Maximizing Resolution and Selectivity: Superficially Porous Column Chromatography Options

Anne Mack
Application Scientist

Stephen Luke
LC Columns Product Manager
Agenda

• Superficially porous particle (SPP) specifications and benefits
• Method development challenges and objectives
• Method development with selectivity
  - Bonded phase
  - Mobile phase pH
• How to ensure the best performance from your SPP column
• When you need more help choosing a column…
SUPERFICICALLY POROUS PARTICLES
SPECIFICATIONS AND BENEFITS
## Current Status of Superficially Porous Particles

<table>
<thead>
<tr>
<th>Status in 2000</th>
<th>Status in 2010</th>
<th>Status in 2015</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Vendors</td>
<td># of Vendors</td>
<td># of Phase chemistries</td>
</tr>
<tr>
<td>Small molecules</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Large molecules</td>
<td>1 (Agilent)</td>
<td>1 (Agilent)</td>
</tr>
</tbody>
</table>

Bell, LC-GC, 2015 June
Majors, LC-GC, 2014 Nov
Superficially Porous Column Technology

Poroshell 120 2.7 µm

- \( d_p = 2.7 \, \text{µm} \)
- Particles
  - 1.7 µm solid core
  - 0.5 µm diffusion path
  - 2.7 µm total diameter
- Efficiency (N) ≈ 90% of sub-2 µm
- \( N \approx 2 \times 3.5 \, \text{µm} \) (totally porous)
- Pressure ≈ 40-50% of sub-2 µm
- 2 µm frit to reduce clogging
- \( P_{\text{limit}} = 600 \, \text{bar} \) for HPLC or UHPLC
Comparing Efficiency and Pressure with Different Types of Columns

<table>
<thead>
<tr>
<th>Particle Size/Type</th>
<th>Pressure</th>
<th>Efficiency</th>
<th>LC Compatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 µm Totally Porous</td>
<td>123 bar</td>
<td>7,800</td>
<td>All 400 bar instruments</td>
</tr>
<tr>
<td>2.7 µm Poroshell 120</td>
<td>180 bar</td>
<td>12,000</td>
<td>All LCs/UHPLCs (up to 600 bar)</td>
</tr>
<tr>
<td>1.8 µm Totally Porous</td>
<td>285 bar</td>
<td>12,500</td>
<td>All LCs/UHPLCs (up to 1200 bar)</td>
</tr>
</tbody>
</table>

Columns: 4.6 x 50mm, Mobile Phase: 60% ACN:40% Water  Flow Rate: 2 mL/min
USP Method for Naproxen Tablets

Method Requirement  N > 4000, Rs better than 11.5

Common Conditions:
Mobile Phase:  50:49:1 MeCN:H2O Acetic Acid
Flow Rate: 1.2 mL/min
Peak 1. Naproxen  2. Butyrophenone

- **4.6 x 150 mm (L1)**
  - Eclipse Plus C18, 5um (TPP)
  - PN 959993-902
  - 20 ul injection
  - Rs = 14.9
  - N= 12,554

- **4.6 x 100 mm (L1)**
  - Poroshell 120 EC-C18, 2.7 um
  - PN 695975-902
  - 13.67 ul injection
  - Rs = 17.0
  - P = 238 bar
  - N= 14,885

- **4.6 x 50 mm (L1)**
  - Poroshell 120 EC-C18, 2.7 um
  - PN 699975-902
  - 6.7 ul injection
  - Rs = 12.6
  - P= 133 bar
  - N= 12,051

2X Faster

4.5X Faster
Larger Diameter SPP

Poroshell 120 2.7 µm
SA = 120 m²/g
Pore size = 120-140Å

Poroshell 120 4 µm
SA = 120 m²/g
Pore size = 120-140Å

- Offers nearly 2X the performance of traditional 5 µm columns with the easy drop-in replacement for current methods
2.7 and 4 µm Poroshell 120 Have Similar Selectivity for Easy Method Transfer and Scaling

Selectivity Comparisons with 4.6 x 50 mm Columns
5-95% CH3CN in 2 min with 0.1% formic acid, 2 mL/min

\[ y = 1.0136x - 0.0033 \]
\[ R^2 = 0.9993 \]

>80 compounds

RT on 2.7 um Poroshell 120 EC-C18 (min)

Linear (RT on 2.7 um Poroshell 120 EC-C18 (min))
Comparison between Poroshell 120 2.7 μm and 4 μm for 4-quinolones in Milk

• PW$_{1/2}$ with 4 μm column increased by 30% compared to 2.7 μm
• Pressure on 4 μm decreased by 45% compare to 2.7 μm column. It is more suitable to use on a < 400 bar LC, while 2.7 μm column is suitable for 600 bar LC.
Long Lifetime with Poroshell 120 2.7 µm Column
>1800 Injections at 550 bar - No Performance Change

Lifetime Test
with Unfiltered, Undiluted Freshly Brewed Green Tea

Peak Width at 1/2 Height, s

Number of Injections

0.01 0.02 0.03 0.04 0.05

Caffeine
Epicatechin
Epicatechin Gallate

P_{max} = 550 bar

A: 0.2% HCOOH in H₂O, B: 0.2% HCOOH in CH₃CN
0.833 mL/min

Time | 0.00 | 1.25 | 2.50
%B   | 10   | 15   | 27

40 °C

Agilent Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm

Sig=210,4nm, Ref=Off

2-µL, 3-mm micro flow cell (PN G1315-60024)

Sample: 2 µL of freshly brewed green tea (brewed from a commercial tea bag in 6 oz of initially boiling water for six minutes)
Other Considerations when Selecting a Column

- Robustness and batch-to-batch reproducibility

Beverage Additives

2010

B10015

2012

B10018

B11041

B11256

B12041
METHOD DEVELOPMENT
CHALLENGES AND OBJECTIVES
Challenges In Method Development

• Worldwide method transfers
  - Instruments and configurations differ from lab to lab
  - More contract labs

• Many chromatographic mode choices
  - RP, SEC, IEX, HILIC, Chiral...
  - RP most common

• Too many columns to choose from
  - Endcapped C18 is a good starting point
Defining the Objective

• How complex is the sample?
• Is high efficiency important?
• Is speed important?
• What are the instrument limitations?
• What is the skillset of the operator?
Examples of Common Separation Goals and Method Performance Criteria

**Good System Suitability Parameters**
- Resolution: ≥2
- Peak shape: USP $T_f$ close to 1 (<2)
- Injection Repeatability: areas, $T_f$, etc. (RSD 0.1 - 0.25%)
- Absolute retention factors: 1 < $k$ < 10
- Relative Retention: $\alpha$ or $k_2/k_1$
- Signal-to-Noise Ratio: >10

**Method Performance Criteria**
- Accuracy
- Precision
  - Ruggedness
  - Robustness
- Selectivity/Specificity
- Linearity
- Range
- Quantitation Limit (LOQ, 10x S/N)
- Detection Limit (LOD, 3x S/N)

**AVOID THESE for System Suitability Criteria:**

*Column efficiency (theoretical plates) & Absolute retention time*

*These inhibit the ability to speed up your method in the future!*
Where to Begin?

Selectivity Impacts Resolution Most

- Change bonded phase
- Change mobile phase

Typical Method Development Parameters

Plates: 5000 10000 15000 20000 25000
Alpha: 1.10 1.35 1.60 1.85 2.1
k': 2.0 4.5 7.0 9.5 12.0

Resolution

$R_s = \frac{N^{\frac{1}{2}}}{4} \cdot \frac{(\alpha-1)}{\alpha} \cdot \frac{k'}{k'+1}$
METHOD DEVELOPMENT WITH SELECTIVITY – BONDED PHASE
Why is Changing the Bonded Phase Effective?

• Different interactions for polar and non-polar compounds.
• Exploit other interactions with bonded phase (e.g., pi-pi)
• Changing the bonded phase can improve selectivity/resolution, reduce analysis time
• Having numerous different bonded phases available on the same particle makes development easier
  - Fast SPP methods make development faster
Poroshell 120 Column Chemistries
Multiple bonded phases for flexibility in method development

Poroshell 120 EC-C18 and C8
• Robust endcapped C18 for best peak shape at pH 2-9

Poroshell 120 StableBond C18 and C8
• Robust chemistries for pH<2

Poroshell HPH-C18 and HPH-C8
• Long lifetime at high pH

Poroshell 120 Phenyl-Hexyl
• Excellent choice for pi-pi interactions
• Selectivity similar to phenyl, diphenyl, or other phenyl-hexyl columns

Poroshell 120 SB-Aq
• Proprietary bonding phase is an excellent choice for polar analytes

Poroshell 120 Bonus-RP
• Embedded polar group provides unique selectivity for polar compounds

Poroshell 120 EC-CN
• Flexible endcapped CN chemistry with Normal and Reversed Phase character

Poroshell 120 HILIC
• Bare silica HILIC for use in hydrophilic interaction chromatography of polar molecules

Poroshell 120 PFP
• Perfluorophenyl chemistry
HSM a Way to Look at Column Orthogonality

<table>
<thead>
<tr>
<th>Poroshell 120</th>
<th>H</th>
<th>S*</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>k'</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC-C18</td>
<td>1.020</td>
<td>0.008</td>
<td>-0.130</td>
<td>-0.004</td>
<td>0.161</td>
<td>6.920</td>
<td>0</td>
</tr>
<tr>
<td>EC-C8</td>
<td>0.877</td>
<td>0.011</td>
<td>-0.232</td>
<td>0.023</td>
<td>0.127</td>
<td>4.840</td>
<td>6</td>
</tr>
<tr>
<td>EC-CN</td>
<td>0.421</td>
<td>-0.057</td>
<td>-0.476</td>
<td>0.002</td>
<td>0.045</td>
<td>0.950</td>
<td>17</td>
</tr>
<tr>
<td>Phenyl-Hexyl</td>
<td>0.752</td>
<td>-0.083</td>
<td>-0.394</td>
<td>0.018</td>
<td>0.136</td>
<td>3.590</td>
<td>13</td>
</tr>
<tr>
<td>Bonus RP</td>
<td>0.686</td>
<td>-0.030</td>
<td>-0.573</td>
<td>0.180</td>
<td>-0.670</td>
<td>3.980</td>
<td>75</td>
</tr>
<tr>
<td>SB-C18</td>
<td>0.956</td>
<td>-0.041</td>
<td>0.168</td>
<td>0.025</td>
<td>0.210</td>
<td>5.440</td>
<td>12</td>
</tr>
<tr>
<td>SB-C8</td>
<td>0.726</td>
<td>-0.087</td>
<td>0.068</td>
<td>0.044</td>
<td>0.087</td>
<td>3.560</td>
<td>15</td>
</tr>
<tr>
<td>SB-Aq</td>
<td>0.581</td>
<td>-0.120</td>
<td>-0.133</td>
<td>0.051</td>
<td>-0.014</td>
<td>2.150</td>
<td>22</td>
</tr>
<tr>
<td>PFP</td>
<td>0.630</td>
<td>-0.520</td>
<td>-0.520</td>
<td>0.430</td>
<td>-0.110</td>
<td>2.300</td>
<td>85</td>
</tr>
</tbody>
</table>

Data provide by Dwight Stoll

$F_s$ factor describes the similarity of two columns. A small $F_s$ indicates that two columns are very similar, while a large factor indicates that two columns are very different. Calculated according to the following equation:

$$F_s = \left[12.5(H_2 - H_1)\right]^2 + \left[100(S_2^* - S_1^*)\right]^2 + \left[30(A_2 - A_1)\right]^2 + \left[143(B_2 - B_1)\right]^2 + \left[83(C_2 - C_1)\right]^2$$

Further details at:

http://www.hplccolumns.org
$F_s = \left[ \frac{12.5(H_2 - H_1)}{2} + 100(S^*_2 - S^*_1) \right]^2 + \left[ 30(A_2 - A_1) \right]^2 + \left[ 143(B_2 - B_1) \right]^2 + \left[ 83(C_2 - C_1) \right]^2 \right]^\frac{1}{2}$
Separation of 8 Steroids with Methanol Gradient

Best Resolution of all analytes with Poroshell 120 Phenyl-Hexyl


40-80 % Methanol/14 min, DAD 260, 80 nm 0.4 ml/min, 2.1 x 100 mm 40 C 0.1% Formic Acid in Water and Methanol, Agilent 1260 Method Development Solution
Poroshell 120 Phenyl-Hexyl vs Poroshell 120 EC-C18

Phenyl-Hexyl alternative selectivity to C18
- recommended for aromatics, especially with methanol
- compatible with highly aqueous mobile for polar compounds.

**Acetonitrile**

\[ Y = 0.8285x + 0.2115 \]

\[ R^2 = 0.9875 \]

**Methanol**

\[ Y = 1.0239x + 0.3264 \]

\[ R^2 = 0.8606 \]
Beta Blockers with Methanol Gradient

Best Resolution of all analytes with Poroshell Bonus-RP

Poroshell 120 EC-C18

Poroshell 120 SB-C18

Poroshell 120 Phenyl Hexyl

Poroshell 120 Bonus RP


10-70 % Methanol/12 min, DAD 260 nm 0.35 ml/min, 2.1 x 100 mm 40 C 10 mM pH 3.8 Ammonium Formate Buffer and Methanol
• Embedded polar group gives unique selectivity for polar compounds compared to C18.

**Graph: Acetonitrile**

- Equation: \( Y = 0.9447x + 0.1211 \)
- \( R^2 = 0.8642 \)

**Graph: Methanol**

- Equation: \( Y = 0.9217x + 0.1131 \)
- \( R^2 = 0.9015 \)
NSAID Separation with a Methanol Gradient

Best Resolution of all analytes with Poroshell 120 PFP

Poroshell 120 PFP

Poroshell 120 EC-C18

Poroshell 120 Bonus RP

Poroshell 120 Phenyl Hexyl


Agilent Technologies
October 16, 2015
METHOD DEVELOPMENT WITH SELECTIVITY – MOBILE PHASE pH
When Does pH Affect Selectivity and Resolution? Compound Type Comparison

- Ionizable compounds – acids and bases can change retention and selectivity most with changes in pH
Change in Retention with pH for Ionizable Compounds is Key to Method Development

• Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)

• Silanols on silica ionize at mid-pH, increasing retention of basic analytes (i.e. possible ion-exchange interactions)

• Choose mobile phase pH to optimize retention and selectivity during method development

• Ensure that your column is compatible with and stable in the mobile phase pH you select
Poroshell HPH-C18 vs Poroshell 120 EC-C18

\[ y = 0.9587x - 0.0476 \]

\[ R^2 = 0.9992 \]

**Method Details**:
- **MP-A** 10mM Ammonium Formate/water adj. to pH3 or 7 using Formic Acid
- **MP-B** ACN or MeOH
- Flow – 0.42 mL/min
- Column Temp. Ambient
- 1 uL injection
- Detection 254 nm

**Gradient**:  
<table>
<thead>
<tr>
<th>time, min</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>stop run</td>
</tr>
</tbody>
</table>
Use of Varied pH can Help Build Separations that are Very Different (poorly correlated)

Retention Time pH 10 Acetonitrile vs. Retention Time pH 3 Acetonitrile

$R^2 = 0.40$

Column: Poroshell HPH-C18 2.7 µm
Change in Retention with pH for Ionizable Compounds is Compound Dependent

More retention for non-charged analytes (i.e. acids at low pH and bases at high pH)

- Acetylsalicylic acid (pKa 3.5)
- Pyridine (pKa 5.2)
- Codeine (pKa 8)
- Procionamide (pKa 9.2)
- Amphetamine (pKa 9.9)
- Caffeine (pKa 14)

Column: Poroshell HPH-C18 2.7 µm

Mobile Phase: 45% Methanol, 55% 20 mM Phosphate Buffer
Selectivity Can be Controlled by Changing pH

Poroshell HPH-C18 4.6 x 50 mm, 2.7 µm

1. Procainamide
2. Caffeine
3. Acetyl Salicylic Acid
4. Hexanophenone Deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone

<table>
<thead>
<tr>
<th>Time</th>
<th>% Buffer</th>
<th>% MeCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

2 ml/min 254 mn

- pH 3: 10 mM HCO₂NH₄
- pH 4.8: 10 mM NH₄HCO₃
- pH 10: 10 mM NH₄HCO₃
HOW TO ENSURE THE BEST PERFORMANCE FROM YOUR SPP COLUMN
Benefits of Installing a Guard Column

Accelerated Lifetime Test
Similac sample (milk substitute diluted 300:1) containing 2 sulfa drugs
Peak width change indicating column failure

By installing a guard column when using dirtier samples, one can extend the life of their column, and utilize more inexpensive guard columns rather than analytical column replacements.
Importance of the Spring Loaded Feature

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.

Spring pushes capillary constantly towards receiving port
Data Collection Rate

Effect of Data Collection Rate using a 2.1 x100 mm Poroshell 120 EC-C18 Column

Data Collection Rate (Hz)

Efficiency, plates

1 ul QC Mix, Uracil, Phenol (k=0.5), 4-Chloronitrobenzene(k=2), Napthalene(k=3.8)
55% MeCN 45 % Water 0.55 ml/min micro flow cell
WHEN YOU NEED MORE HELP CHOOSING A COLUMN...
On-Line Tool “The Navigator”
A Column and Sample Prep Selection Tool

http://navigator.chem.agilent.com
Literature on Poroshell 120 Columns

- There are continuous updates and additions to Poroshell 120 Columns Literature.
- Brochures, app notes, flyers and other documents are updated and added often!

5990-5951EN

5990-5951EN

5991-4893EN
AGILENT SMALL MOLECULE LC COLUMNS OVERVIEW:
A FAMILY OF PHASE CHOICES TO PERFECT EVERY SEPARATION

**High pH**
- Best for high pH mobile phases—good for method development
- High performance over a wide pH range
- Best lifetime and peak shape at high pH
- Alternatives selective to alkyl, phenyl, cyanogen
- High conductivity for LC/MS applications and recommended for EKC 1064

**Polar Compounds**
- Excellent selectivity to alkyl, phenyl, cyanogen
- High efficiency and long life at high pH
- Good performance on guard columns

**Neutral and Aqueous**
- Excellent performance on guard columns
- High efficiency and long life at neutral pH

**ZORBAX Eclipse Plus**
- 1.8 um, stable to 1000 bar
- 5 um, stable to 450 bar

**ZORBAX StableBond**
- 1.8 um, stable to 1200 bar
- 5 um, stable to 450 bar

**ZORBAX Eclipse XDB**
- 1.8 um, stable to 1200 bar
- 5 um, stable to 450 bar

**ZORBAX Extend C18**
- 1.8 um, stable to 1000 bar
- 5 um, stable to 450 bar

**ZORBAX Extend C8**
- 1.8 um, stable to 1000 bar
- 5 um, stable to 450 bar

**ZORBAX Extend C4**
- 1.8 um, stable to 1000 bar
- 5 um, stable to 450 bar

**ZORBAX Extend C18**
- 1.8 um, stable to 1000 bar
- 5 um, stable to 450 bar

Learn more at
www.agilent.com/chem/discoverporoshell

Choose the right LC column for your analysis with the LC Columns and Sample Prep Navigator at
www.agilent.com/chem/lsq
Agilent is Here to Help

Visit [www.agilent.com/chem/cstechsupport](http://www.agilent.com/chem/cstechsupport)

Agilent Technologies

Technical Support Resources

Agilent is committed to helping you succeed with your application. Send us an email with your questions and a technical support representative will contact you as soon as possible, usually within the day, provided you are emailing during business hours in your region. However, please allow up to 48 hours for a reply.

Helpful Tip: Provide as much information as possible regarding the instrument you are using, your sample type, the column you are using now and what you are trying to achieve.

TECHNICAL SUPPORT CONTACTS

US, Canada – please call 800-227-9776 or email LC-column-support@agilent.com for LC columns support and sampsupport@agilent.com for sample prep support

Mexico, Brazil, Argentina and other countries in S. America – please contact your preferred distributor (see [www.agilent.com/harvicontacts](http://www.agilent.com/harvicontacts) for more information)

Europe, Middle East and Africa, please contact your distributor (see [www.agilent.com/chem/contactus](http://www.agilent.com/chem/contactus) or email esmece@agilent.com

India – please send an email to columns_helpdesk@agilent.com

Singapore, Malaysia, Australia, Korea, the Philippines: contact your local distributor (see [http://www.chem.agilent.com/en-US/Contact-Us/Pages/Singapore.aspx](http://www.chem.agilent.com/en-US/Contact-Us/Pages/Singapore.aspx) or send an email to cce-sing@agilent.com

Japan – please call 0120-477-111 or email email_japan@agilent.com

China – please call 800-820-3278-4
Conclusions

• Resolution is a common goal during method development
• Selectivity is a main driver of resolution
• Superficially porous particle columns (e.g., Poroshell 120) offer…
  - 12 chemistries, including high pH stable options
  - Faster method development
  - Higher sample throughput
  - Maintain method ruggedness
  - Compatibility with any LC system
Thank You

Questions?