 and HC2. Conditions for each separation, using reduced amounts of TFA with FA or AcOH, are very amenable for ultrafast analysis of mAb and suggest profiling alternatives for LC/MS analysis.



Conditions, Figure 6A

Eluent: A: H_2O + 0.5% AcOH:0.05% TFA (v/v)
B: *n*-propanol:ACN:H_2O (80:10:10) + 0.5% AcOH:0.05% TFA (v/v)

Gradient:

Time (min)	% B
0	25
10	35
12	35
14	90
18	25

Conditions, Figure 6B

Eluent: A: H_2O + 0.05% FA:0.05% TFA (v/v)
B: *n*-propanol:ACN:H_2O (80:10:10) + 0.05% FA:0.05% TFA (v/v)

Gradient:

Time (min)	% B
0	25
7	50
8	90
9	25

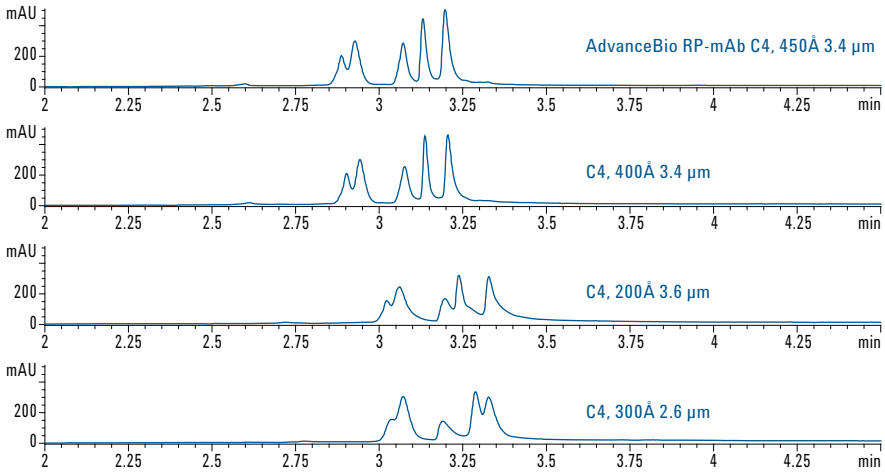
Figure 6. MS-friendly mobile phase compositions for ultrafast LC/MS characterization of reduced and alkylated antibodies using an Agilent ZORBAX RRHD 300SB-C8, 2.1 × 100 mm column. In the top panel (A) separation, acetic acid was added to the eluent to enhance MS signal intensity. The bottom panel (B) was performed with a more common water:acetonitrile mobile phase, but with a reduced amount of TFA and addition of formic acid to reduce signal suppression.

Use columns based on wide pore superficially porous particles

Because large molecules diffuse much more slowly than small molecules, the typical flow rate is lower for protein separations to allow the protein to enter and exit the pores sufficiently. Therefore, a column with a shorter diffusion path for protein migration becomes much more desirable. The Agilent AdvanceBio RP-mAb column offers this shorter diffusion distance due to its superficially porous particle structure and makes it possible to operate steeper gradients at higher flow rates without extra band effects. Figure 7 provides a good example of how a fast, high resolution mAb fragment analysis can be achieved using Poroshell technology and how the porosity of the particle has an impact on the quality of the separation.

Alternatively, sub-2 µm particles in shorter column lengths are an attractive choice to obtain higher sensitivity and less band broadening during a shorter run time, while still delivering excellent resolution.

Agilent Rapid Resolution High Definition (RRHD) sub-2 µm columns are optimized for performing rapid separations of proteins during very short run times. Using an intact monoclonal antibody, systematic gradient optimizations were performed under various column flows to evaluate separation speed and the subsequent resolution effects towards the mAb separation.



Method parameters

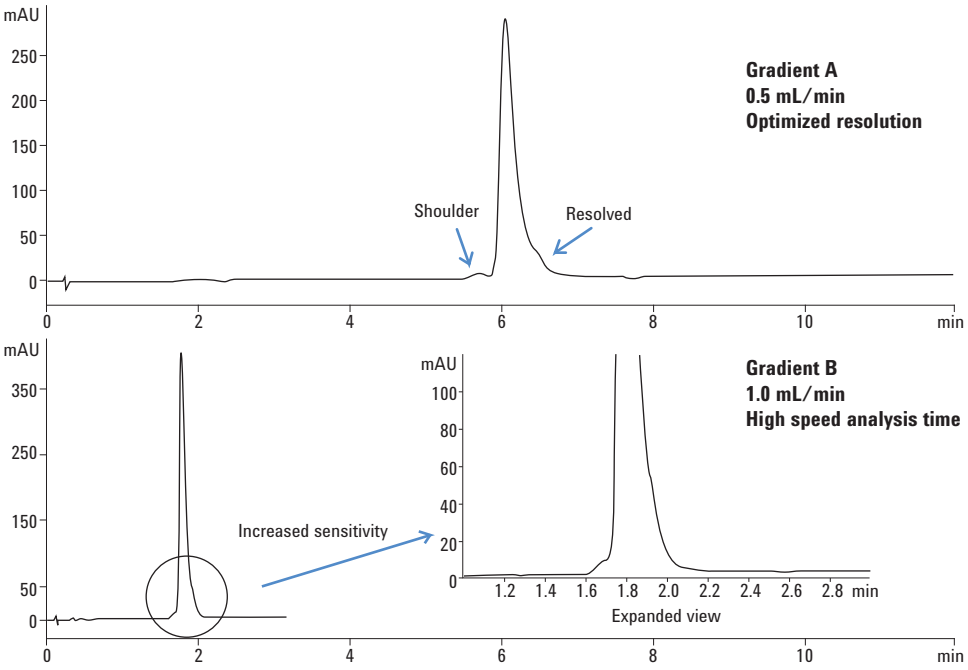
Column dimensions:	2.1 × 100 mm
Mobile phase A:	0.1% TFA in water
Mobile phase B:	*n*-propanol:ACN:Mobile phase A (80:10:10)
Flow rate:	0.8 mL/min
Gradient:	5-40% B in 5 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Sample:	1 µL injection of Fc/Fab, papain digested humanized recombinant Herceptin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

Figure 7. The Agilent AdvanceBio RP-mAb column offers shorter diffusion distance and steeper gradients without extra band effects.

Two gradients were then chosen to highlight the separation efficiency for fast mAb profiling. Specifically, the gradient in Figure 8 (top chromatogram) was optimized to highlight the ultra high resolution of an intact mAb and its shoulder peaks during a fast run time of 7 minutes, while the gradient in the Figure 8 bottom chromatogram was selected to highlight an ultra fast separation in under 2.0 minutes with increased sensitivity and slightly less resolution. Both separations deliver excellent results and highlight different aspects of an mAb separation that are desirable for biotherapeutic profiling.

Table 2. Optimized gradients for resolution or speed.

Gradient A for optimized resolution		Gradient B for high speed analysis	
Time (min)	%B	Time (min)	%B
0	25	0	25
10	35	3	35
12	35	4	90
14	90	5	25
18	25		



Conditions

Column:	Agilent ZORBAX RRHD 300SB-C8, 1.8 µm, 2.1 × 50 mm (p/n 857750-906)
Eluent:	A: H_2O:IPA (98:2) + 0.1% TFA (v/v) B: IPA:ACN:H_2O (70:20:10) + 0.1% TFA (v/v)
Injection volume:	1 µL (2 mg/mL)
Flow rate:	0.5 or 1 mL/min
Gradient:	Multi-segmented and linear elution
Temperature:	80 °C
Detector:	UV, 225 nm
System:	Agilent 1290 Infinity LC System with auto injector, binary pump, thermostatted column compartment, and diode array detector

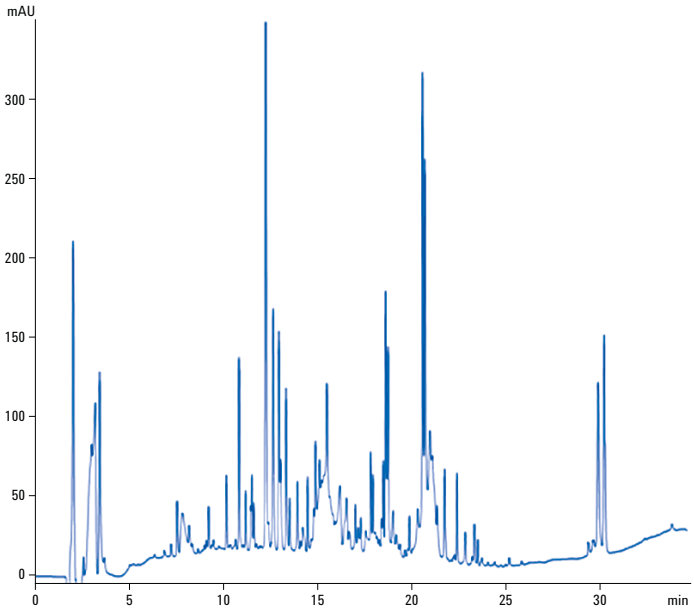
Figure 8. Two chromatographic comparisons showing optimized gradient separations of intact mAbs on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 1.8 µm column. The top chromatogram shows the ultrahigh resolution obtained during a longer run time and slower flow rate, while the bottom chromatogram ,with expanded view, shows increased sensitivity with adequate resolution in a very fast analysis time (see Table 2), useful for mAb screening.

Ensure columns and conditions of your method enable full sequence coverage

For full resolution of a peptide map, the column needs to have some key attributes:

- An optimal pore size for the molecular weight range of the peptides – 120Å is ideal
- A phase that has good selectivity to resolve peptide mapping fragments, so they can be identified by MS; an end-capped, densely C18-bonded phase will provide excellent retention and selectivity of hydrophobic, hydrophilic, and basic peptides
- A column that enables fast separations with high resolution

The Agilent AdvanceBio Peptide Mapping column was specifically designed to improve separations for peptide mapping and provides the increased resolution, higher sensitivity, and greater selectivity needed for high efficiency peptide profiling, such as erythropoietin (rhEPO) mapping and enhanced glycopeptide mining. Furthermore, the 2.7 µm superficially porous particles enables the use of a longer column, 2.1 × 250 mm, at a higher flow rate to deliver better separation performance of the rhEPO at a fraction of the backpressure compared to sub-2 µm columns. Figure 9 is an example of an optimized EPO peptide map. The separation displays excellent resolution, selectivity and peak shape across the entire gradient profile, making this separation highly amenable to ESI-MS analysis, and resulting in 100% sequence coverage (Figure 10).



Conditions

Column:	AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm (p/n 651750-902)
Sample:	Digested EPO, recombinant humanized
Sample concentration:	2.0 mg/mL
Eluent:	A: water (0.1% FA) B: ACN (0.08% FA), 0 to 28 minutes, 3 to 45% B, 28 to 33 minutes, 45 to 60% B, 33 to 34 minutes, 60 to 95% B
Injection volume:	5 µL
Flow rate:	0.4 mL/min
Temperature:	55 °C
Detector:	UV, 220 nm

Figure 9. Reversed-phase rhEPO peptide map using 2.1 × 250 mm Agilent 2.7 µm AdvanceBio Peptide Mapping Column.

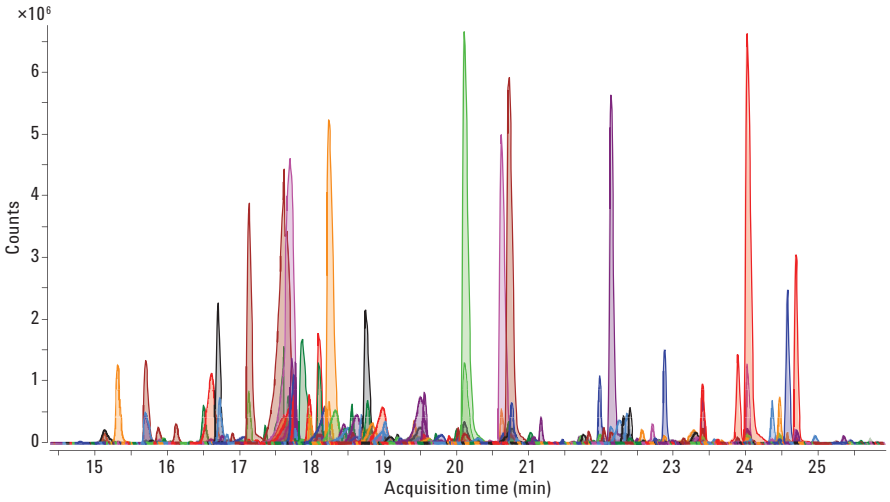


Figure 10. 100% rhEPO sequence coverage achieved using the 2.1 × 250 mm AdvanceBio Peptide Mapping column and ESI-MS. Data generated using Molecular Feature Extractor (MFE) in Agilent Masshunter qualitative MS analysis software.

Drug development

Purification and characterization of proteins are critically important to drug development, with a variety of analysis techniques routinely used. In addition to increasing the information derived in characterization, reproducibility and column lifetime become more important during the development stage.

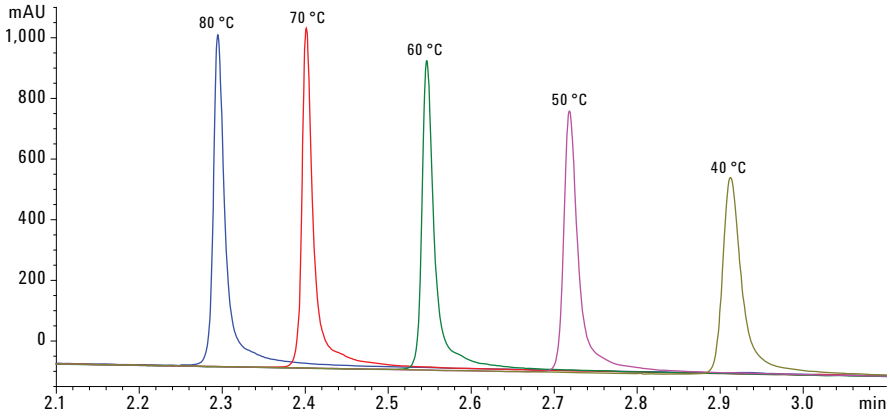
Due to the heterogeneity in hydrophobic structure of mAbs, reversed-phase separation is becoming an option for monitoring purity and stability during manufacturing, formulation and storage. Agilent ZORBAX RRHD columns have smaller, 1.8 µm particles, reproducible performance, and selectivity options that enable robust separation performance, with fast analysis times for mAb impurity characterization using reversed-phase. LC/UV is an excellent choice for mAbs and peptide mapping.

Tips for better drug development

Elevating column temperature can enhance peak shape performance, decrease retention, and improve sensitivity

Solvent viscosity, protein diffusivity, and mobile phase polarity depend strongly on temperature. The manipulation of column temperature is a critical variable in the separation of hydrophobic peptides and proteins. The monoclonal antibody (mAb) separation shown in Figure 11 provides an excellent example of the effects of temperature on peak shape and retention when temperature is elevated. At lower temperatures, the mAb and mAb fragments exhibits poor peak shape thus eluting at too high of the organic mobile phase to provide an adequate profile. Alternatively under identical chromatographic conditions, but at higher temperatures, the separation delivers excellent peak shape during a short elution time and provides increased sensitivity and resolution.

A) 0.1% TFA in water:IPA (98:2)
B) IPA:ACN:Mobile phase A (70:20:10), 1.0 mL/min, 10-70% B in 5 min, 5 µL injection of humanized recombinant Herceptin Variant IgG1 intact from Creative Biolabs (1 mg/mL), 254, 8 nm; Ref = Off, AdvanceBio RP-mAb Diphenyl, 2.1 × 100 mm, 3.5 µm



A) 0.1% TFA in water
B) *n*-propanol:ACN:Mobile phase A (80:10:10), 1.0 mL/min, 5-50% B in 5 min, 1 µL injection of Fc/Fab, papain digested humanized recombinant Herceptin Variant IgG1 from Creative Biolabs (2 mg/mL), 60 °C, 220, 8 nm; Ref = Off, AdvanceBio RP-mAb Diphenyl, 2.1 × 100 mm, 3.5 µm

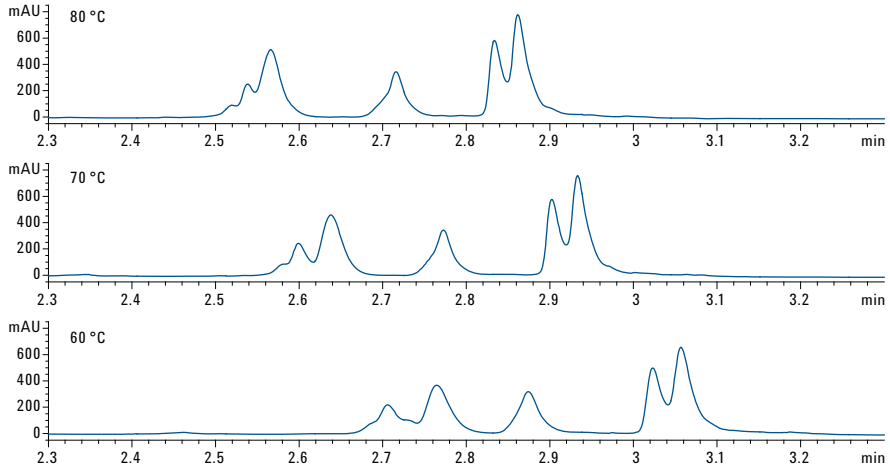
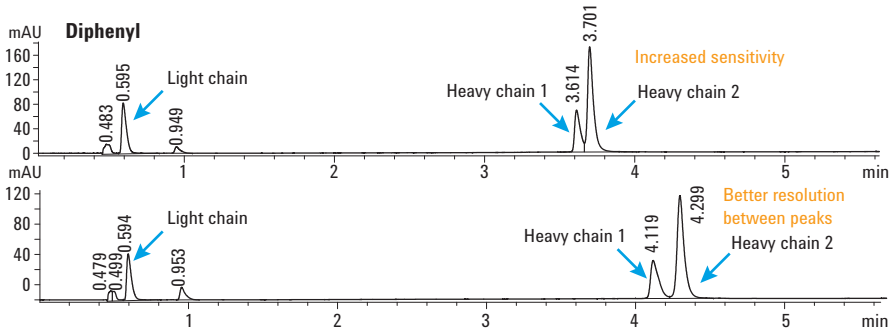


Figure 11. Temperature-dependent performance of intact monoclonal antibody separations on an Agilent AdvanceBio RP-mAb column. Top) Increasing column temperature improves peak shapes and speeds retention times. Bottom) mAb profiling is improved with higher column temperature, as fragment peaks are better resolved.

Adjust gradients for best analysis

The chromatographic comparisons in Figure 12 show two optimized high-speed separations of reduced and alkylated monoclonal antibody. The ZORBAX RRHD 300-Diphenyl, 2.1 × 100 mm column and chromatographic conditions enabled well-resolved separations of the reduced mAb light chain and two heavy chain variants. The top panel chromatogram details a separation with narrow bands and high resolution of the heavy chains. In comparison, the separation displayed in the bottom panel has been optimized to obtain better resolution of the heavy chains, but with a slight increase in peak width. In this separation, the two heavy chains display near baseline resolution. In contrast between the two separations, the diphenyl phase enabled enhanced separation control for resolving the two heavy chain peaks with minor changes to the gradient slopes.



Conditions

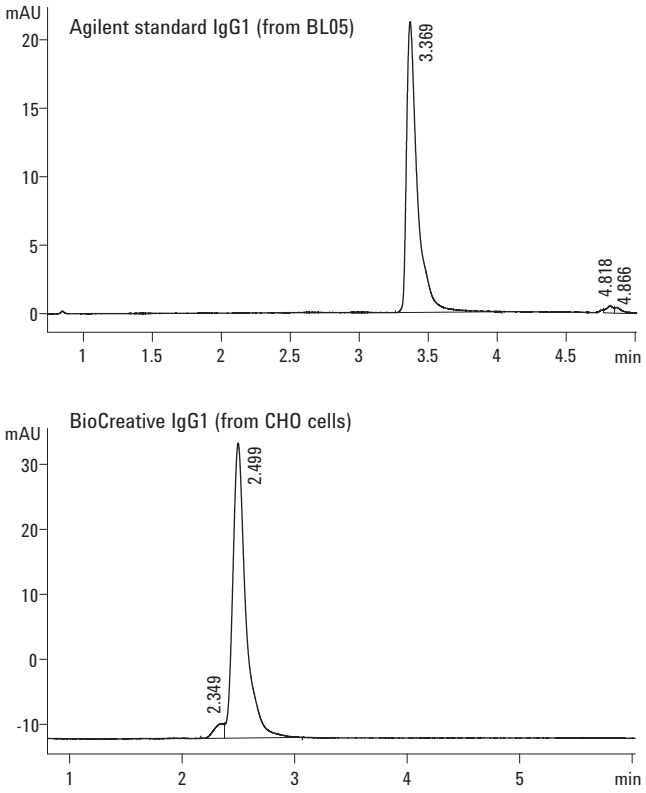
Column:	Agilent ZORBAX RRHD 300-Diphenyl, 2.1 × 100 mm, 1.8 µm (p/n 858750-944)
Sample:	Reduced monoclonal antibody (IgG1) BioCreative IgG1
Sample concentration:	1.0 mg/mL
Eluent:	A: 0.1% TFA in water B: 80% *n*-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Injection volume:	2 µL
Flow rate:	0.5 mL/min
Gradient:	First condition: 0 minutes 1% B, 2 minutes 20% B, 5 minutes 70% B Second condition: 0 minutes 1% B, 2 minutes 20% B, 5 minutes 50% B
Temperature:	74 °C
Detector:	UV, 280 nm

Figure 12. Comparison of two ultrafast separations of reduced monoclonal antibodies achieved on an Agilent ZORBAX RRHD 300-Diphenyl, 2.1 × 100 mm LC column under different optimized gradient conditions. The top panel separation delivered narrow peak widths with shorter retention times. The bottom panel separation displays higher resolution between the two heavy chain peaks, but with less efficiency.

Under systematic gradient methods, we identified gradients for each mAb to deliver high speed separations with optimum resolution. The separation displayed in the top panel of Figure 13 was optimized for the Agilent standard antibody expressed from CDH media and using gradient conditions shown in Table 3, gradient A. The separation was completed in under 4 minutes and exhibits excellent resolution of the intact peak with a very narrow band width. In comparison, the bottom chromatogram was optimized for the humanized mAb expressed from a CHO cell line using the gradient conditions shown in Table 3B, gradient B. In this separation, the gradient was optimized with a shallower gradient curve that resolved a front shoulder from the mAb base peak. Both separations in Figure 13 were developed to facilitate high throughput run to run mAb characterization with high efficiency. Each separation finished with a fast 90% isopropanol wash and rapid re-equilibration to enable repeated injection sequences.

Table 3. Optimized gradients for best selectivity.

Gradient A for Agilent standard IgG1		Gradient B for BioCreative IgG1	
Time (min)	%B	Time (min)	%B
0	10	0	5
2.5	25	5	25
4.5	35	7	25
4.56	90	8	90
5	90	9	5
6	10		



Conditions

Column:	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm (p/n 858750-909)
Sample:	Standard IgG1 from CDH media (Agilent), IgG1 from CHO (BioCreative)
Sample concentration:	1.0 mg/mL
Eluent:	A: 0.1% TFA in water B: 70% iso-propyl alcohol, 20% ACN, 10% water and 0.1% TFA
Injection volume:	2 µL
Flow rate:	1.0 mL/min
Gradient:	see Table 3
Temperature:	75 °C
Detector:	UV, 280 nm

Figure 13. UHPLC separations optimized for two monoclonal antibodies on an Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm LC column. The separations were performed at 1.0 mL/min and 75 °C. The top panel was optimized for a mAb expressed from CDH media, while the bottom chromatogram was optimized for a humanized mAb expressed by a CHO cell line. Each separation was followed with a fast 2 minute equilibration post run time.

Compare different selectivities for the best protein separation

Alternative selectivities provide additional tools to enhance protein separation for mAbs, showing great promise in facilitating biotherapeutic analyses.

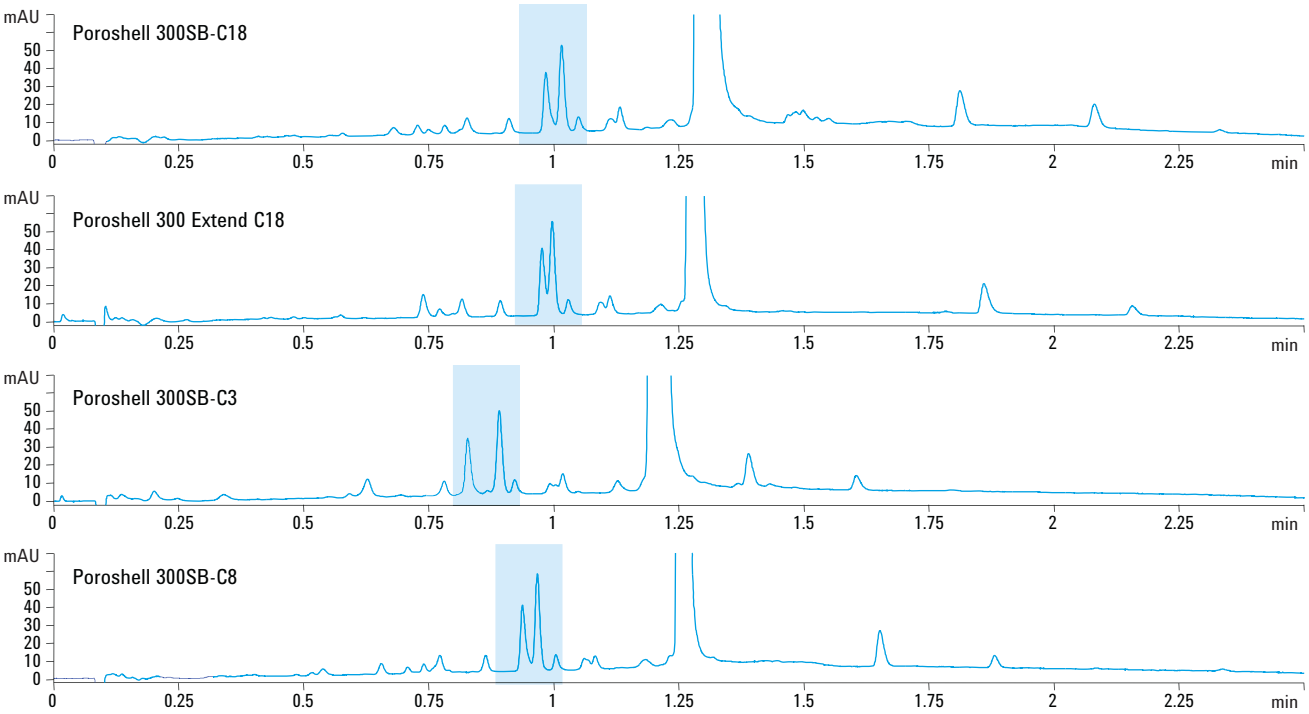
Figure 14 shows that selectivity differences, coupled with the high resolving power of Poroshell 300 columns, can help achieve very favorable improvements in separation.

Select a column phase that will last

The mobile phases routinely used for reversed phase analysis are acidic, containing trifluoroacetic acid or formic acid. These mobile phases provide the longest lifetimes on most HPLC columns. Column choice should take into consideration not only performance related to delivering the best peak shapes, but also performance in terms of lifetime, based on the conditions being used.

For typical peptide mapping applications, 0.1% TFA and formic acid may be used. AdvanceBio Peptide Mapping columns, made with a densely bonded phase, will deliver exceptional peak shapes and perform very well in these conditions.

When working with higher concentrations of TFA or other modifiers, consider StableBond technology, which as been created to remain stable in low pH environments. One note to remember: Acetic acid is sometimes used in lieu of trifluoroacetic acid or formic acid. At elevated temperatures, acetic acid will significantly limit column lifetime.



Conditions

Column:	Agilent Poroshell 300, 2.1 × 75 mm, 5 µm (p/n 660750-902)
Sample:	Degraded insulin
Eluent:	A: water + 0.1% TFA B: ACN + 0.08% TFA
Flow rate:	1.75 mL/min
Gradient:	5% B hold 0.3 minutes, 5 to 65% B, 2.7 minutes
Temperature:	45 °C

Figure 14. Changing the bonded phase of an Agilent Poroshell 300 improves resolution of the critical pair of peaks to improve accuracy of analysis.

A ZORBAX RRHD 300SB-C3 column was evaluated for lifetime at low pH during repeated injection sequences. Column packed bed stability, phase stabilization, and inlet frit performance are all critical for continued operation at elevated temperatures and pressures during repeated mAb analysis. To evalute this performance, 1,000 repeated injections of ribonuclease A, cytochrome C and lysozyme were performaned. A chromatographic view of this separation is shown in Figure 15. Figure 16 shows the lifetime plot.

Conditions

Column:	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50 mm (p/n 857750-909)
Eluent:	Mobile phase A: H_2O + 0.1% TFA (v/v) Mobile phase B: ACN + 0.1% TFA (v/v)
Injection volume:	2 µL
Flow rate:	1.25 mL/min
Gradient:	% Solvent B Time (min) 10 0 70 2.5 90 2.6 90 3.0 10 5.0
Temperature:	75 °C
Pressure:	900 bar
System:	Agilent 1290 Infinity LC System with auto injector, binary pump, thermostatted column compartment, and diode array detector

Fast separation standard proteins and their degradation products using an Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50 mm, 1.8 µm column.

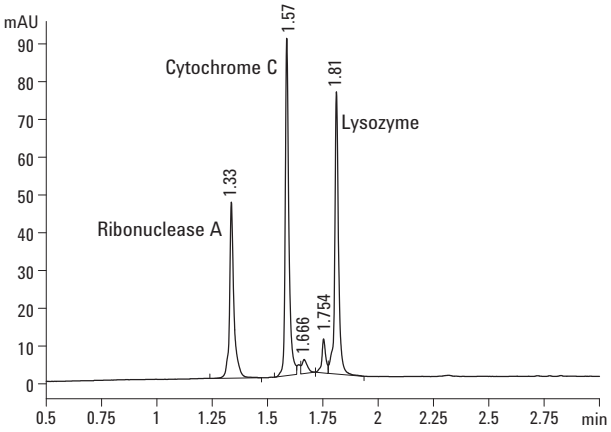


Figure 15. High speed separation of ribonuclease A, cytochrome C and lysozyme for lifetime stability monitoring using an Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50 mm LC column.

Life test of Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50 mm, 1.8 µm

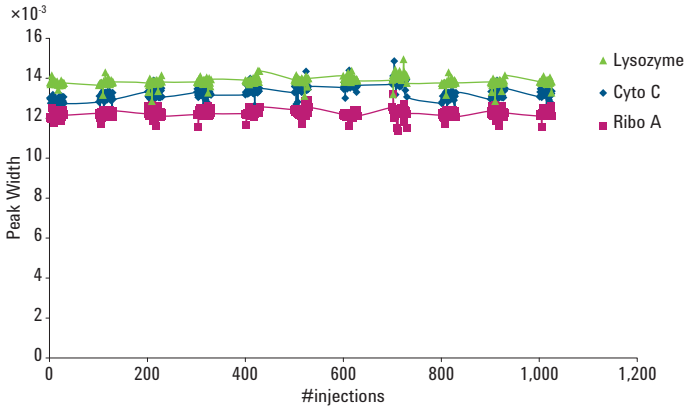


Figure 16. The lifetime plot of an Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50 mm LC column. The graph displays a continuous series of protein peak width recordings of ribonuclease A, cytochrome C, and lysozyme plotted during every 100th run interval over the course of 1,000 injections.

Quality Control

QC methods need to be focused on reliability and robustness. In addition, analytical speed becomes even more important.

Tips for better QC

Evaluating column reproducibility

ZORBAX RRHD 300Å columns exhibit outstanding batch-to-batch reproducibility, stemming from a long heritage of QC controls in manufacturing that ensure consistent performance. Figure 17 and Table 4 show the results obtained after 200 consecutive injections done to examine the reproducibility of a ZORBAX RRHD 300SB-C18 column. The integrity of peak shape, asymmetry, retention time and efficiency remained the same after the injections, without cleaning the column.

Table 4. Two hundred injections of insulin demonstrates the reproducibility of Agilent ZORBAX RRHD 300SB-C18 1.8 µm LC column.

	Heavy Chain peak 1			Heavy Chain peak 2		
Inj.#	RT (min)	Peak width	Symmetry	RT (min)	Peak width	Symmetry
1	6.845	0.043	1.74	6.912	0.048	0.611
50	6.871	0.043	1.75	6.935	0.049	0.609
150	6.864	0.040	1.70	6.924	0.049	0.617
200	6.867	0.041	1.76	3.928	0.051	0.592

Conditions

Columns:	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm
Sample:	Reduced monoclonal antibody (IgG1) (1.0 mg/mL)- Agilent BL05 IgG1
Sample injection:	2 µL
Mobile phase A:	0.1% TFA in water
Mobile phase B:	80% *n*-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Gradient:	0 minutes–1% B, 2 minutes–20% B, 5 minutes–50% B, 7 minutes–50% B, 8.0 minutes–90% B, 8.3 minutes–1% B, hold for 2 minutes
Temperature:	75 °C
Flow rate:	0.4 mL/min
Detection:	UV 280

Column reproducibility - 200 injections of reduced monoclonal antibody using Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm column

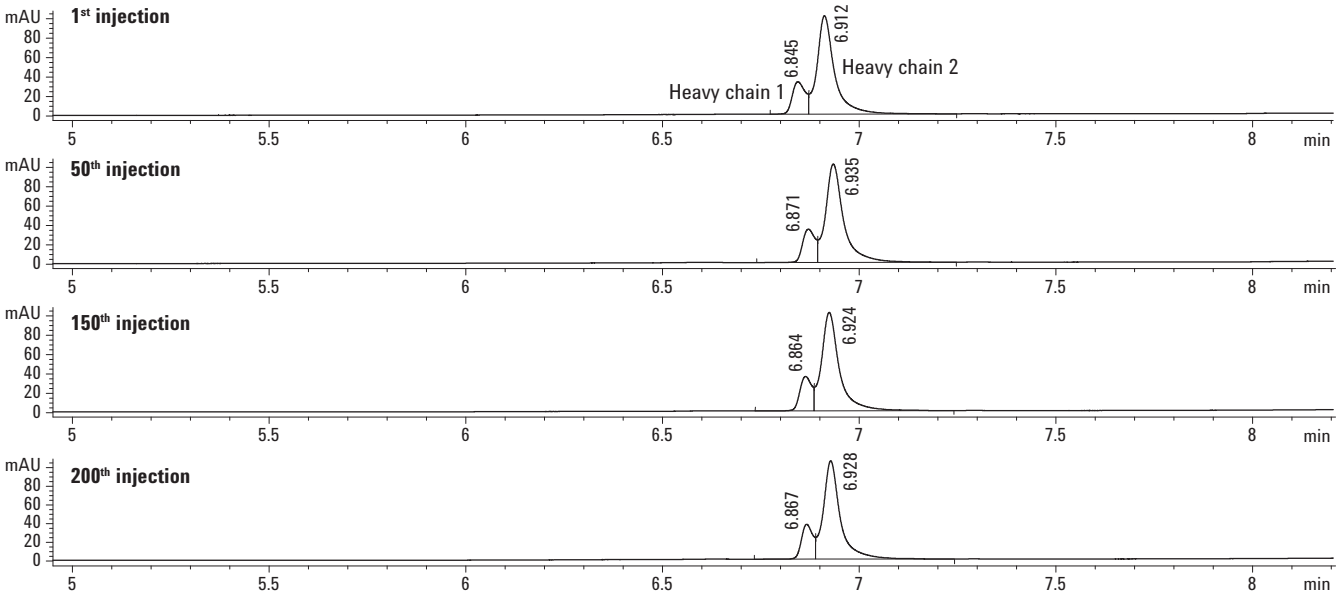


Figure 17. Two hundred injections reveal the reproducibility of the Agilent ZORBAX RRHD 300SB-C18 1.8 µm LC column.

Improve analytical speed with flow rate and shorter columns

Flow rate can also be manipulated to provide a fast separation in shorter column lengths. Peak asymmetry and efficiency remain unchanged, a feature of sub-2 µm particles that facilitates rapid separations. Figure 18 and Table 5 explain the effect of flow rate in the analysis of recombinant human erythropoietin, and Figure 19 and Table 6 on the separation of insulin.

Table 5. Effect of flow rate on retention time, asymmetry, and peak width in the separation of rEPO protein.

Flow rate (mL)	Pressure (bar)	Retention time (min)	Asymmetry	Peak width
0.5	350	1.64	0.60	0.047
1.0	680	1.41	0.85	0.030
1.5	890	1.26	0.84	0.030

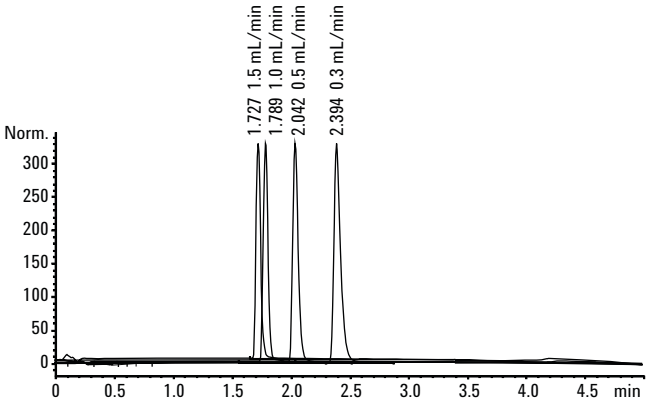
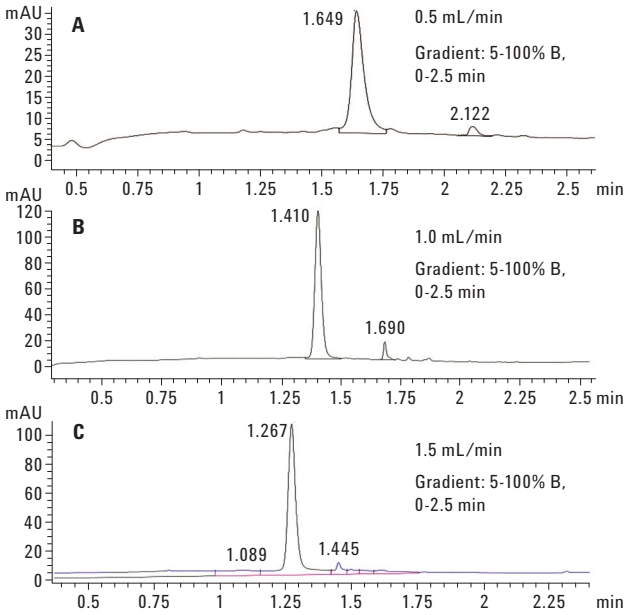


Figure 18. Different flow rates can be selected to separate insulin protein on the Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 50 mm, 1.8 µm LC column.

Table 6. Effect of flow rate on retention time, asymmetry, and peak width in the separation of insulin.

Flow rate (mL)	Pressure (bar)	Retention time (min)	Asymmetry	Plate count
0.3	230–150	2.39	0.80	8,815
0.5	350–250	2.04	0.82	8,390
1.0	680–520	1.78	0.88	8,034
1.5	890–670	1.72	0.88	8,060



Conditions

Column:	Agilent ZORBAX 300SB-C18, 2.1 × 50 mm, 1.8 µm, (p/n 857750-902)
Sample:	Insulin, oxidized insulin chain A and chain B from bovine pancreas (Sigma-Aldrich Corp.)
Sample concentration:	1 mg/mL
Eluent:	A: 0.1% TFA in distilled water B: 80% ACN + 0.01% TFA in distilled water
Injection volume:	3 µL
Flow rate:	see Table 6
Gradient:	5 to 100% B, 0 to 4 minutes
Pressure:	~ 650 bar
Detector:	UV, 280 nm
System:	Agilent 1290 Infinity LC System

Figure 19. Different flow rates can be selected to separate rEPO protein on the Agilent ZORBAX RRHD 300SB-C18, 2.1 × 50 mm, 1.8 µm LC column.

AdvanceBio RP-mAb

The only reversed-phase columns focused on the unique challenges of monoclonal antibody characterization

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
- **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

Agilent AdvanceBio RP-mAb columns

Bonded phase	Pore size	Temperature 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

ZORBAX Rapid Resolution High Definition (RRHD) 300Å, 1.8 µm

The 1.8 µm particles offer UHPLC performance, displaying greater sensitivities with enhanced peak shapes and greater resolution when compared to conventional 300Å 3.5 µm columns. Performance is extremely reproducible, a hallmark of ZORBAX manufacturing quality control. These columns are specially loaded for stability to 1200 bar premium UHPLC, so you can push flow rates with confidence.

ZORBAX RRHD 300Å, 1.8 µm is available in multiple phases, including unique diphenyl, for precise selectivity and performance profiles. Thus, the StableBond coating has excellent low pH stability and thermal flexibility, the 300SB-C18 column is stable to 90 °C, offering flexibility with temperature elevation to improve separation, and special bonded phases such as the ZORBAX StableBond C8 enable high resolution mAb separations with faster run times.

Agilent ZORBAX Rapid Resolution High Definiton (RRHD) 300 Å columns

Bonded phase	Particle size	Pore size	Temperature limits	pH range	Endcapped	Pressure Limit (bar)
300SC-C18	1.8 µm	300Å	90 °C	1.0 – 8.0	No	1200 (18,000 psi)
300SB-C8	1.8 µm	300Å	80 °C	1.0 – 8.0	No	1200 (18,000 psi)
300SB-C3	1.8 µm	300Å	80 °C	1.0 – 8.0	No	1200 (18,000 psi)
300 Diphenyl	1.8 µm	300Å	80 °C	1.0 – 8.0	Yes	1200 (18,000 psi)

Poroshell 300

This column's unique particle technology improves reversed-phase analysis for large intact proteins. While Poroshell 300 has a 300Å pore size, it behaves like a column that has a larger pore size, and is more accommodating for larger (50 kDa, 150 kDa for mAbs) biomolecules. Poroshell 300 frits are less susceptible to clogging, when compared to columns with small particles. The superficially porous particles on a Poroshell 300 enable higher flow rates and faster analysis times without less increase of backpressure, while still achieving excellent resolution. A Poroshell 300 offers unique advantages for large intact proteins, due to its superficially porous particle, which behaves more like a larger pore column, with significant speed advantages.

Agilent Poroshell 300 column specifications

Bonded phase	Particle size	Pore size	Temperature limits	pH range	Endcapped	Pressure limit
300SB, C18, C8, C3	5 µm	300Å	90 °C	1.0 – 8.0	No	400 bar (6,000 psi)
300Extend C18	5 µm	300Å	40 °C above pH 8, 50 °C below pH 8	2.0 – 11.0	Yes	400 (6,000 psi)

AdvanceBio Peptide Mapping

AdvanceBio Peptide Mapping columns have an ideal 120Å pore size for identifying a wide molecular weight range of peptide fragments. They are tested with a challenging peptide mix to ensure performance and reproducibility for peptide mapping. The Agilent unique superficially porous particle technology enables higher flow rates and better resolution of the full peptide sequence.

Agilent AdvanceBio Peptide Mapping columns

Bonded phase	Particle size	Pore size	Temperature limits	pH range	Endcapped	Pressure limit
EC-C18	2.7 µm superficially porous	120Å	60 °C	2.0 – 8.0	Double	600 bar (9,000 psi)

Ordering Information

Agilent AdvanceBio RP-mAb columns, 3.5 µm					
Description	Size (mm)	Part number	Description	Size (mm)	Part number
C4	2.1 × 50	799775-904	SB-C8	4.6 × 50	789975-906
C4	2.1 × 75	797775-904	SB-C8	4.6 × 100	785975-906
C4	2.1 × 100	795775-904	SB-C8	4.6 × 150	783975-906
C4	2.1 × 150	793775-904	Diphenyl	2.1 × 50	799775-944
C4	4.6 × 50	799975-904	Diphenyl	2.1 × 75	797775-944
C4	4.6 × 100	795975-904	Diphenyl	2.1 × 100	795775-944
C4	4.6 × 150	793975-904	Diphenyl	2.1 × 150	793775-944
SB-C8	2.1 × 50	789775-906	Diphenyl	4.6 × 50	799975-944
SB-C8	2.1 × 75	787775-906	Diphenyl	4.6 × 100	795975-944
SB-C8	2.1 × 100	785775-906	Diphenyl	4.6 × 150	793975-944
SB-C8	2.1 × 150	783775-906			

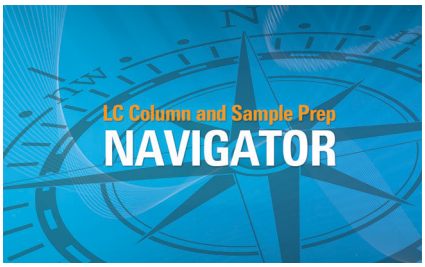
Agilent Poroshell 300, 5 µm					
Description	Size (mm)	300SB-C18	300SB-C8	300SB-C3	300Extend-C18
		USP L1	USP L7		USP L1
Capillary	0.5 × 75		5065-4468		
MicroBore	1.0 × 75	661750-902	661750-906	661750-909	671750-902
Narrow Bore	2.1 × 75	660750-902	660750-906	660750-909	670750-902
Guard cartridge, 4 /pk.	2.1 × 12.5	821075-920	821075-918	820975-924	
Guard hardware kit		820888-901	820888-901	820888-901	
MicroBore Guard, 3/pk.	1.0 × 17	5185-5968	5185-5968	5185-5968	5185-5968

Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300Å columns, 1.8 µm						
Description	Size (mm)	300SB-C18	300SB-C8	300SB-C3	300-Diphenyl	300-HILIC
		USP L1	USP L7		USP L11	
Narrow Bore	2.1 × 100	858750-902	858750-906	858750-909	858750-914	959758-901
Narrow Bore	2.1 × 50	857750-902	857750-906	857750-909	857750-914	959757-901

Agilent AdvanceBio Peptide Mapping columns, 2.7 µm	
Description	Part number
4.6 × 150 mm	653950-902
3.0 × 150 mm	653950-302
2.1 × 250 mm	651750-902
2.1 × 150 mm	653750-902
2.1 × 100 mm	655750-902
4.6 mm Fast Guard	850750-911
3.0 mm Fast Guard	853750-911
2.1 mm Fast Guard	851725-911

Agilent AdvanceBio Columns

Agilent is working to improve the accuracy and productivity of biochromatography with the AdvanceBio Family of biocolumns. AdvanceBio columns feature unique technological advances to enhance UHPLC performance and biochromatographic testing to ensure reproducible results. AdvanceBio columns are engineered to help biopharma scientists get more information from every characterization.



Navigate your way to a successful result

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With a wealth of biocolumns and small molecule columns available, Agilent has introduced the LC columns and Sample Prep NAVIGATOR to help you choose the right column for your application.

The NAVIGATOR presents four easy search options:

- By part number cross-reference for LC columns and sample prep products to find the best Agilent replacement
- By column recommendations based on method
- By compound drop down list
- By USP method. In addition, the tool offers column support to optimize chromatography, sample prep product recommendations, and quick access to technical support resources and other tools.

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