So ... You Think You Know How to Run Solid Core-Superficially Porous and sub-2u LC Columns

Agilent Technologies, Inc.
WHAT IS HPLC?
WHAT IS UHPLC?

- High Pressure Liquid Chromatography?
- High Price Liquid Chromatography?
- I Have No Idea?
- High PERFORMANCE Liquid Chromatography!
- ULTRA High PERFORMANCE Liquid Chromatography!!
What Worked in the Past
May Not Be a Best Practice Today!

- **400 bar Instruments**
  - 250 mm length
  - 5 µm particles
  - 150 mm length
  - 3.5 µm particles

- **Non-Porous Particles**
  - Micro-Bore Capillary Columns
  - Narrow-Bore Capillary Columns
  - Well-Plate Samplers
  - 1000 bar Instruments
  - 1.8 µm particles

- **1300 bar Instruments**
  - Superficially Porous Particles – 300A, 5µm
  - 15 mm length
  - Overlapped Injections
  - Superficially Porous Particles – 120A, 2.7µm

- **1000 bar Instruments**
  - 10 µm particles
LC Separation Improvement
Why Develop Smaller Particles?
Better Performance!

Smaller particle sizes yield flatter curves, minima shift to higher flow rates
One Benefit of UHPLC
Faster Analysis

Original method
ZORBAX LC column
Extend-C18
4.6 x 150 mm, 5 μm
3 μL inj.

Mobile phase: (70:30) MeOH: 50 mM pyrrolidine buffer
Flow = 1.0 mL/min, Temp. : ambient

3x faster

ZORBAX RRHT column
Extend C18
4.6 x 50 mm, 1.8 μm
1 μL inj.
Higher efficiencies using SPP

Additional efficiency can be generated through the use of superficially porous particles (SPP) rather than a totally porous particle (TPP)

<table>
<thead>
<tr>
<th>SPP particle</th>
<th>For</th>
<th>Maximum pressure</th>
<th>Typical pressure</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9 µm</td>
<td>Highest UHPLC performance</td>
<td>1300 bar</td>
<td>Similar to sub-2 µm totally porous</td>
<td>~120% of sub-2 µm totally porous</td>
</tr>
<tr>
<td>2.7 µm</td>
<td>UHPLC performance at lower pressures</td>
<td>600 bar</td>
<td>50% of sub-2 µm totally porous</td>
<td>~90% of sub-2 µm totally porous</td>
</tr>
<tr>
<td>4 µm</td>
<td>Improved HPLC performance</td>
<td>600 bar</td>
<td>Typically &lt; 200 bar</td>
<td>~200% of 5 µm totally porous</td>
</tr>
</tbody>
</table>
### Techniquen / product

<table>
<thead>
<tr>
<th>Technique / product</th>
<th>Performance</th>
</tr>
</thead>
</table>
| 1.9 µm              | Highest UHPLC performance  
Pressure rating: 1300 bar  
Typical pressure: Similar to sub-2 µm totally porous  
Efficiency: ~120% of sub-2 µm totally porous |
| UHPLC 2.7 µm        | UHPLC performance at lower pressure  
Pressure rating: 600 bar  
Typical pressure: 50% of sub-2 µm totally porous  
Efficiency: ~90% of sub-2 µm totally porous |
| HPLC 4 µm           | Improved HPLC performance  
Pressure rating: 600 bar  
Typical pressure: Often < 200 bar  
Efficiency: ~200% of 5 µm totally porous |
# Higher Performance for Any LC

<table>
<thead>
<tr>
<th>Instruments</th>
<th>We recommend…</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UHPLC only</strong></td>
<td></td>
</tr>
</tbody>
</table>
| **Maximum pressure**: High (> 600 to 1000+ bar) | InfinityLab Poroshell 120 1.9 µm
| **Dispersion volume**: Very low                  | InfinityLab Poroshell 120 2.7 µm                  |
| **HPLC and UHPLC**   |                                                   |
| **Maximum pressure**: Low to high (400 to 1000+ bar) | InfinityLab Poroshell 120 2.7 µm
| **Dispersion volume**: Medium to very low         | InfinityLab Poroshell 120 4 µm                    |
| **HPLC only**        |                                                   |
| **Maximum pressure**: Low to mid (400 to 600 bar) | InfinityLab Poroshell 120 4 µm
| **Dispersion volume**: High to low                 | InfinityLab Poroshell 120 2.7 µm                  |
Improved Performance by Using Poroshell 120 4µm and Totally Porous 5um columns

- 50% improvement in resolution over the 5um totally porous column
- Backpressure still below 200 bar
Comparison of 3.5μm and Poroshell 2.7μm and 4.0μm

- Poroshell 120 4μm offers higher performance than totally porous 3.5μm columns with 600 bar stability.
- Poroshell 120 2.7μm offers the highest performance.
Higher Efficiencies: TPP vs. SPP
Seed column feedback

“…the efficiency of the new Poroshell column was superior to Zorbax once achieved a good resolution in the separation of isomers of estradiol, essential for the validation of a method for monitoring such analytes. Thus, we will start to use the new Poroshell column in the ongoing validations in anabolic”
- Residue Laboratory Veterinary Medication LANAGRO / MG
Fast LC
Aromatic acids

Figure 2. A 250 mm, 5 μm Agilent ZORBAX analysis of aromatic acids is improved by transferring to a high-performance 50 mm, 1.9 μm Agilent InfinityLab Poroshell column; minimum resolution is improved, while saving significant time, sample, solvent, and money.
Ultra-fast LC
Aromatic acids

Figure 4. Additional time, solvent, and money can be saved by operating the highly performing 50 mm, 1.9 µm Agilent InfinityLab Poroshell column near its pressure limit without compromising method performance.
High resolution LC
Tanshinones in Danshen (Salvia miltiorrhiza)

High efficiency column increases the number of peaks that can be resolved ($n_c$) and improves the accuracy of fingerprinting.

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Figure 3. Tanshinones fingerprint profiling on an Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 150 mm, 1.9 µm column. Peaks 10 (cryptotanshinone) and 13 (tanshinone IIA) were identified using reference standards.
The advantage of longer columns
Total phenolic acids in Danshen (Salvia miltiorrhiza)

Figure 2. Comparison of the Salvia Total Phenolic Acids fingerprint profiling on Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 1.9 μm and 2.1 × 100 mm columns.
The advantage of a scalable family of particles
Aromatic acids

Figure 3. Similar selectivity among Agilent InfinityLab Poroshell particle sizes allows analysts to choose their column configuration based on instrument pressure limits or method performance requirements without needing to do additional method development.
No Substitute for Chemistry Improved Resolution!

1. 3,4 Dimethoxyphenol
2. 2,6 Dimethoxyphenol
3. 3,5 Dimethoxyphenol
4. 2,6 Difluorophenol
5. 2,4 Difluorophenol
6. 2,3 Difluorophenol
7. 3,4 Difluorophenol
8. Degradation Product 2,6 Dimethoxyphenol
9. 3,5 Difluorophenol
10. 2,6 Difluorophenol
11. 2,6 Dichlorophenol
12. 4 Chloro 3 methyl phenol
13. 4 Chloro 2 methyl phenol
14. 3,4 Dichlorophenol
15. 3,5 Dichlorophenol

<table>
<thead>
<tr>
<th>Time</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>16</td>
<td>15</td>
</tr>
</tbody>
</table>

Flow Rate 2 ml/min
270 mn 4.6 x 150 mm
Smaller Particles in Smaller Volume Columns Reduces Dispersion Within the LC Column

a) Longitudinal Diffusion (dispersion)

b) Radial Diffusion (dispersion)
Effect of Water Contaminants on LC

**PARTICLES**
- Damage pump and injector
- Plug column and frits
- Increase back pressure

**BACTERIA**
- Plug column and frits
- Increase back pressure
- Release ions and organics *(see effect of ions and organics)*

**ORGANICS**
- Lead to ghost peaks, baseline drift, poor repeatability
- Interfere with analytes
- Reduce column lifetime
- Increase MS background
- Suppress signal

**IONS**
- Form adducts
- Suppress signal
- Complicate mass spectra

The Measure of Confidence

Agilent Technologies
Plugged Packing

Beware of buffered mobile phases
Buffers usually contain insoluble material – filter
Buffer solubility decreases with increasing % organic* - avoid 100%B with buffer salts

Effect of Flushing on MS background

Milli-Q® water on Monday (after weekend)

Fresh Milli-Q® water after discarding several liters
Split Peaks from Injection Solvent Effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 μm
Mobile Phase: 82% H₂O : 18% ACN
Injection Volume: 30 μL
Sample: 1. Caffeine 2. Salicylamide

A. Injection Solvent
100% Acetonitrile

B. Injection Solvent
Mobile Phase

Tip: Injecting in a solvent stronger than the mobile phase can cause peak shape problems such as peak splitting or broadening
Trick: Keep Organic Concentration in Sample Solvent ≤ Mobile Phase
Peak Tailing

Symmetry > 1.2

Some Peaks Tail:
- Secondary - Retention Effects.
- Residual Silanol Interactions.
- Small Peak Eluting on Tail of Larger Peak.

Chemistry Problem

All Peaks Tail:
- Extra-Column Effects.
- Build up of Contamination on Column Inlet.
- Heavy Metals.
- Bad Column.

System Problem
Effect of Strong Sample Solvent

- 2.1mm I.D.
- 35uL injection
- Sample Solvent Strength (50%) > Mobile Phase

50 mm

150 mm

Direction of Flow
What Length Column for New Methods?

- Flow rate vs. Gradient time vs. Peak capacity
- For small molecules (MW < ~1000)
- Different Column Lengths
- Broken lines are isobar (800 bar)
Shorter Columns with Fast Gradients Yield Higher Peak Capacity

Shorter Gradient (5 min)

Peak Capacity:
- 258 for 50 mm
- 240 for 100 mm
- 221 for 150 mm
Longer Columns with Long Gradient Times Yield Greater Peak Capacity

Long Gradient (40 min)

Peak Capacity:
- 422 for 50 mm
- 510 for 100 mm
- 525 for 150 mm
Separation of Licorice Root on RRHD Columns –
3X Column Length Produces Moderate increase in Retention with Major Improvement in Resolution – Why?

Mobile Phase: 10-100% B /30 min  A: 0.1% formic acid (fa)  B: acetonitrile with 0.1% fa
F=0.4 mL/min  Ambient temperature  280nm UV

1290 Infinity

RRHD SB-C18 2.1 x 50 mm, 1.8um  
Pmax=366 bar  
n_c = 424

RRHD SB-C18 2.1 x 100 mm , 1.8um  
Pmax=595 bar  
n_c = 485

RRHD SB-C18 2.1 x 150 mm, 1.8um  
Pmax=768 bar  
n_c = 589
Increased Peak Capacity and Change in Gradient Retention Yields More Information

Mobile Phase: 10-100% B /30 min  
A: 0.1% formic acid (fa)  
B: acetonitrile with 0.1% fa  
F=0.4 mL/min  
Ambient temperature  
280nm UV

1290 Infinity

50 mm
7 peaks  
Rs: 0

100 mm
8 peaks  
Rs: 1.37

150 mm
9 peaks  
Rs: 2.40

Increasing peak capacity led to increased Rs for this sample.
# Agilent UHPLC Column Selection Guidelines

<table>
<thead>
<tr>
<th></th>
<th>Fast Analysis</th>
<th>High Rs (N)</th>
<th>400 bar LC “Fitness”</th>
<th>1000+ bar UHPLC “Fitness”</th>
<th>ID’s 4.6, 3.0 &amp; 2.1 mm</th>
<th>Dirty Samples?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poroshell 120, 600 bar</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️ 2um frit</td>
</tr>
<tr>
<td>RRHT, 600 bar</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️ 50mm</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
</tr>
<tr>
<td>RRHD, 1200 bar</td>
<td>✔️</td>
<td>✔️</td>
<td>✗ (3.0 &amp; 2.1)</td>
<td>✔️</td>
<td>✗</td>
<td>✔️</td>
</tr>
</tbody>
</table>
Today’s Chromatography - Higher Performance Requires More Attention to Details

• Higher Performance Column Capabilities
  • Higher Theoretical Plates (N) Yield Narrower Peaks
  • Need Better Control of Flow Path and Lower Dispersion
  • Need Higher Data Acquisition Rate

• New Instrument Capabilities for More Efficiency
  • Better Flow Control
  • Lower Extra Column Volume
  • Lower Dispersion

• Need to Optimize Older Instruments
  • Decrease Extra Column Volume
  • Improve Connections
  • Increase Data Rate
Which Instruments May Need to be Adapted?

1290 Infinity II LC

READY TO GO

1100 through 1260

MAY NEED OPTIMIZATION
Optimize LC for Lower Volume and Sharp, Efficiency Peaks

Adjust Data Collection Rate

• Set to fastest setting that does not compromise S/N
• Most often the fastest setting will not be necessary

Reduce extra column volume

• With Agilent LC’s change “green” tubing (0.17mm) to “red” tubing (0.12 mm)
• Change needle seat to lower volume
• Choose a lower volume flow cell
• Choose lower volume for column heater
Lower Tubing Volume Lowers Dispersion
Easiest Path is to Decrease Diameter

Band Color Indicates Diameter

Optimal Diameter for Solid-Core and Sub-2u

<table>
<thead>
<tr>
<th>Color</th>
<th>i.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>0.075 mm (0.003 inches)</td>
</tr>
<tr>
<td>Red</td>
<td>0.12 mm (0.005 inches)</td>
</tr>
<tr>
<td>Green</td>
<td>0.17 mm (0.007 inches)</td>
</tr>
<tr>
<td>Blue</td>
<td>0.25 mm (0.01 inches)</td>
</tr>
<tr>
<td>Clear</td>
<td>0.50 mm (0.02 inches)</td>
</tr>
</tbody>
</table>
Where May Instrument Modifications Be Necessary?

- Pump
- Autosampler
- Column Comp.
- Detector

Extra-column volume
Data rate
Tubing Choices based on Column I.D.

**4.6 mm ID columns**
- High pressure Gradient pump
- Std or Well Plate sampler
- 3 μL heat exchanger
- Thermostatted Column compartment
- Rapid Resolution HT Column
- Diode Array detector
- Waste
- 0.17 x 400 mm capillary
- 0.17 x 150 mm capillary
- 0.17 x 105 mm capillary
- 0.17 x XXX mm capillary

**2.1 mm ID columns**
- High pressure Gradient pump
- Std or Well Plate sampler
- 3 μL heat exchanger
- Thermostatted Column compartment
- Rapid Resolution HT Column
- Diode Array detector
- Mass Spectrometer
- Cell Inlet Capillary
- Cell Outlet Capillary
- DAD equipped with a 5uL or 1.7 μL flow cell
- 0.12 x XXX mm Capillary
- 0.12 x 400 mm capillary
- 0.12 x 150 mm capillary
- 0.12 x 105 mm capillary
- 0.12 x XXX mm PEEK Capillary

* Pieces to upgrade, in kits
System dispersion not optimized

Resolution 0.961

Peak width 0.038 min

Peak width 0.037 min

System dispersion optimized

Resolution 1.902

Peak width 0.018 min

Peak width 0.019 min
Comparison of 1290 Gradient Performance

LC/UV systems extra column volume is reduced by 60% (from 9.7 to 3.9 µL)
LC/MS system extra column volume is reduced by 64% (from 8.7 to 3.1 µL)

**Default 1290 LC**

- Solvent Tray
- Diode Array Detector
- Column Compartment
- Autosampler
- Binary Pump

**Optimized 1290 LC**

- Solvent Tray
- Binary Pump
- Autosampler
- Column Compartment
- Diode Array Detector

Needle Seat Capillary: 0.12 x 100 mm = 1.1 µL
ALS → TCC Capillary: 0.12 x 340 mm = 3.8 µL
TCC → DAD Capillary: 0.12 x 220 mm = 2.5 µL
Flow Cell V(σ) 1.0 µL = 2.3 µL
TCC → MS Capillary: 0.12 x 340 mm = 3.8 µL
2.1 x 50 mm Column = 172.3 µL
Void Volume of Column = 103.9 µL

Needle Seat Capillary: 0.11 x 100 mm = 0.9 µL
ALS → TCC Capillary: 0.08 x 220 mm = 1.1 µL
TCC → DAD Capillary: 0.08 x 220 mm = 1.1 µL
Flow Cell V(σ) 0.6 µL = 0.8 µL
TCC → MS Capillary: 0.08 x 220 mm = 1.1 µL
2.1 x 50 mm Column = 172.3 µL
Void Volume of Column = 103.9 µL
Optimized LC Improves Gradient Resolution

Column: RRHD Eclipse Plus C18, 2.1 x 50mm, 1.8um  Gradient: 25-95% CH$_3$CN in 1.2 min, Flow Rate: 0.4 mL/min  LC: Agilent 1290 Infinity Sample: Alkylphenones

Default LC/UV System, 9.7 µL extra column volume, $P_{\text{max}} = 300$ bar

$Rs_{5,6} = 1.77$
$n_c = 44.0$

Optimized LC/UV System, 3.9 µL extra column volume, $P_{\text{max}} = 310$ bar

$Rs_{5,6} = 2.25$
$n_C = 55.7$

>20% improvement in gradient Rs and peak capacity with optimized LC
What Happens If the Connections Poorly Made?

Wrong ... too long

Ferrule cannot seat properly

If Dimension X is too long, leaks will occur

Wrong ... too short

Mixing Chamber

If Dimension X is too short, a dead-volume, or mixing chamber, will occur
Influence post-column capillary connections

One bad capillary connection!

130 mA

Fixed!

160 mA

mA

mA

min

min
Most commonly used fittings in UHPLC are non-adjustable 2-piece or 3-piece metallic fittings. Since different manufacturers of column hardware have different design in column end fittings, as shown in Figure 1, a new set of tubing and fittings needs to be installed for every brand of column to guarantee that the stem length, namely the length between the bottom of the ferrule and the end of tubing, fits the column end fitting.

The spring-loaded design constantly pushes the tubing against the receiving port, delivering a reproducible connection with no dead volume for consistent chromatographic performance.

Stem length is adjustable through the spring, which makes the fitting compatible with all types of LC columns.
Most Versatile, Secure High Pressure Fitting
Fast Analysis of Analgesics on Poroshell 120 – Demonstrating “Fitness” for 400 bar LCs


Conditions: Column: Poroshell 120 SB-C18, 4.6 x 50mm, 2.7um    Mobile Phase: 0.2% Formic Acid 80% water:20% ACN
Temperature: 25 °C, Detection: 275 nm, Sample Injection: 2ul
UHPLC Durability Allows Enhanced Performance at High Flow

- **Column:** 2.1x50 Eclipse plus C18
  - **Flow:** 0.3 ml/min
  - **Peak Capacity:** 35 (PW = 5 sigma)
  - **Pressure:** 204 bar

- **Column:** 2.1x50 Eclipse plus C18
  - **Flow:** 0.6 ml/min
  - **Peak Capacity:** 53
  - **Pressure:** 395 bar

- **Column:** 2.1x50 Eclipse plus C18
  - **Flow:** 1.2 ml/min
  - **Peak Capacity:** 72
  - **Pressure:** 735 bar

- **Column:** 2.1x50 Eclipse plus C18
  - **Flow:** 1.8 ml/min
  - **Peak Capacity:** 84
  - **Pressure:** 1050 bar

- **Column:** 2.1x100 Eclipse plus C18
  - **Flow:** 0.6 ml/min
  - **Peak Capacity:** 69
  - **Pressure:** 862 bar
1. First the green dots: the peak is defined by 24 values and shows a well-integrated profile.

2. Second the red dots/line: the peak is only described by six measurements, the peak area is smaller (limit of detection is smaller) and the calculated efficiency is only 89% of the blue-lined peak.

3. Third the yellow dots/line: the peak is severely distorted, retention time and peak area are unreliable and a calculated efficiency shows only 39% of the reality.

- Savitzky-Golay (1964): 12-15 points per peak
- Dyson (1998): 100 points per peak
Detectors
For narrow peaks, high data rates!!!
Maintaining Resolution at High Analysis Speed

80Hz versus 10Hz (20Hz) Data Rate
- Peak Width: \(-55\%\) \((-30\%\)
- Resolution: \(+90\%\) \((+30\%\)
- Peak Capacity: \(+120\%\) \((+40\%\)
- App. Column Eff.: \(+260\%\) \((+70\%\)

<table>
<thead>
<tr>
<th>Data Rate</th>
<th>Peak Width</th>
<th>Resolution</th>
<th>Peak Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 Hz</td>
<td>0.300</td>
<td>2.25</td>
<td>60</td>
</tr>
<tr>
<td>40 Hz</td>
<td>0.329</td>
<td>2.05</td>
<td>55</td>
</tr>
<tr>
<td>20 Hz</td>
<td>0.416</td>
<td>1.71</td>
<td>45</td>
</tr>
<tr>
<td>10 Hz</td>
<td>0.666</td>
<td>1.17</td>
<td>29</td>
</tr>
<tr>
<td>5 Hz</td>
<td>1.236</td>
<td>0.67</td>
<td>16</td>
</tr>
</tbody>
</table>

Sample: Phenones Test Mix
Column: Zorbax SB-C18, 4.6x30, 1.8um
Gradient: 50-100%ACN in 0.3min
Flow Rate: 5ml/min
Comparison of Peak Efficiency on Poroshell 120 EC-C18 with Different Data Collection Rates

Column: Poroshell 120 EC-C18, 4.6 x 100mm  
Instrument: 1200 SL 2ul flow cell  
Flow Rate: 2.00 ml/min  
Sample: 2ul injection of 3B  
Mobile Phase: 60:40 MeCN:Water
Summary

• The New Generation of UHPLC Instruments (1290 Infinity) are providing an Optimum Platform for Sub-2u and Poroshell Type Columns

• ZORBAX RRHD – Rapid Resolution High Definition – LC columns for the 1290 Infinity LC are designed for the demands of this UHPLC and any other UHPLC.

• RRHD columns are made from the same ZORBAX 1.8um particles and bonded phases in the ZORBAX RRHT columns for the RRLC.

• Both RRHT and RRHD Columns operate over the entire pressure range available in all UHPLC instruments.

• Superficially Porous (Poroshell) columns deliver Many Benefits of sub-2u particles at dramatically lower pressure
Basic Instructions for Using HPLC/ UHPLC Columns Effectively

1. Install and run the column only in the flow direction marked on the column

2. **Use only high quality, chromatography grade solvents**

3. Filter all aqueous buffers and all samples through a mobile phase appropriate 0.2μm filter before use.

4. Replace bottles of mobile phase buffer every 24-48 hours – do not add mobile phase to the bottle.

5. Use an in-line filter to prolong column life.
   1. 5067-1551 for 2.1 or 3.0mm ID RRHT columns or 5067-1553 for 4.6 mm ID RRHT columns
   2. In-line filter ships with 1290 pump for use with RRHD columns and can be ordered separately – part number 5067-4638.
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