Reverse Phase

Agilent Technologies

How proteins separate on reverse-phase HPLC

- RP chromatography separates proteins through the interaction of the “hydrophobic foot” of the protein with a non-polar surface of the particle
- RP columns are nearly always based on silica particles
  - Mechanical stability, easy to make, surface can be modified, excellent peak shape & efficiency)
- Solvent
  - Organic modifier: Acetonitrile, isopropanol, methanol
  - Ion pair additive: Trifluoroacetic acid (TFA), Formic Acid, or Acetic.
- Gradient separation

Analysis & characterization of protein drugs by RP-HPLC

- RP HPLC plays a very important role in the analysis & characterization of protein therapeutic drugs
  - Verification of fidelity of primary sequence
    - Using peptide map with comparison to a reference standard
  - Determination of deamidation & oxidation
    - By means of peptide map comparison of native & stressed protein
    - Using an intact protein
  - Determination & confirmation of disulfide bonds
    - By peptide mapping comparing native & reduced protein
  - Characterization of glycosylation
    - Peptide mapping can determine glycopeptide (in conjunction with MS)

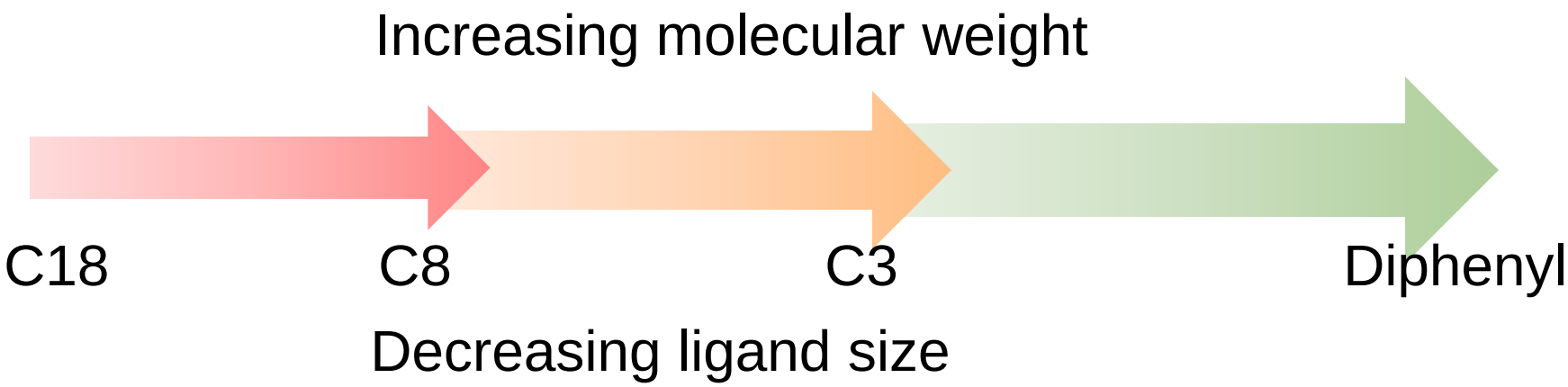
Choose The Initial Bonded Phase:
C18 C8 C3

Buy Them All and Try Them All…..

Not really feasible, right?

How do you narrow it down?

Molecular Weight



- Larger molecular weights separate better with smaller ligands
  - Upper Mw of C18 = 70kDa
- Not much difference between C8, C3 & diphenyl
  - Upper Mw of C3/C8/DP = 180kDa

Pore Size

Generally speaking, the “go to pore” size for most protein applications using RP is 300 A.

Smaller pore sizes are available for smaller proteins and peptides

Larger pore sizes are available for larger proteins and synthetic molecules

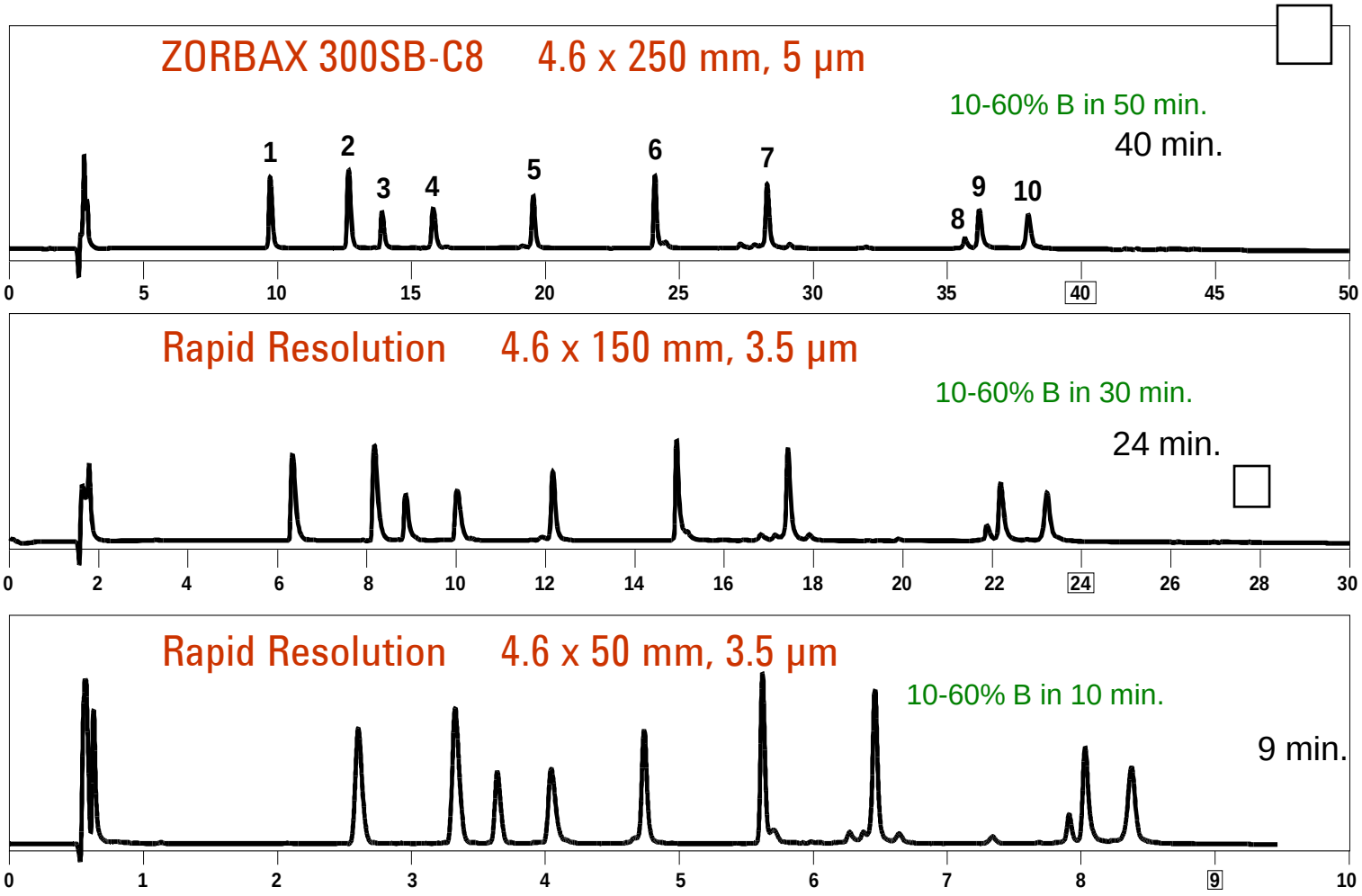
About Particle Size

Smaller particle sizes will increase resolution power

Coupled with smaller column lengths, decrease time and increase throughput

Agilent offers many options down to 1.8 um for RP

As Column Volume Decreases, Decrease Gradient Time (tg) to Keep Gradient Retention (k*) and Resolution Constant



Initial Separation Conditions

Mobile Phase: A: 95% H2O/5% ACN, 01.% TFA

B: 5% H2O/95% ACN, 0.1% TFA

Gradient: 0-60% B in 60 min

Temp: 35- 40 C

Flow Rate: 1ml/min

Optimize Organic Modifier

In order of increasing elutropic force and decreasing polarity:

Water

Methanol

Acetonitrile

N-Propanol

THF

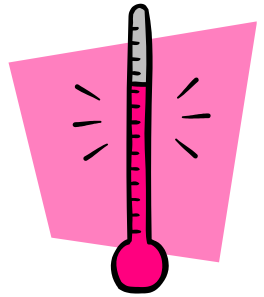
About Ion Pair Agents

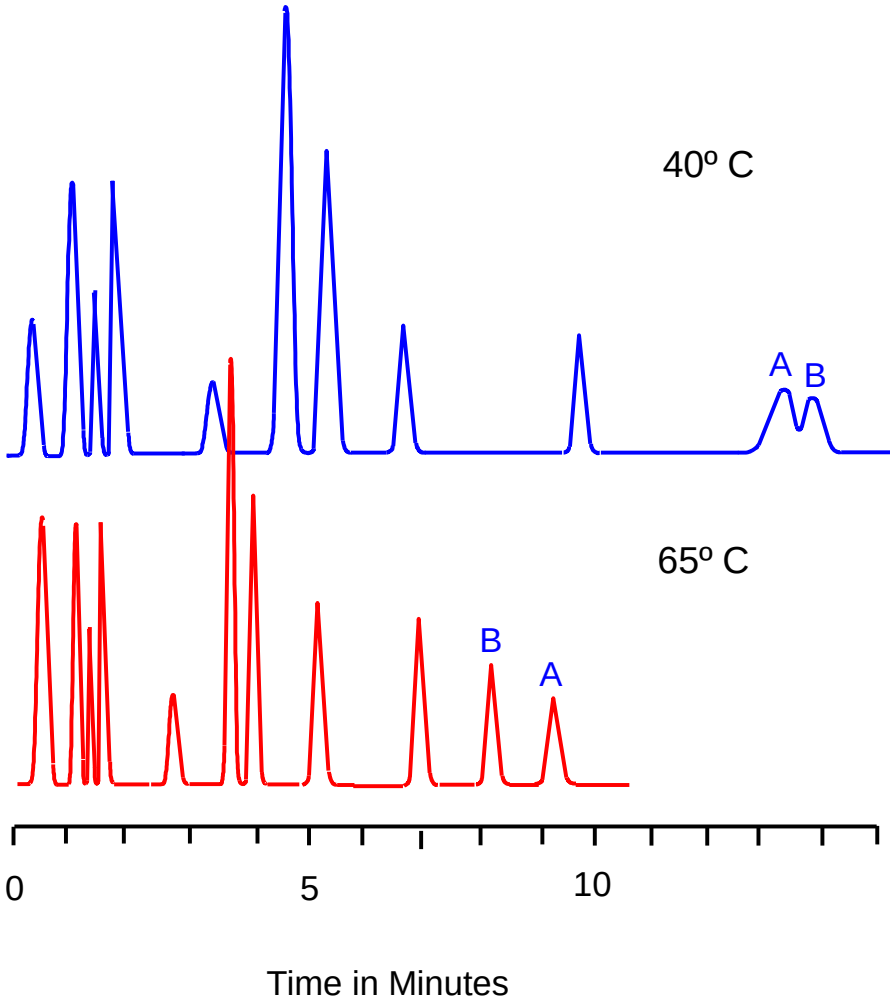
- Typically TFA is used
- If LC/MS is used, can substitute Formic or Acetic acid

Optimize Temperature

- Higher column temperature can dramatically improve resolution and recovery
- Check manufacturer specs for compatibility
- Agilent Zorbax Stablebond columns are rated to 80 C

Effect of Column Temperature on Retention k and Selectivity α





1. Column temperature can be used to fine tune a separation by affecting both the retention k and selectivity α.
2. About a 1% increase in T leads to a 1 to 2% decrease in k.
3. Increase in T leads to decrease in Pressure due to decrease in mobile phase viscosity.
4. Increase in T also leads to decrease in peak widths.
5. Also, use of a thermostat column compartment improves retention time precision.

Optimize Mobile Phase pH

- Start with acidic pH first
- If not ideal, move to mid or high pH, check manufacturers specs for limits
- Selectivity will change because acidic amino acids will become negatively charged and basics may lose their charge
- Ammonium hydroxide is an excellent mobile phase for high pH separations using LC/MS

High pH Can be Used for Separating Hydrophobic or Other Low-Solubility Peptides

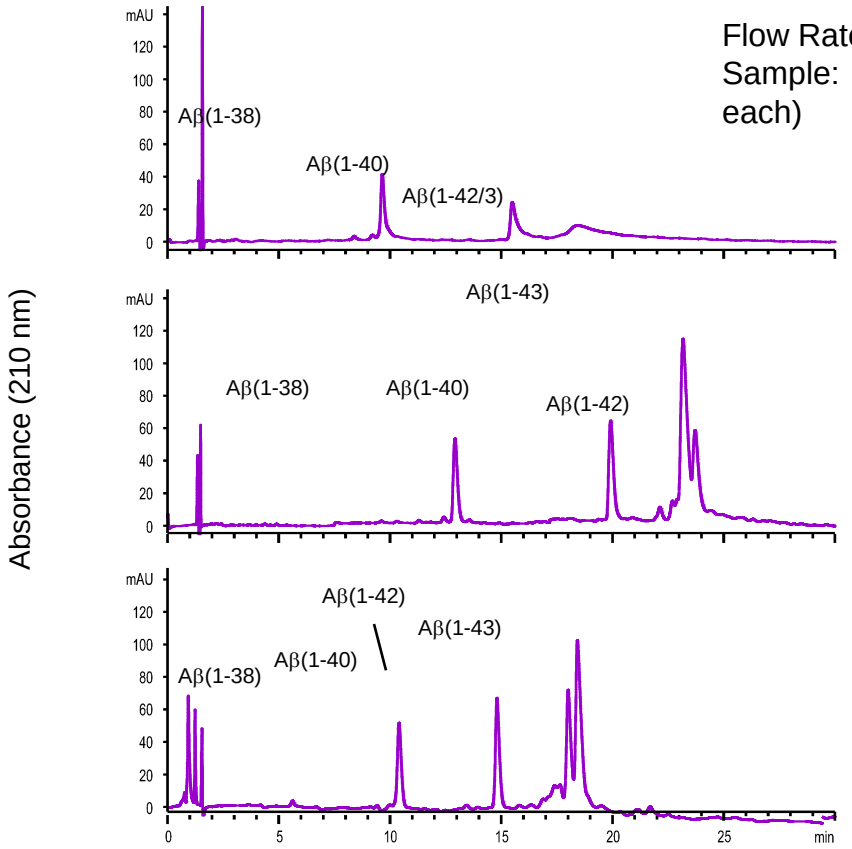
Comparison of Aß Peptide RP-HPLC Separations at Low and High pH

Column: ZORBAX 300Extend C18 2.1 x 150 mm, 5 µm
Flow Rate: 0.25 mL/min
Sample: 5 µL sample (100 pmol each)

TFA Conditions, 25°C
A- 0.1% TFA in water
B- 0.085% TFA in 80%AcN
33-45%B in 30 min.

TFA Conditions, 80°C
A- 0.1% TFA in water
B- 0.085% TFA in 80%AcN
29-41%B in 30 min.

NH_4OH Conditions, 25°C
A- 20 mM NH_4OH in water
B- 20 mM NH_4OH in 80%AcN
26-38%B in 30 min.

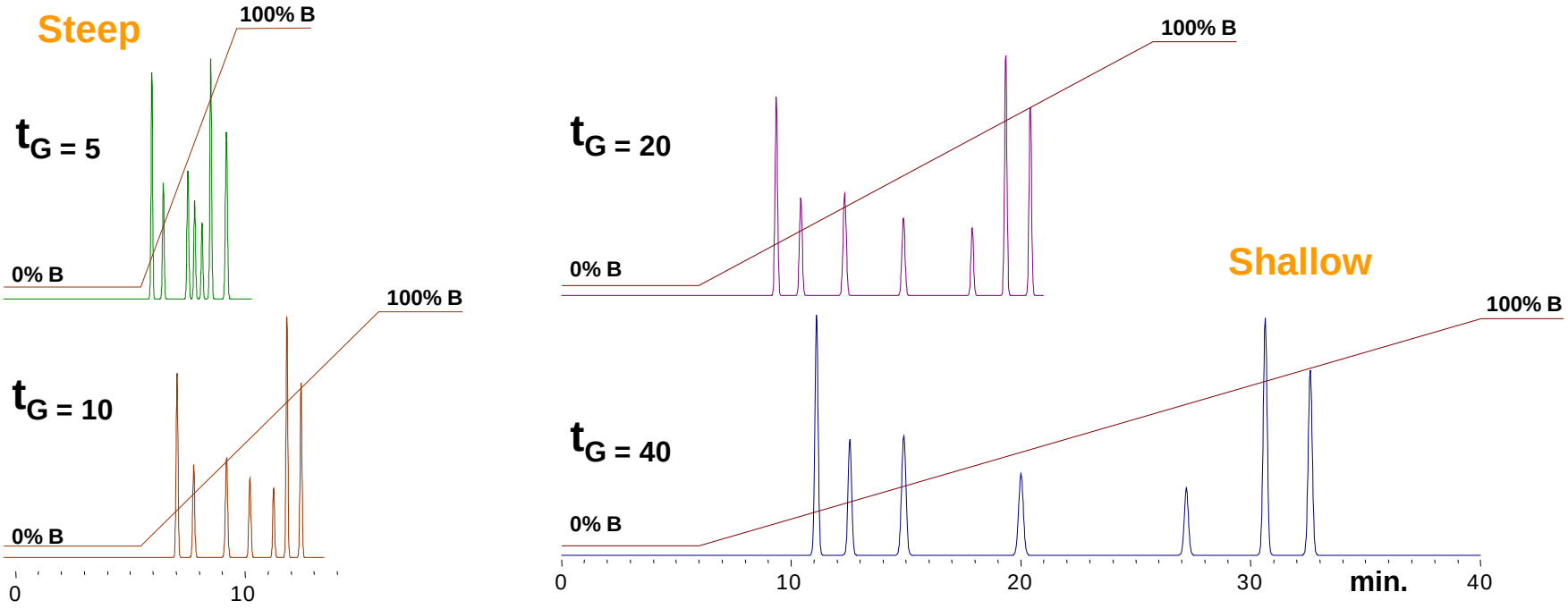


Adjust Gradient Slope to Optimize Resolution

Accomplished by changing:

- gradient time
- % change in organic modifier over time

Gradient Slope



Agilent RP Column Choices for Biomolecules

Zorbax Silica

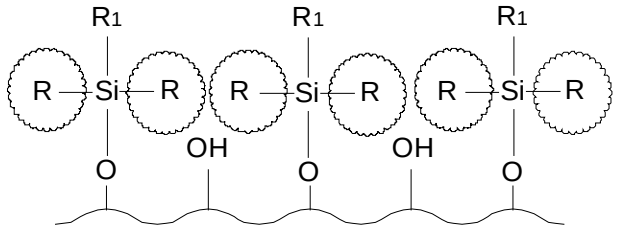
Zorbax Stablebond 300

300 A pore size

available in C-3, C-8, C-18, and CN

non-endcapped

particle sizes: 3.5, 5, and 7



NEW!

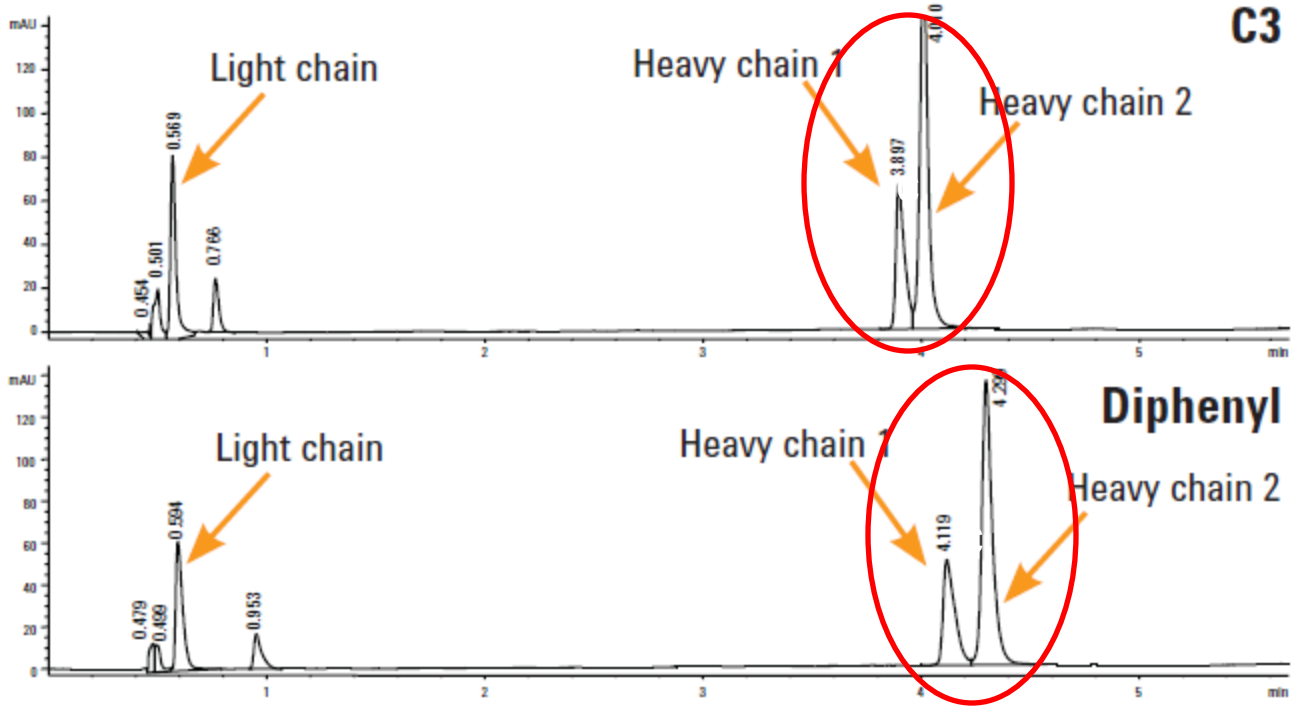
Zorbax 300 SB RRHD for Proteins and Peptides!

- Stablebond 300 silica
- C-18, C-8, C-3, HILIC and **unique diphenyl** bonded phase
- 1.8 um particle size
- 1200 Bar pressure limit for uHPLC

Diphenyl Selectivity

- Diphenyl provides alternate selectivity to C3
  - In addition to difference in hydrophobicity
- C18, C8 and C3 work on a purely hydrophobic interaction
- DP has π-π interactions also
- Additional affinity for aromatic amino acids and double bonds
  - *Orthogonality*

C3 vs Diphenyl



- Reduced IgG1
- 0.1% TFA gradient, 74°C
- Less hydrophobic DP column shows baseline resolution

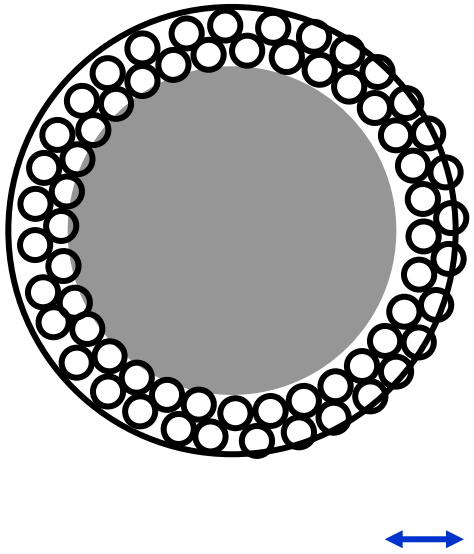
Poroshell 300

300 A pore size

Stablebond chemistry

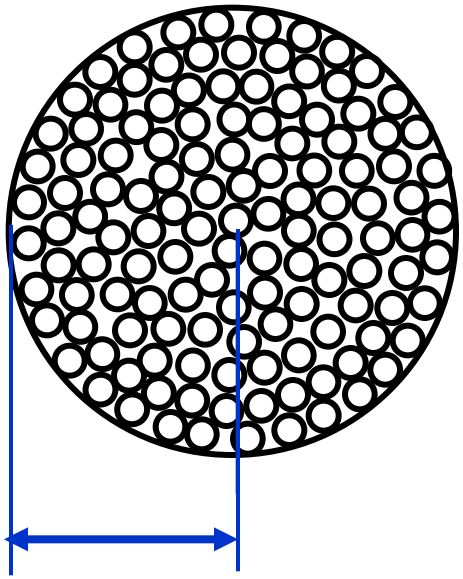
available in C-3, C-8,C-18, and C-18 Extend

5 um particle size



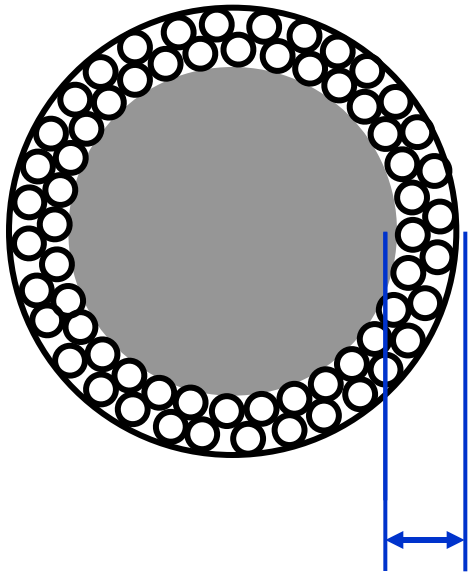
Comparison of Diffusion Distance
Totally porous silica vs. superficially porous silica

5 µm
Totally Porous Particle



2.5 µm

5 µm
Superficially Porous Particle



0.25 µm

Required diffusion distance
for a macromolecule
reduced 10 fold !

AdvanceBio Peptide Mapping Column

A superficially porous column with a 2.7um particle and C18 functionality which enables separation of hydrophilic through hydrophobic peptides to give superior resolution across the gradient range to efficiently resolve peptide fragments.

What are the compelling features?

- Peptide Mapping Resolving Power/Peak Capacity
- Enhance Resolution across a Wide Dynamic Range
- Fast Analysis Times
- Lower Pressure Drops than sub 2um Columns
- Quality Checked for Peptide Performance

Peptide mapping

Peptide mapping is the single most important technique in analytical characterization of protein drugs. It is used to…

- Development (characterization)
  - Confirm primary structure by comparison of a product to a reference protein (detect point mutations, mis-translations & confirm genetic stability)
  - Identify location of disulfide bonds
  - Characterize & analyze degradation processes such as deamidation & oxidation
  - Isolate digest fragments for sequencing or further identification
  - Identify sites of glycosylation
- Quality control
  - Drug substance identity test
  - Drug substance purity test

AdvanceBio Peptide Mapping Column

Primary Benefit – Reduce Peptide Mapping Time without Losing Resolution

What is It? - A superficially porous column with a 2.7um particle and C18 functionality which enables separation of hydrophilic through hydrophobic peptides to give superior resolution across the gradient range to efficiently resolve peptide fragments.

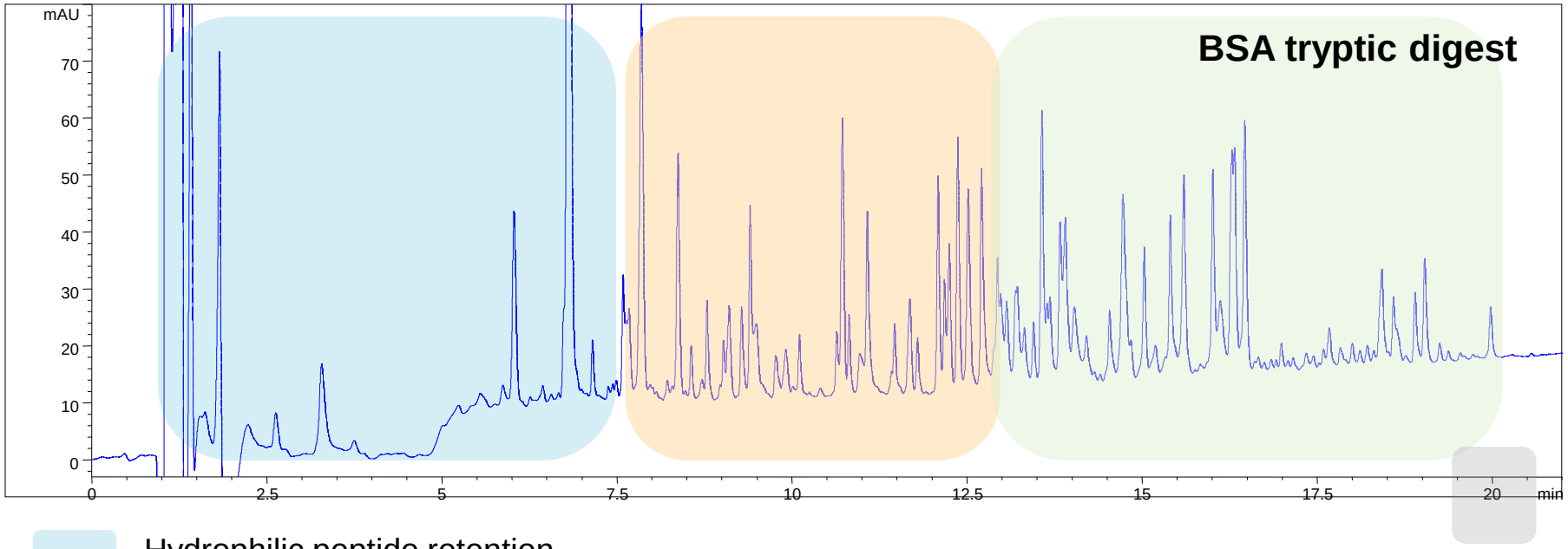
Major Features

- High Resolution of the Peptide Map
- Fast Analysis Times
- Lower Pressure Drops than sub 2um Columns
- Quality Checked for Peptide Performance

AdvanceBio Peptide Mapping Column Highlights

Peptide Mapping

2.1 x 150mm AdvanceBio Peptide Mapping Column
Mobile phase**:** A-water (0.1%TFA), B- ACN (0.08%TFA), 40 C, flow: 0.52mL/min



Hydrophilic peptide retention

Narrow Peaks w baseline resolution

Hydrophobic peptide retention

Reduced and fast analysis time

Critical and desired peptide mapping components to achieve fast, selective and highly efficient peptide separations across a wide dynamic range.

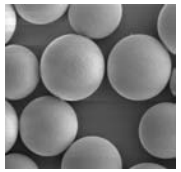
PLRP-S

Polystyrene divinylbenzene bead

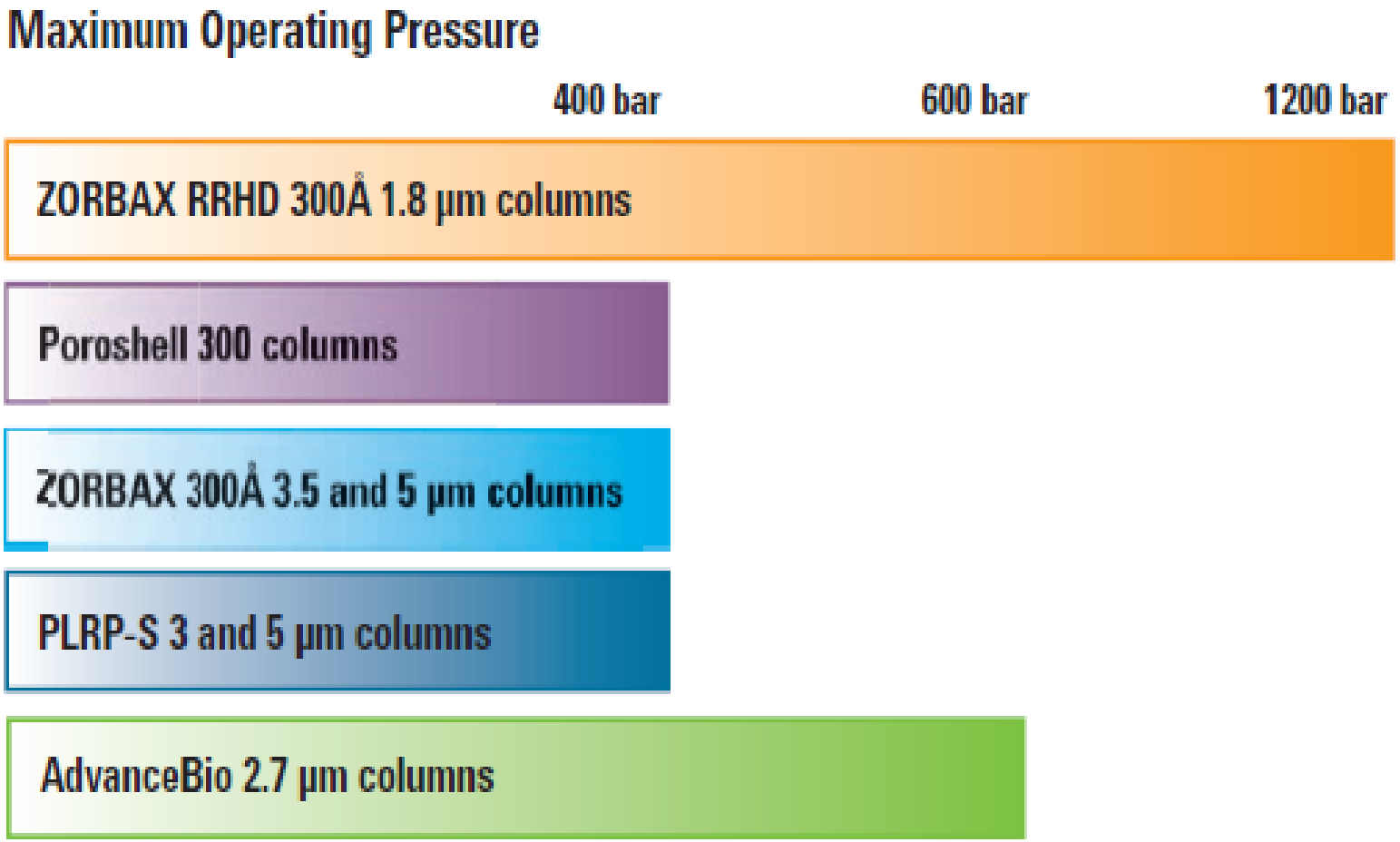
Available in 100, 300, 1000, and 4000A pore sizes

Particle sizes 3, 5, 8, 10, and higher

Various geometries from Nano, Capillary to preparative



Match to System Capabilities



Thank you!