U/HPLC and LC-MS columns
*Which to use?*

Dr Lee May May
Senior Technical Manager, Asia Pacific
Advanced Analytics

**Which column should I use?**

- Chromolith®
- Purospher®
- Superspher®
- ZIC®-HILIC
- LiChrospher®
- UHPLC core-shell

- 5 µm
- 3 µm
- 2 µm (Sub 2 µm)
- monolith
Which column should I use?

This is a typical question for analysts involved in method development for novel compounds or new samples.

In real-life practice...
- Look into column stock to see what's available!
- Start with the usual e.g. RP-18??

But to work through the suitable column, one should:
- Understand the analytes and the sample matrix
- Define the sample preparation steps
- Try out on TLC first if possible
- Consider an appropriate HPLC detector
- Work through mobile phase selection
- Select the HPLC column

Which HPLC column should I use?

Sample content defines the column chemistry

- Structure of sample components?
- Number of compounds present?
- Sample matrix?
- pKa values of sample components?
- Concentration range?
- Molecular weight range?
- Solubility?
- Other pertinent data?

Column Chemistry
(bonded phase, bonding type, endcapping, carbon load)

We have Liquid Chromatography (LC) columns for:
(1) Analytical HPLC/UHPLC
(2) LC-MS
(3) Preparative LC
(4) Other hyphenated techniques like LC-NMR-MS
Selection of column chemistry

- Very hydrophobic Compounds
- Non Polar Compounds
- Closely Related Compounds
- More Polar Compounds
- Polar and Very Polar Compounds

- Too Much Retention on RP-18
- Retention too short or inadequate Separation on RP-18
- Poor Peak Shape (Basic Compounds)
- Not Enough RP
- HILIC Mode

- Basic Compounds
- H-Bonding, Phenolic, Acidic or Hydroxylated Compounds
- Electron-Acceptors
- Nitroaromatics, Heteroaromatics
- Strong Dipole

- C30
- C18
- C8
- C4
- F5 (PFP)
- RP-Amide
- ES-Cyano
- Phenyl-Hexyl
- Biphenyl
- RP-Amide
- F5* (PFP)
- ZIC-HILIC
- HILIC (Si)
- NH2
- OH5
- ES-Cyano
- Diol

* F5: no HILIC mechanism, but suitable for separation of polar compounds
** Suitable for 100% aqueous mobile phases and therefore as well for the separation of more polar compounds

Column Technologies and Specifications
U/HPLC Column Technologies: particle-structure types

Modern packing materials for LC columns have progressed from irregular pieces to spherical beads with a narrow size distribution. There are currently 3 popular structure types:

- Porous particle
- Fused core/core shell particles
- Monolithic structure

The technology behind such packing engineering offers the analyst different performance in chromatographic separation.

Schematics from https://www.bioanalysis-zone.com/2016/02/08/chapter-2-advances-in-lc-separations-for-proteomics/

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U/HPLC Column Types
Attributes of different packing materials

**Porous**
- Traditional format widely accepted
- Available in different particle and pore sizes
- Widest range of chemistries
- Requires full sample preparation
- Comparatively higher back pressure

**Fused core**
- Commercially available since 2006
- Narrow particle size distribution for symmetrical peaks
- Shorter path length for narrow peak width
- Higher plate, N
- Lower sample loading
- Higher back pressure

**Monolithic**
- Silica monoliths available since 2001
- Very low backpressure
- Higher flowrates possible for shorter runs
- Minimal sample prep
- Higher loading capacity
- Rugged long lifetime
- Limited chemistries compared to Porous
- Restricted column length dimensions
U/HPLC columns – size of particle (analytical scale)

Typical particle size for analytical columns would include 1.8, 2, 3, 5 and 10 mm. The trend is towards smaller particle size since *efficiency and resolution* would be enhanced (below left). Particle size is however inversely proportional to pressure (below right) and flowrate so this can be an issue with conventional HPLC.

http://www.chromacademy.com/hplc_UHPLC_method_transfer_essential_guide.html
U/HPLC columns – pore size of particle

The pore size has an effect on the retention and resolution of the analytes. A larger pore size will enable larger solute molecules to be retained longer because of greater interaction with the surface area of the particles. Choose a pore size of 150Å or less for sample MW ≤2000. Choose a pore size of 300Å or greater for sample MW > 2000.

Same particle size but different pore size!

Small Pore Diameter
Larger Surface Area

Large Pore Diameter
Smaller Surface Area


U/HPLC column material types

Common material types include Silica, Polymeric resin, Zirconia and Alumina particles. These have specific properties that in turn impact performance and operating conditions.

Silica
- Typical pH operating range is 2-8. Below 2, the functionalized groups detach (bleed). Above 8, the silica would dissolve
- Typical operating temperature limit is 60ºC
- Compatible with most organic solvents
- Able to withstand high pressure

Polymeric resin
- Typically wider pH range of 1-13
- Very narrow particle size distribution
- Susceptible to swelling with some organic solvents

Zirconia
- Full pH range of 1-14
- Good thermal stability – operating temperature limit 200ºC
- Does not shrink or swell – compatible with solvents
- High mechanical strength

Alumina
- Typical pH 3-13
- Not as thermally or mechanically stable as Zirconia
Supelco LC Column Trademarks

- LiChrosorb®
  Particulate Silica Irregular
- Chromolith®
  Monolithic Silica
- Superspher®
  Particulate Silica spherical
- Chromolith® CapRod®
  Monolithic Silica capillary
- Sequant®
  Particulate Silica/polymeric spherical
- LiChrospher®
  Particulate Silica spherical
- Purospher® STAR
  Particulate Silica spherical
- Aluspher®
  Particulate Alumina spherical
- ChiraDex®
  Particulate Silica spherical

U/HPLC column chemistries
HPLC column by USP specifications
Purospher® STAR Particulate U/HPLC and LC-MS columns

Purospher® STAR RP-18

Chromatographic Characterization

Tanaka Test
The Tanaka test compares the quality and performance of HPLC columns. The more symmetrical the hexagon appears and the larger its area, the more balanced the stationary phase is in the sum of its chromatographic properties.

A: $k'$ (Pentyl benzene) 9.59
B: $\alpha$ (Pentyl-/ Butyl benzene) 1.51
C: $\alpha$ (Triphenylene/ o-Terphenyl) 1.63
D: $\alpha$ (Caffeine/ Phenol) 0.44
E: $\alpha$ (Benzylamine/ Phenol; pH7.6) 0.23
F: $\alpha$ (Benzylamine/ Phenol; pH2.7) 0.02
Purospher® STAR RP-18e

Best in class - for almost all RP- applications

Purospher® STAR RP-18 endcapped, 5µm

Competitor-L

Competitor-N

Competitor-Z

Competitor-S

Competitor-P

Competitor-D

Chromolith® Monolithic columns
**Chromolith® - a single fused piece**

*Merck is the first in world to make monolithic silica columns!*
*We have the patented monolithic silica technology.*
*We are a one-stop solution provider for monolithic silica column users.*

**Chemically pure silica particles**

**SEM of a cross section of a silica monolith**

**Mesopores: 13, 15 nm**

**Macropores: 1.15-1.5-2 mm**

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**Chromolith® excellent batch-to-batch reproducibility**

Production of Chromolith® columns is tightly controlled and fulfils the requirements for QA/QC laboratories.

**Column**

Chromolith RP-18e HR 100-4,6mm

**Mobile phase:**
A = LiChrosolv Acetonitrile 0,1%TFA
B = Water + 0,1%TFA Milli-Q

**Gradient:**
2 min 0%A, 10 min 30%A

**Flow rate:**
1ml/min

**Detection:**
UV 210 nm

**Temperature:**
25°C

**Injection Vol:**
2 μl

**Compounds:**
1. Norepinephrine
2. Octopamine
3. Epinephrine tartrat
4. Dopamine
5. DOPA
6. Norephedrine
7. Ephedrine
8. N-Methylphenphedrine

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**Supelco.**
Chromolith® columns vs. conventional particulate columns +

Making changes to compendial methods...

Blockbuster drugs - off patent

Aripiprazole

Esomeprazole

Olmesartan

Raloxifene
Esomeprazole

**From USP**

<table>
<thead>
<tr>
<th>From USP</th>
<th>Official October 1, 2016</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample solution:</strong></td>
<td>After 30 min in pH 6.8 phosphate buffer, pass a portion of the solution under test through a suitable filter. Transfer 5 mL of the filtrate to a suitable glassware containing 1.0 mL of 0.25 M sodium hydroxide. Mix well. Protect from light. Buffer, Mobile phase, System suitability, and Chromatographic system: Proceed as directed in the Assay. Analysis: Samples: Standard solution and Sample solution Calculate the percentage of esomeprazole (C_{31}H_{21}N_{5}O_{5}S) dissolved:</td>
</tr>
<tr>
<td><strong>Chromatographic system:</strong></td>
<td>(See Chromatography (621), System Suitability)</td>
</tr>
<tr>
<td><strong>Mode:</strong></td>
<td>LC</td>
</tr>
<tr>
<td><strong>Detector:</strong></td>
<td>UV 302 nm</td>
</tr>
<tr>
<td><strong>Column:</strong></td>
<td>4.6 mm x 15 cm, 5-µm packing L7</td>
</tr>
<tr>
<td><strong>Column temperature:</strong></td>
<td>30°C</td>
</tr>
<tr>
<td><strong>Flow rate:</strong></td>
<td>1.5 mL/min</td>
</tr>
<tr>
<td><strong>Injection volume:</strong></td>
<td>20 µL</td>
</tr>
<tr>
<td><strong>System suitability:</strong></td>
<td>Tailing factor: NMT 2.0 Relative standard deviation: NMT 2.0%</td>
</tr>
</tbody>
</table>

**Esomeprazole Magnesium (API Assay)**

**HPLC**

**Performance criteria to be met:**
- RS: NLT 3 between omeprazole RS A and esomeprazole
- RRT: 0.8 for and 1.0 for omeprazole RS A and esomeprazole

**Purospher® STAR RP-8 endcapped (5 µm) 150 x 4.6 mm**

- Flow rate: 0.8–1 mL/min
- Injection size: 50 µL
- Injection volume: 50 µL
- Flow rate: 1.0 mL/min
- Column Pressure: 87 Bar (1261 psi)

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Retention Time (min)</th>
<th>Plates</th>
<th>Resolution</th>
<th>RRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Omeprazole Related Substance A</td>
<td>7.46</td>
<td>11978</td>
<td>-</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>Omeprazole</td>
<td>8.69</td>
<td>12271</td>
<td>4.2</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Up-to-date Information about Pharmacopoeia

What changes are allowed in a USP monograph?
Can we change the column material?
Are we allowed to use a different column dimension?
Is it allowed to scale down to smaller ID columns to save solvent?
Is there a possibility to speed up the separation?

The answer is YES to all these questions...but how?

USP permitted changes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Allowed Range</th>
<th>OK for</th>
<th>Isocratic</th>
<th>Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>±0.2</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Buffer concentration</td>
<td>±10%</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>The lesser of ±30% relative or ±10% absolute for minor components</td>
<td></td>
<td>Yes</td>
<td>NR*</td>
</tr>
<tr>
<td>UV wavelength</td>
<td>0, but error ±3 nm OK</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Column length and particle size</td>
<td>$L/d_p = -25%$ to $+50%$ or $N = -25%$ to $+50%$</td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Column diameter</td>
<td>OK if linear velocity constant</td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Flow rate</td>
<td>OK if linear velocity constant, plus additional ±50%; exceptions</td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Injection volume</td>
<td>OK if performance OK</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Column temperature</td>
<td>±10 °C</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*NR = not recommended, but not explicitly prohibited.
See text for abbreviations and discussion. Based on data of reference 1.

http://www.chromatographyonline.com/method-adjustment-usp-way-1aa
Esomeprazole Magnesium (API Assay)

**UHPLC**

Purospher® STAR RP-8 endcapped (2 μm) 100 x 2.1 mm

- Injection volume: 5 μL (10X decrease)
- Flow rate: 0.3 mL/min
- Column Pressure: 300 Bar (4320 psi)

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Retention Time (min)</th>
<th>Plates</th>
<th>Resolution</th>
<th>RRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Omeprazole Related Substance A</td>
<td>3.5</td>
<td>11746</td>
<td>-</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>Omeprazole</td>
<td>4.1</td>
<td>12928</td>
<td>4.2</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Alternative HPLC Procedure – monolithic column**

Chromolith® HighResolution RP-18 endcapped
100 x 4.6 mm

- Injection volume: 20 μL (2.5x decrease)
- Flow rate: 1.0 mL/min
- Column Pressure: 50 Bar (725 psi)

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Retention Time (min)</th>
<th>Plates</th>
<th>Resolution</th>
<th>RRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Omeprazole Related Substance A</td>
<td>4.6</td>
<td>9998</td>
<td>-</td>
<td>0.82</td>
</tr>
<tr>
<td>2</td>
<td>Omeprazole</td>
<td>5.6</td>
<td>10925</td>
<td>4.7</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Polar Analytes: What are the options?

Sequant® ZIC®-HILIC Columns

- Better retention of polar and hydrophilic compounds
- Orthogonal selectivity to reversed-phase
- Robust bonded stationary phase
- Extended pH stability for ZIC® pHILIC
**What about polar compounds?**

Improved selectivity with hydrophilic and polar compounds

HILIC can replace methods for:

1. RP columns and ion-pairing reagents in the mobile phase
2. RP columns and highly or completely aqueous mobile phases
3. Ion-exchange methods

HILIC: Column: ZIC-HILIC 3.5 µm, 100x4.6 mm; Mobile Phase A: 5 mM ammonium acetate 0.1% (v/v) formic acid (pH 4); Mobile Phase B: MeCN mit 0.1% (v/v) formic acid; Gradient: 5% A to 95% A in 15 min; Inject. Vol.: 20 µL; Flow rate: 0.6 mL/min

**Sequant® ZIC®-HILIC**

Complementary Zwitterionic Columns

Same bonding type, weak ionic interactions by 1:1 charge balance

Positive charge more accessible

Phosphorylcholine (PC)

Negative charge more accessible

Sulfobetaine (SB)
Sequant® ZIC®-HILIC
Separation of Positive Species
On complementary zwitterionic HILIC columns

Toluene (void marker), uracil and cytosin (neutrals), plus BTMA (arrow) with eluent 80:20 acetonitrile / 25 mM aqueous NH₄Ac buffer, pH 6.8. Columns 100x4.6 mm, flow 0.5 mL/min, temp. 23 °C, detection UV at 254 nm.

Separation of Aminoglycosides
Selectivity benefits from repulsion to zwitter

Gradient LC-MS with ESI+ SIM Detection
Streptomycin (STR), gentamicin (GEN), paromomycin (PAR), tobramycin (TOB), and neomycin (NEO) on ZIC-cHILIC 100x2.1 mm at flow 0.4 mL/min. Gradient start 50%B; 0-7 min 50-95%B; 7-8 min 95%B, 8-16 min 50%B. Eluents: A) ACN +1% FA; B) 100mM NH₄Ac +3% FA.
Cisplatin

**Chromatographic Conditions**
- **Column:** SeQuant™ ZIC®-HILIC (5 μm, 200 A) 150x2.1 mm
- **Injection:** 1 μl
- **Detection:** UV @ 305nm
- **Flow Rate:** 0.1 mL/min
- **Mobile Phase:** Buffer: Ammonium formate 25mM pH 6.5. Mx 1,4-dioxane and Buffer 80:20 (v/v)
- **Temperature:** Ambient
- **Diluent:** Mobile phase without buffer
- **Sample:** Cisplatin

**Chromatographic Data**

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Retention Time (min)</th>
<th>Resolution</th>
<th>Asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cisplatin</td>
<td>25.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Monohydrated cisplatin</td>
<td>47.8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Supelco HPLC, UHPLC and LC-MS columns**

<table>
<thead>
<tr>
<th>Functionality</th>
<th>USP</th>
<th>High purity silica particles</th>
<th>Conventional silica particles</th>
<th>Monolithic columns</th>
<th>Non-silica based materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP-18</td>
<td>L1</td>
<td>Purospher RP-18 HC</td>
<td>LiChrospher RP-18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP-18 polar endcapped</td>
<td>L1</td>
<td>Purospher RP-18e</td>
<td>LiChrospher RP-18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP-8 endcapped</td>
<td>L7</td>
<td>Purospher STAR RP-8e</td>
<td>LiChrospher RP-8e</td>
<td>Chromolith RP-8e</td>
<td>CapRod RP-8e</td>
</tr>
<tr>
<td>RP-8</td>
<td>L7</td>
<td>Purospher STAR RP-8</td>
<td>LiChrospher RP-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl</td>
<td>L11</td>
<td>Purospher STAR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>L10</td>
<td>LiChrospher CN</td>
<td>Superspher CN</td>
<td>Chromolith CN</td>
<td></td>
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<tr>
<td>Diol</td>
<td>L20</td>
<td>LiChrospher Diol</td>
<td>Superspher Diol</td>
<td>Chromolith Diol</td>
<td></td>
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<tr>
<td>Si</td>
<td>L3</td>
<td>Purospher STAR Si</td>
<td>LiChrospher Si</td>
<td>Chromolith Si</td>
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<tr>
<td>NH2</td>
<td>L8</td>
<td>Purospher STAR NH2</td>
<td>LiChrospher NH2</td>
<td>Chromolith NH2</td>
<td></td>
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<tr>
<td>ZIC</td>
<td>ZIC HILIC</td>
<td>ZIC cHILIC</td>
<td>ChiraDex</td>
<td>ZIC-pHILIC</td>
<td></td>
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<tr>
<td>Chiral modifications</td>
<td>L45</td>
<td>Chiraphase NT</td>
<td>ChiraSep DNBPG</td>
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<td></td>
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</tbody>
</table>
Pharma QC Requirements

- Reproducibility
  - Repeatability & Robustness
- Peak Symmetry
  - Accuracy & Precision
- Efficiency / Matrix tolerance
  - Time & Cost savings
- pH Stability
  - Flexibility

Validation Kits with 3 different batches available

Best column choice for Pharma QC

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Reproducibility</th>
<th>Peak Symmetry</th>
<th>Selectivity (modifications available)</th>
<th>Efficiency</th>
<th>pH-Stability (&gt; pH 7)</th>
<th>Matrix tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiChrospher®</td>
<td>*****</td>
<td>**</td>
<td>***</td>
<td>**</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>Supelcosil®</td>
<td>****</td>
<td>**</td>
<td>*****</td>
<td>**</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>Purospher® STAR</td>
<td>*****</td>
<td>*****</td>
<td>****</td>
<td>*****</td>
<td>*****</td>
<td>***</td>
</tr>
<tr>
<td>Ascentis® / Discovery®</td>
<td>****</td>
<td>***</td>
<td>****</td>
<td>****</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>Ascentis® Express / BIOshell®</td>
<td>*****</td>
<td>*****</td>
<td>****</td>
<td>*****</td>
<td>**</td>
<td>****</td>
</tr>
<tr>
<td>Chromolith®</td>
<td>***</td>
<td>**</td>
<td>****</td>
<td>***</td>
<td>***</td>
<td>*****</td>
</tr>
<tr>
<td>Chromolith® HR</td>
<td>****</td>
<td>****</td>
<td>**</td>
<td>****</td>
<td>***</td>
<td>*****</td>
</tr>
</tbody>
</table>
Centre of Analytical Sciences, CAS
Application Lab for Training and Discovery

Centre of Analytical Sciences (CAS)
Location – International Business Park, Singapore

International Business Park is a high-tech business park managed by JTC Corporation in Jurong East, Singapore. Established in 1992, the International Business Park is Singapore’s first business park.
Centre of Analytical Sciences (CAS)

Thermo Dionex Bio compatible UHPLC with UV DAD and fluorescence detectors.

Fume hood and solvent cabinet for sample preparation work.

Metrohm volumetric Karl Fischer titrator, Sartorius analytical balances, Milli-Q water, Mettler pH/ion meter and filtration tools.

CAMAG Linomat 5 HPTLC auto-spotter.

Centre of Analytical Sciences (CAS)

Vortex mixer, magnetic stirrer hotplate and Leica light microscope.

WFA instruments: Pharo 300, Nova 60, Multy, Move 100, RQFlex, Turbiquant.

Class 2 BSC and autoclave in Bio room for handling pathogens and other biologicals.

CO₂ incubators and shakers; also inverted light microscope (not shown).
Centre of Analytical Sciences (CAS)
Small group training – Instrumental HPTLC workshop

Thank you!
Supelco columns for small and large molecules
A wide range of column technologies to meet customer needs

Purospher STAR (120 Å)
- Optimal Reproducibility
- Outstanding Peak Symmetry
- Stable from pH 1.5 - 10.5 (RP-18)

Ascentis (100 Å) / Discovery
- Wide range of phase chemistries

Titan (80 Å)
- UHPLC columns

SeQuant (100, 200 Å)
- Orthogonal selectivity to RP for separation of polar compounds
- Zwitterionic functionality designed for HILIC mode
- Highly suitable for LC/MS use

Discovery BIO (300 Å)
- Three choices for reverse phase mode

Ascentis Express (90, 160 Å)
- BiOshell (160, 400, 1000 Å)
- Fast separations with very high efficiency
- Wide range of phases
- Available in micro format

Validation Kits with 3 different batches available