# Scale Up to the Drug Discovery Fast Lane with Superficially Porous Particle Preparative LC Columns

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Small Molecule Preparative LC Columns
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# Learning objectives

**In today's webinar, you will learn:**

- Characteristics of columns that drive throughput
- What is a superficially porous particle
- How columns packed with superficially porous particles can be beneficial to the purification workflow

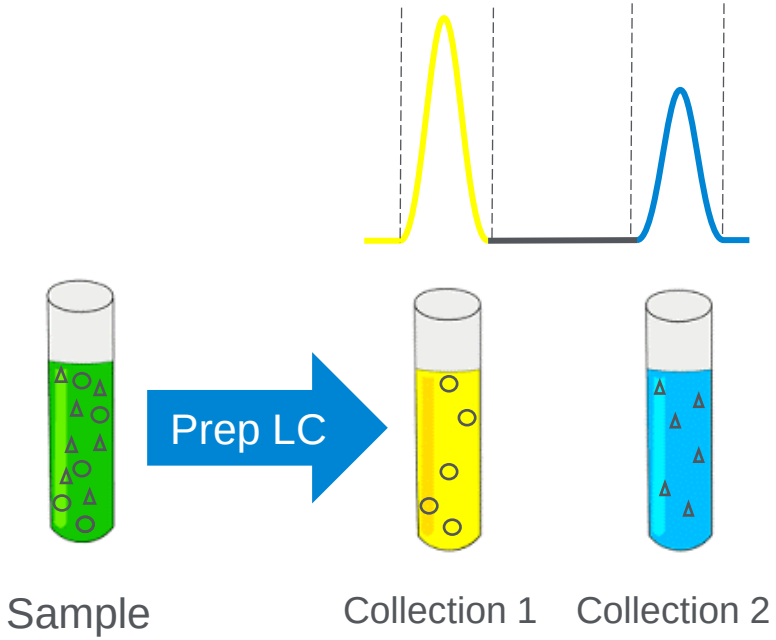
**This webinar is intended for:**

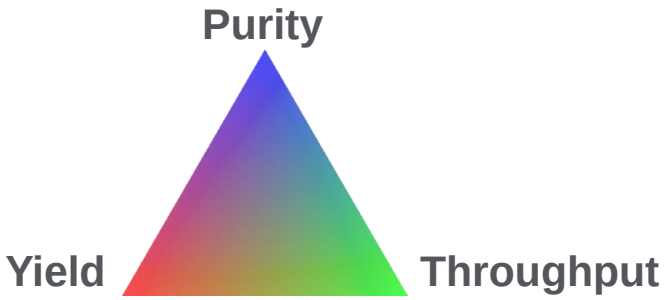
- Purification scientists
- Users of reversed phase preparative LC in small molecule drug discovery labs
- Chemists and lab managers trying to drive high resolution and throughput

# What is Prep LC?

## Workflow and understanding what is important in discovery environments

# What is Prep LC and why do we do it?




## Goal of preparative LC

- Collect one or more compounds from a sample

## Why do we do it?

- To obtain a “pure” product
- For compound characterization

## The focus

- To get required purity (purity)
- To get required amount (yield)
- In the required timeframe (throughput)

# Small molecule purification environments

## High Throughput

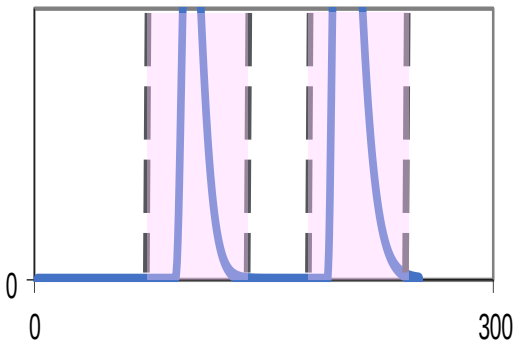
| Objectives | Workflow | Applications |
|---|---|---|
| • Purifying many different samples<br>• Small amounts of material for preliminary bioactivity testing or further characterization<br>• Target – main component | • Not much time can be spent on method development – analytical screen to determine best column, then utilize focused gradient to improve separation (if necessary)<br>• Requires small amounts of many different samples, typically 1-2 injections per sample | • Combinatorial chemistry libraries<br>• Reaction cleanup<br>• Open access purification for medicinal chemists |

## Bulk Purification

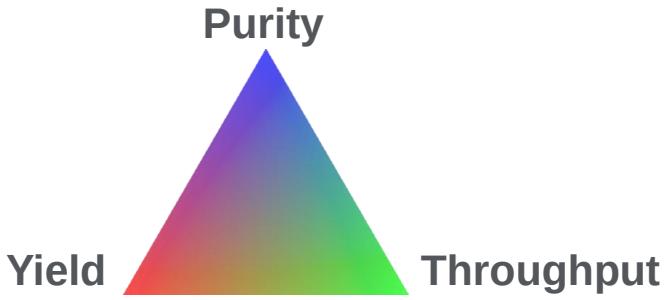
| Objectives | Workflow | Applications |
|---|---|---|
| • Purifying one sample<br>• Significant amounts of material for more in-depth testing and characterization<br>• Target – main component or an impurity | • Considerable time is spent optimizing method to maximize yield, purity and minimize solvent consumption<br>• Many injections of the same sample | • Purification of target compounds (API), Natural products, etc..<br>• Impurity isolation for Identification, structure elucidation and activity testing |

# Objectives of prep LC: purity, yield and throughput

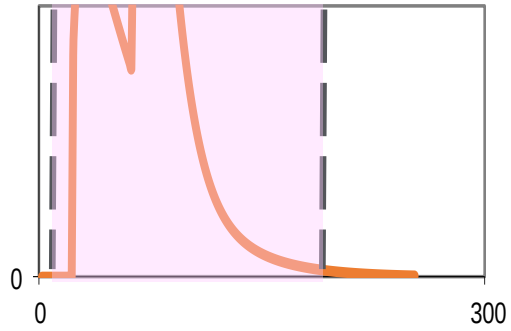
- **Purity**
- **Yield**
- **Throughput**




Longer run times to have enough separation between peaks




- **Throughput**
- **Yield**
- **Purity**



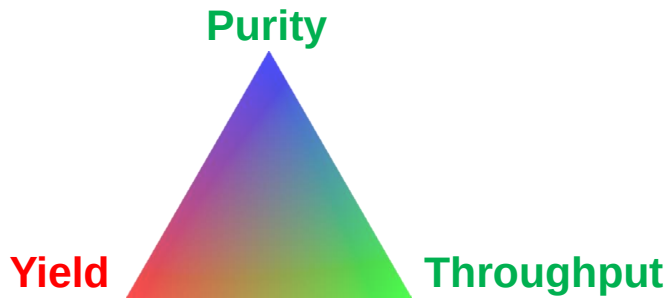

Shorter run times, but collections contain impurities

You can optimize 2 of the parameters by sacrificing the third
The most important parameter depends on the application

# Objectives based on purification environment

## High Throughput

| Objectives | Priority |
| --- | --- |
| • Purifying many different samples<br>• Small amounts of material for preliminary bioactivity testing or further characterization | • Purity – Yes<br>• Throughput – Yes<br>• Yield – No |




## Bulk Purification

| Objectives | Priority |
| --- | --- |
| • Purifying one sample<br>• Significant amounts of material for more in-depth testing and characterization<br>• Optimizing method to maximize yield, purity and minimize solvent consumption | • Purity – Yes<br>• Yield – Yes<br>• Throughput - No |

# Steps in developing a prep method

Agilent InfinityLab

| 1. Check sample formulation | 2. Screen column phases | 3. Optimize the separation | 4. Determine maximum sample load | 5. Determine the prep column size | 6. Scale conditions to prep column | 7. Purify the compound(s) |
|---|---|---|---|---|---|---|
| Solvent, concentration, additives | Run analytical (scalar) columns | Maximize the resolution between the target compound and nearest peaks | Calculate resolution as a function of mass and volume loading | Use the analytical column loading data | Use linear scale up factor | Measure purity and amount of compound(s) obtained |
| Confirm LC method compatibility | Select optimum column phase | Confirm conditions compatible with compound stability/next step | Optimum loading to achieve objectives | Confirm compatible with prep instrument | Confirm compatible with prep instrument | Confirm objectives met |

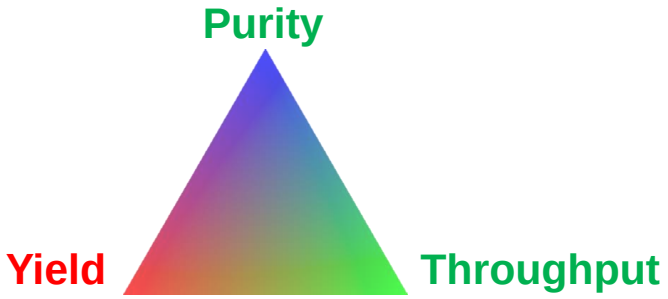
# Steps 1 and 2: high throughput purification

Agilent InfinityLab

**1. Check sample formulation** | **2. Screen column phases** | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | 6. Scale conditions to prep column | 7. Purify the compound(s)

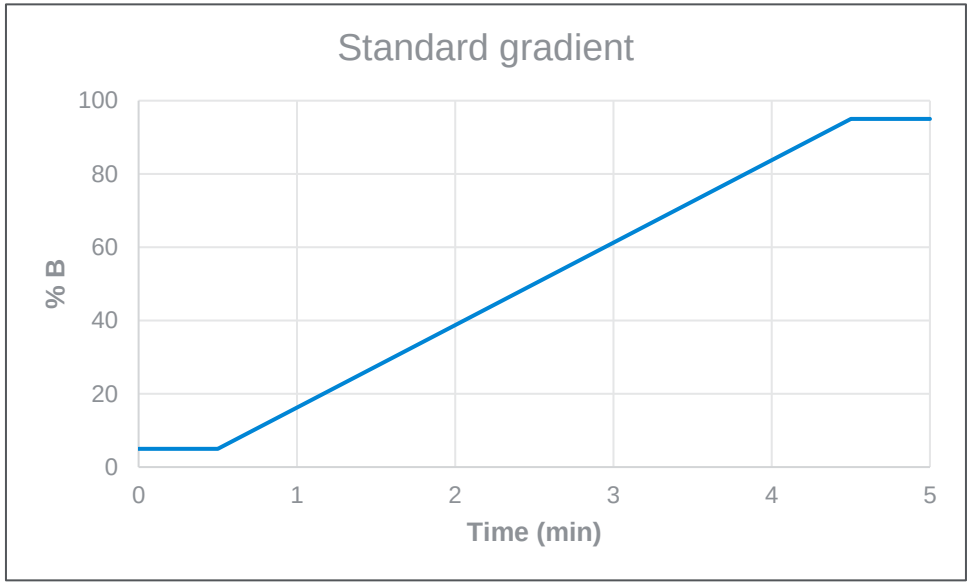
## 1. Check sample formulation

- Samples are crude mixes and can have complicated matrices




## 2. Screen column phases

- Screening combinations of columns/mobile phases using a generic gradient
- Column chemistries are ones that have historically high success rate
- The goal is to identify a column/mobile phase combination that will have the highest chance of separation



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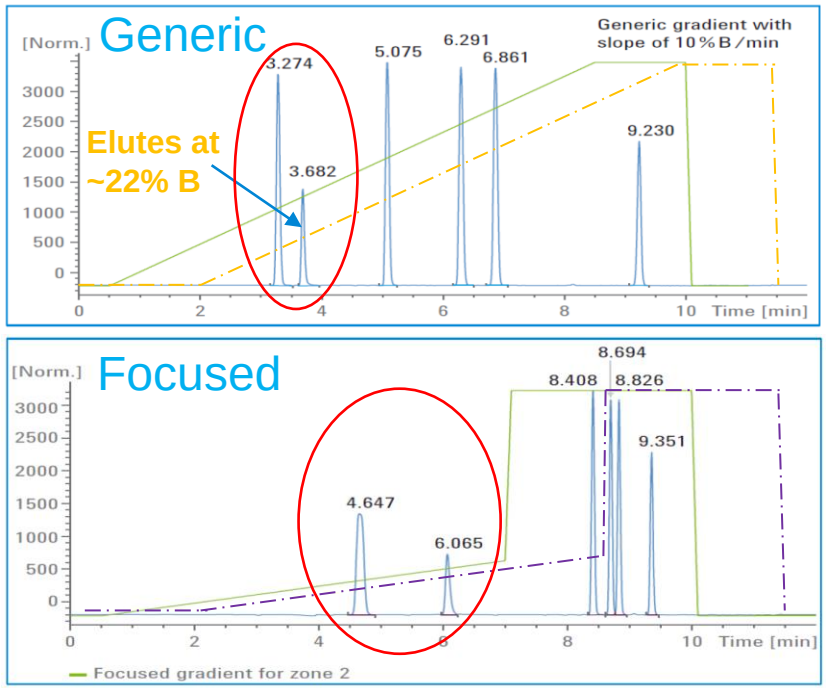
# Step 3: Optimize separation (high throughput purification)

Agilent InfinityLab

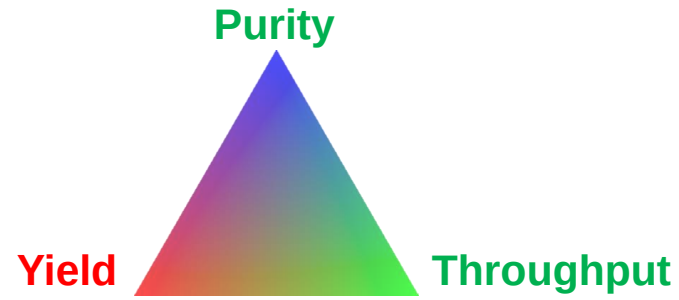
1. Check sample formulation
2. Screen column phases
**3. Optimize separation**
4. Determine max sample load
5. Determine prep column size
6. Scale conditions to prep column
7. Purify the compound(s)

- Running a focused gradient can tremendously improve the critical pair's separation with minimal method development
- Focused gradient – reducing the slope of the original gradient specific to the peak(s) of interest




**Generic gradient (Adjusted):**
5% B: 0 to 2.0 min
85% B: 10.0 min
85% B: 10 to 11.5 min
**Slope: 10% B/min**

**Focused gradient (Adjusted):**
5% B: 0 to 2.0 min
25% B: 8.5 min
85% B: 8.6 to 11.6 min
**Slope: 3.1% B/min**




**Want more info? Visit http://explore.agilent.com/preparative-lc-primer**

# Steps 4 and 5: high throughput purification

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1. Check sample formulation | 2. Screen column phases | 3. Optimize separation | **4. Determine max sample load** | **5. Determine prep column size** | 6. Scale conditions to prep column | 7. Purify the compound(s)

## 4. Determine max sample load

- Generally not done for high throughput work since only small amount of sample is required

## 5. Determine prep column size

- Instead, a column is chosen based on anticipated load. The most popular column IDs for discovery work are 21.2 mm and 30 mm ID.

| Column ID | Flow Rate | Difficult Separation (1.2 < α < 1.5) |
|---|---|---|
| 21.2 mm | 20 mL/min | 20 – 70 mg |
| 30 mm | 40 mL/min | 40 – 140 mg |

# Steps 6 and 7: high throughput purification

Agilent InfinityLab

1. Check sample formulation | 2. Screen column phases | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | **6. Scale conditions to prep column** | **7. Purify the compound(s)**

## 6. Scale conditions to prep column

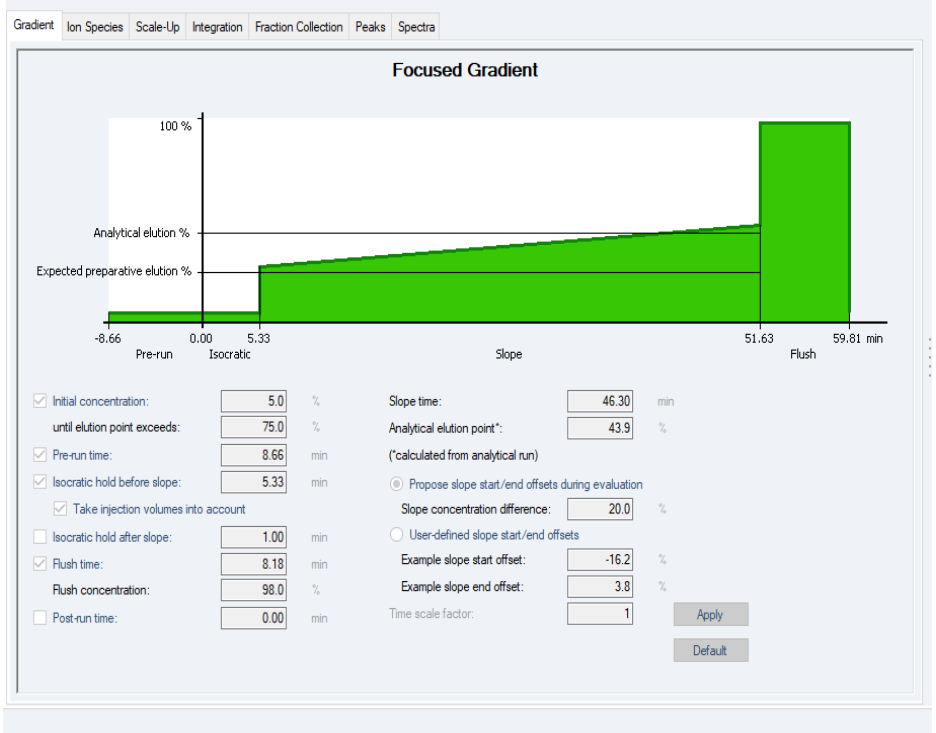
- Premade focused gradients
- A generic gradient of 10-50% B would have 4 focused gradients:
  10-20%, 20.1-30%, 30.1-40%, 40.1-50%
- User can select gradient based on peak's elution percentage

### Manual calculation

$$f_p = f_a \left(\frac{d_p}{d_a}\right)^2 , V_p = V_a \left(\frac{d_p}{d_a}\right)^2$$

$f_a$ = Analytical flow rate
$d_p$ = Prep internal diameter
$d_a$ = Analytical internal diameter
$V_a$ = Analytical injection volume

### Automated calculation




## 7. Purify the compound(s)

- Generic trigger settings to accommodate most collections
- Always a clean waste bottle in case a compound is missed
- MS detection is typically used to minimize number of collections made

# Needs for a high throughput environment

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**High Throughput**

**What's important?**

- Collecting enough material (10-100 mg)
- High purity
- Minimal method development

**Ways to improve throughput:**

- Screening (step 2) – analytical columns
- Size (step 5) - preparative columns

# Superficially Porous Particles

## What they are and how they work

# Understanding performance by understanding band broadening

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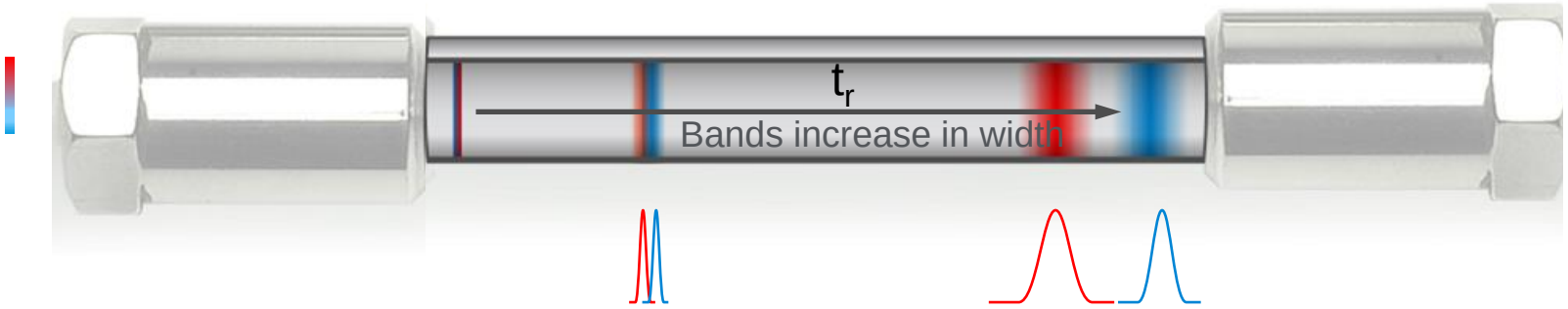
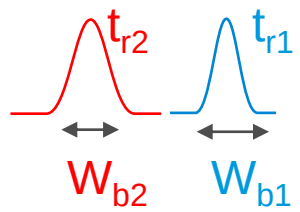
Sample enters enter the column in a defined injection volume (single band)

Components of the sample interact and move through the column at different speeds

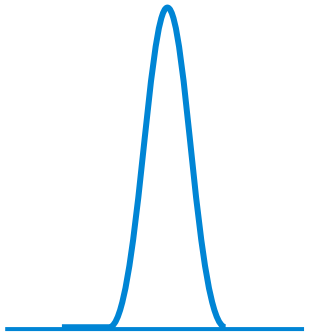
The separation of the bands increases as they move through the column

Components exit column in discrete bands for detection

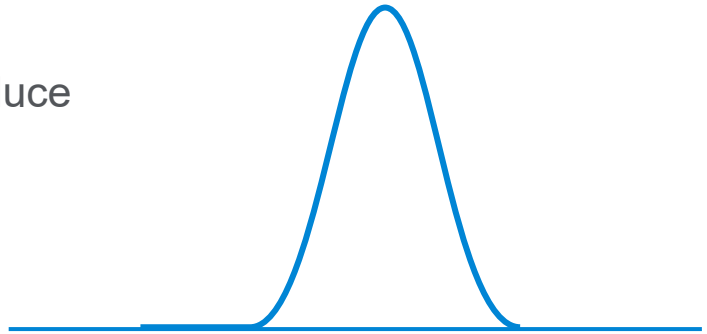



Why do some columns produce peaks like this?



…and other columns produce peaks like that?



**How do I get the best performance from my column?**



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# Van Deemter curves

- Van Deemter curves provide a simple way to compare columns
- Uses plate height (measure of band broadening), which is inversely proportional to plate number (N)

## Experimental

- Sample
  - Void volume marker (e.g., uracil)
  - Neutral analyte (naphthalene, toluene, etc.)
- Isocratic method that produces a symmetrical peak
- Inject sample across a wide range of flow rates
- Plot plate height (H) against linear velocity (u)

## Equations

$$N = 5.54 \left( {}^{t_r}/_{W_{1/2}} \right)^2$$

L = length of the column
$w_{1/2}$ = peak width at half height
$t_r$ = retention time of analyte

$$H = {}^{L}/_{N}$$

$$u = {}^{L}/_{t_0}$$

L = length of the column
$t_o$ = retention time of void volume marker

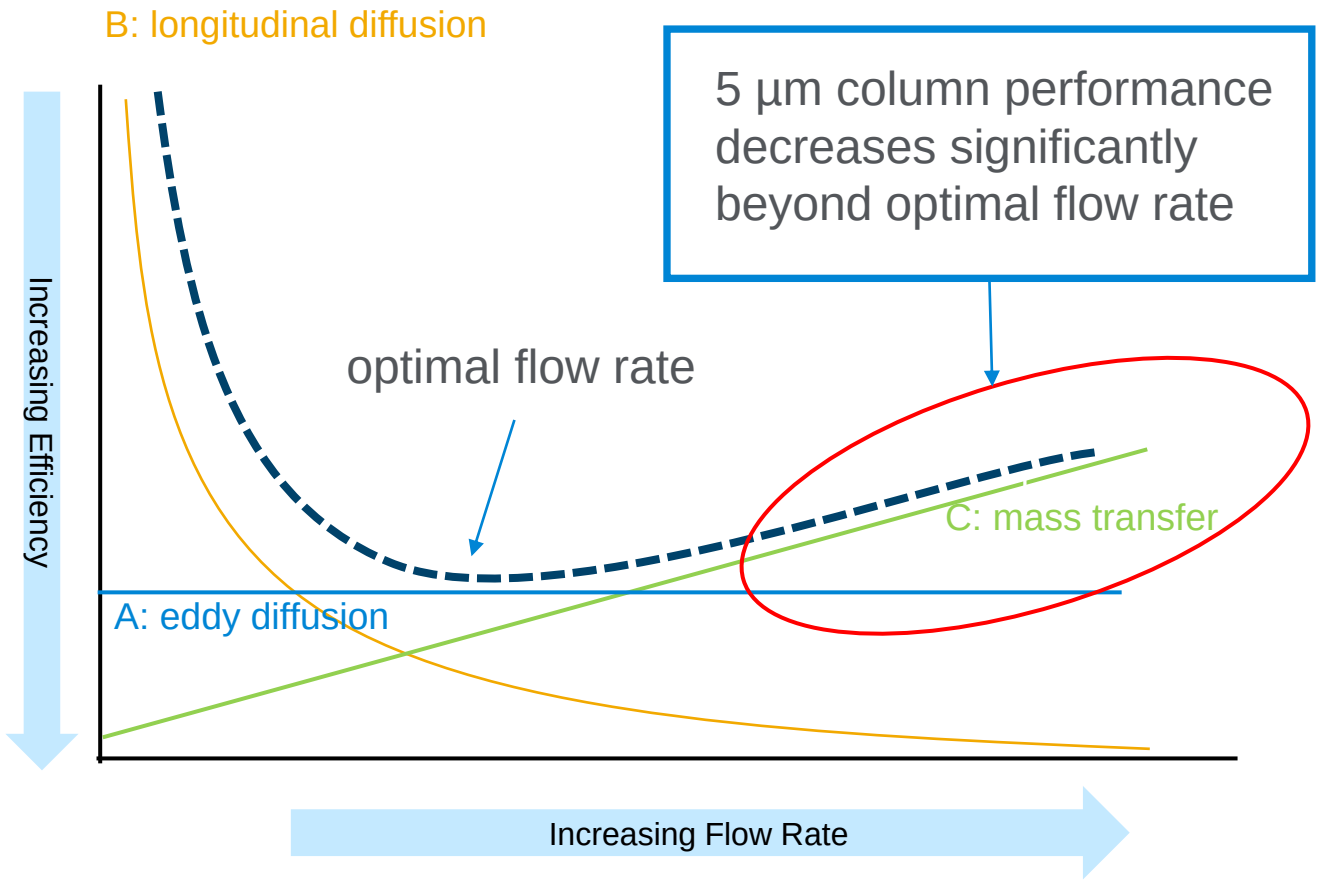
# Van Deemter equation – significance of A, B and C terms

$$H = A + {}^{B}/_{u} + Cu$$

Where:

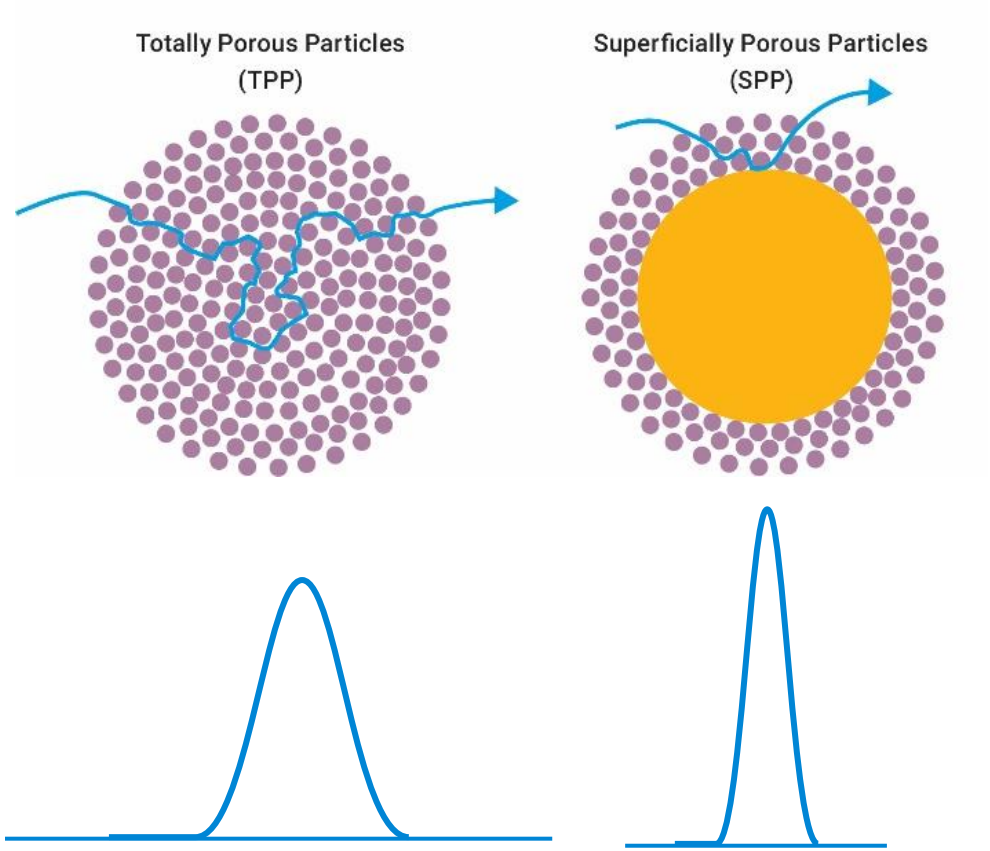
- **A term**: eddy diffusion
  - Flow path of analyte through stationary phase particles
  - Particle size, size distribution and packing quality
- **B term:** longitudinal diffusion
  - Diffusion in the mobile phase
  - Only significant at very low flow rates
- **C term:** mass transfer resistance
  - Analyte traveling in/out of particle
  - **Significant at mid to high flow rates**

## Van Deemter plot for 5 µm TPP column

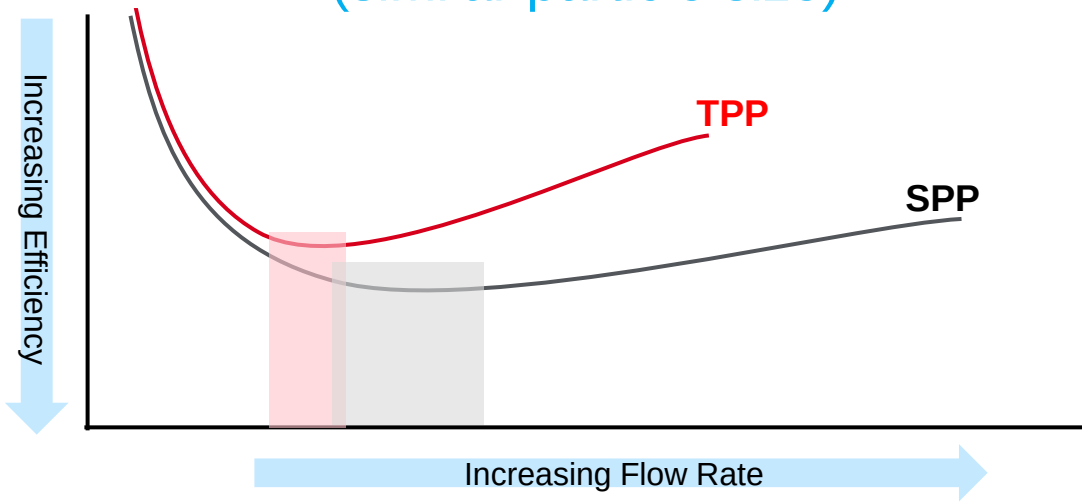


# Superficially porous particles

- Superficially porous particle (SPP) – Solid core and porous outer layer
- Solid core shortens diffusion path, resulting in a narrower chromatographic peak over totally porous particle (TPP) column

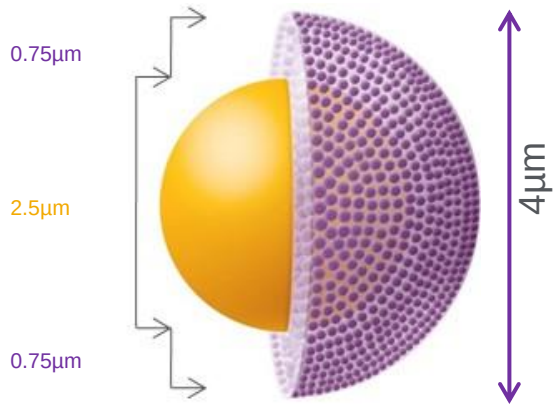



## van Deemter plots for SPP and TPP columns (similar particle size)



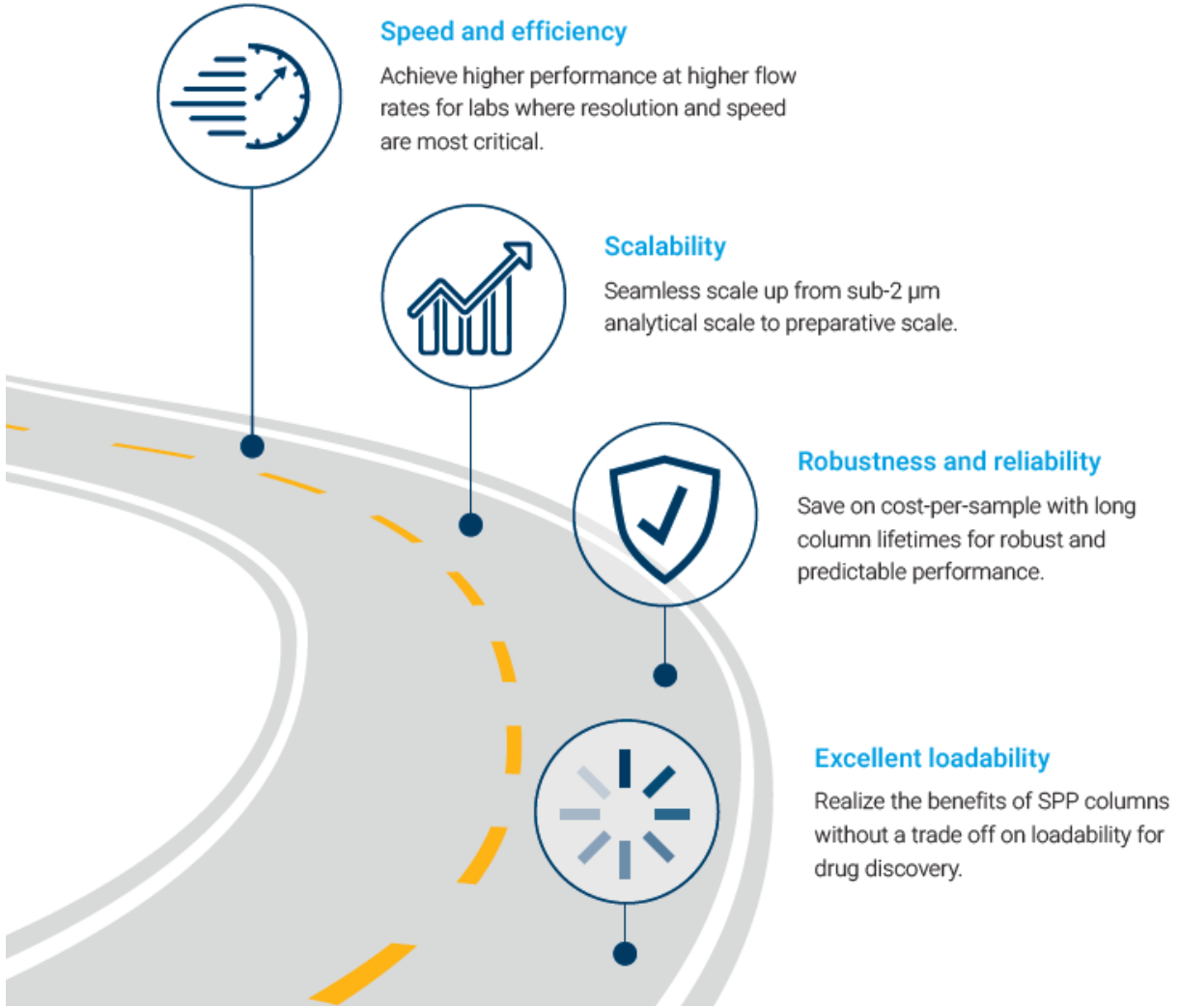

- SPP columns have smaller C terms
- Can run them at higher flow rates while maintaining column efficiency

# Agilent InfinityLab Poroshell 120 preparative LC columns




## Made with 4 µm Poroshell particles

- Symmetrical particles with smooth surfaces (↓ A term)
- Narrow particle size distribution (↓ A term)
- Short analyte diffusion path in/out of the porous layer (↓ C term)

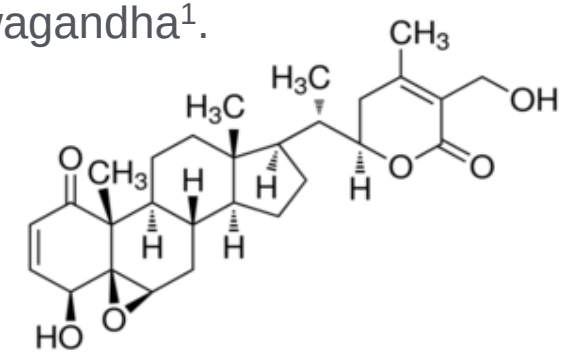


# Generic purification methods on SPP and TPP columns

## Separation of withaferin A in ashwagandha

- Discovery labs regularly purify large batches of samples.
- They require small amounts (10 to 100 mg) of high purity fractions for downstream workup and characterization
- Purification of bioactive components in natural products represents a similar challenge to that of drug candidates in crude mixtures – both have complicated matrices.
- Withaferin A (WFA) is the most bioactive withanolide in ashwagandha[1].

Withaferin A[2]




| General Run Conditions | |
|---|---|
| **Instrument** | Agilent 1290 Infinity II autoscale preparative LC system |
| **Sample** | Ashwagandha extract in 2:1 ethanol:water, 100 mg/mL |
| **Mobile Phase** | A: Water + 0.1% formic acid |
| | B: Acetonitrile + 0.1% formic acid |
| **Injection Volume** | 1 mL filtered extract |

| | Optimized | Elevated |
|---|---|---|
| **Agilent InfinityLab Poroshell 120 SB-C18 21.2 x 50, 4 µm** | | |
| **Flow rate:** | 25 mL/min | 37.5 mL/min |
| **Gradient:** | 5 – 95% B in 15 min | 5 – 95% B in 10 min |
| **TPP C18, 19 x 150, 5 µm** | | |
| **Flow rate:** | 17 mL/min | 25.5 mL/min |
| **Gradient:** | 5 to 95% B in 18 min | 5 to 95% B in 12 min |

Link to application note: 5994-3518EN

1. M. H. Mirjalili et al. *Acta Chromatographica 25(**2013**)4, 745-754*
2. *https://www.sigmaaldrich.com/content/dam/sigma-aldrich/structure3/198/mfcd10687098.eps/_jcr_content/renditions/mfcd10687098-medium.png*

# WFA separation at optimal and 1.5x optimal flow rate

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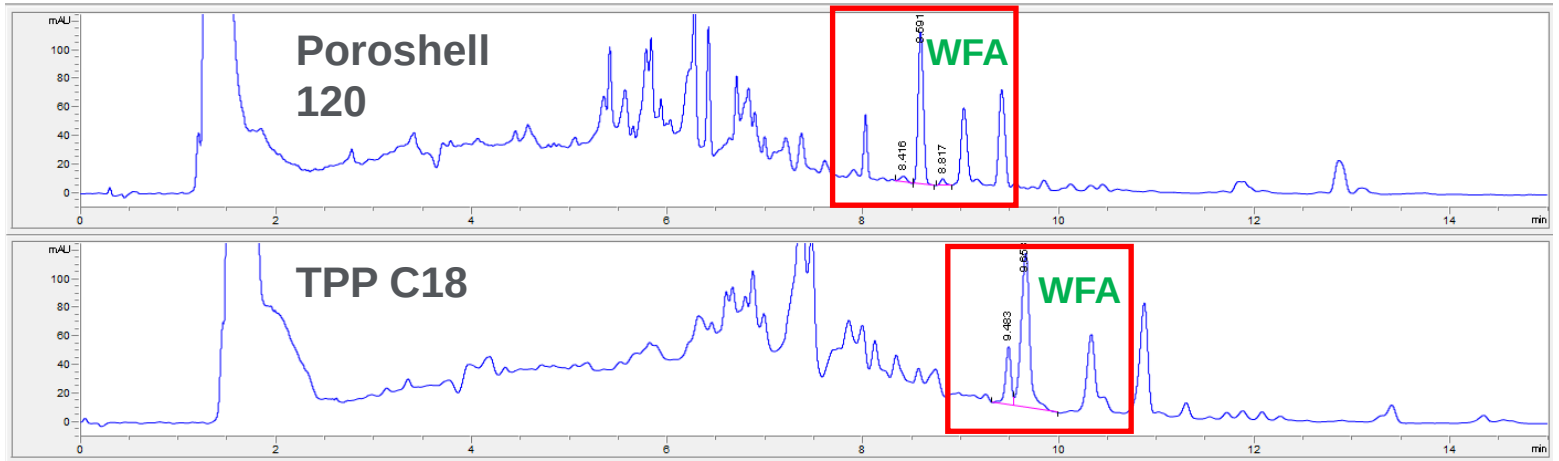
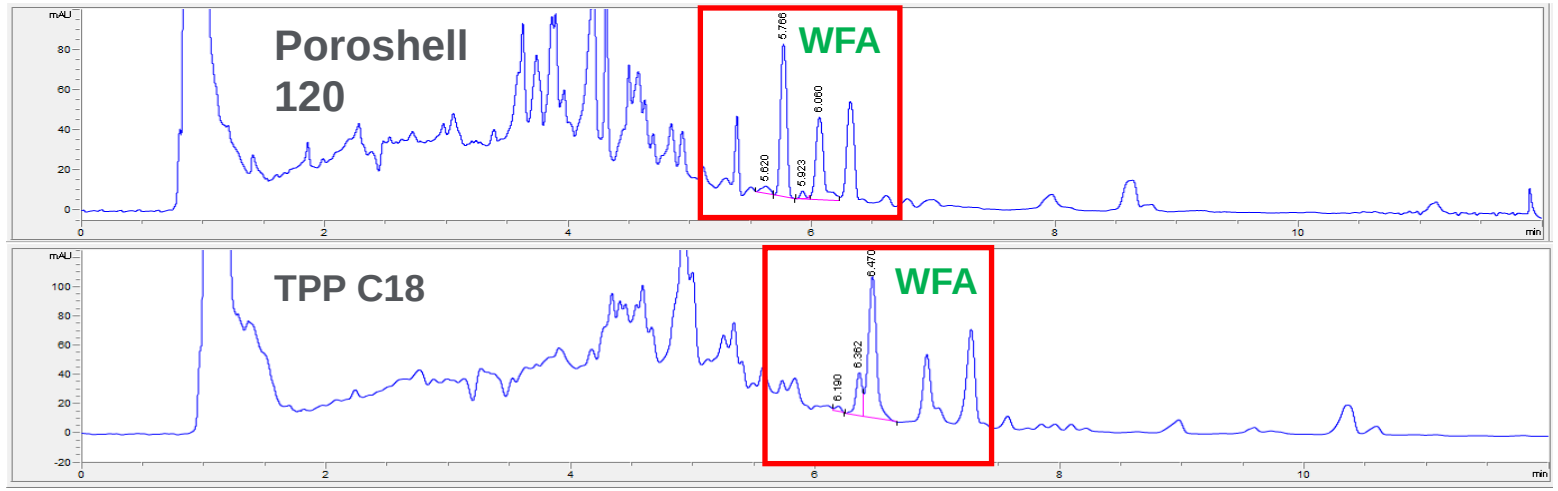
**Optimal flow rate**




- Separates WFA from impurities
- Best overall resolution

- Separates WFA from leading impurity
- Trailing impurity elutes in WFA tail

**1.5x optimal flow rate**




- Still WFA from impurities
- Best overall resolution

- Impurities have almost completely coeluted



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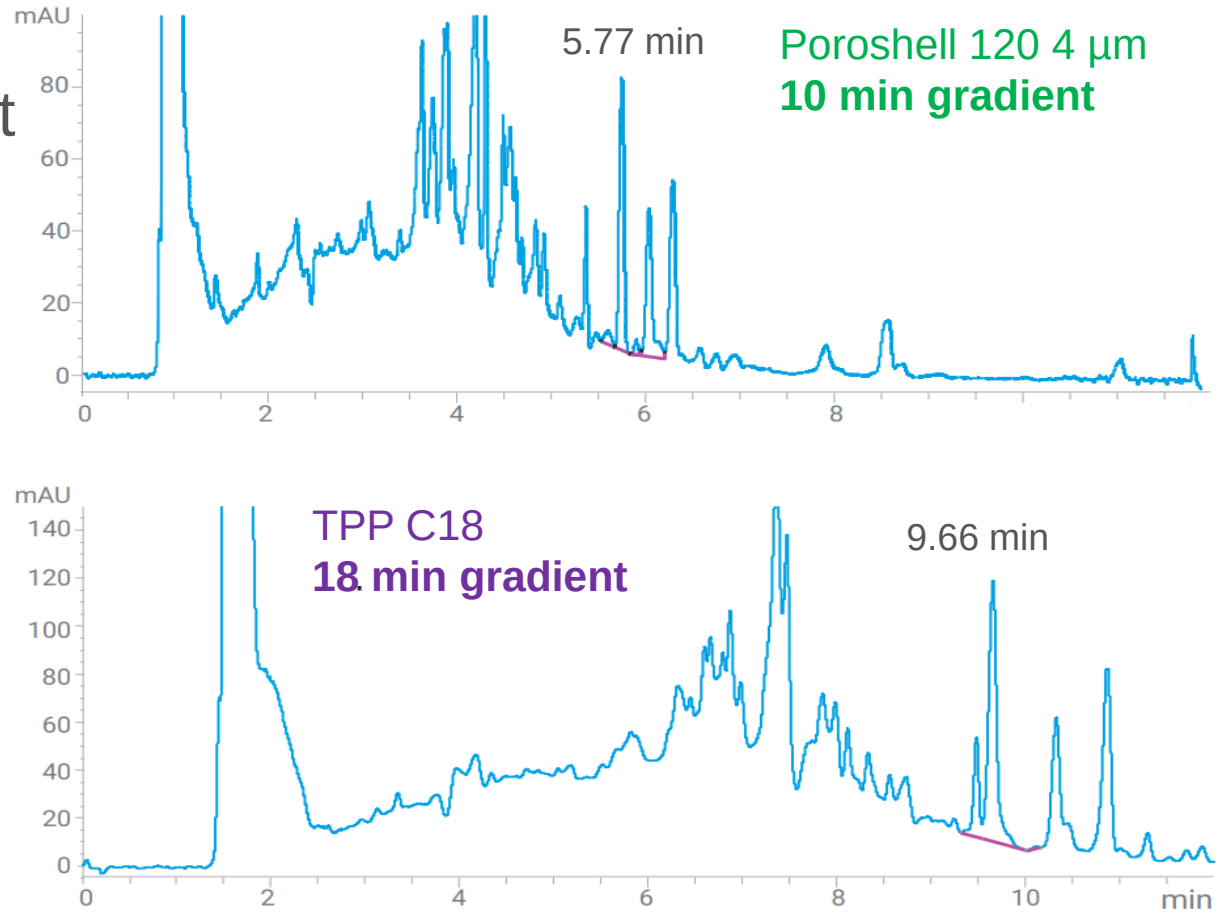
# Comparing best methods

## 1.5x optimal SPP flow rate vs optimal TPP flow rate

- SPP column maintains separation at high flow rate using a generic gradient
- TPP column loses resolution between optimal and high flow rates
- The SPP method is 45% faster than the TPP method



# Integrating SPP columns into the purification workflow

## Overcoming challenges

# Challenges integrating SPP into preparative LC

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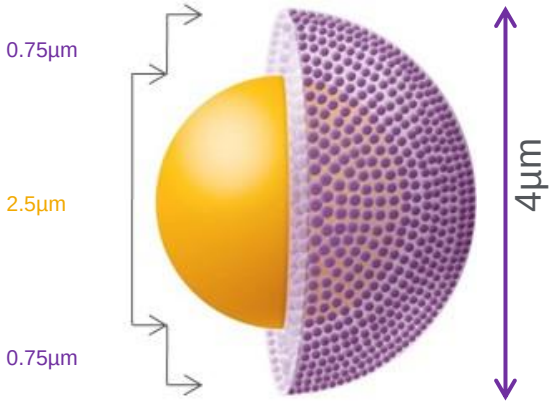


**= How?**

## Instrument compatibility

- Running conventional SPP sizes may exceed system pressure limits
- System volume causes enough band broadening to negate SPP performance benefits

## Sample loading limitations

- SPP phases lack the surface area necessary for adequate sample loading

# Addressing challenges – pressure and particle size

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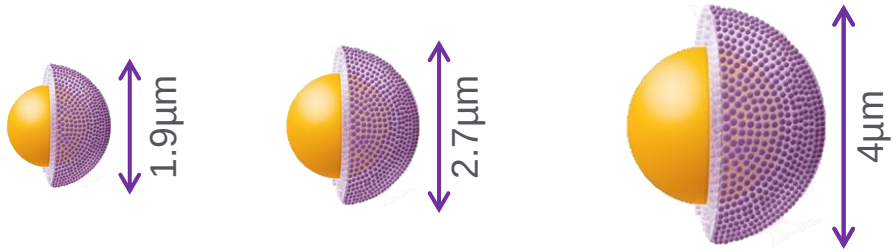
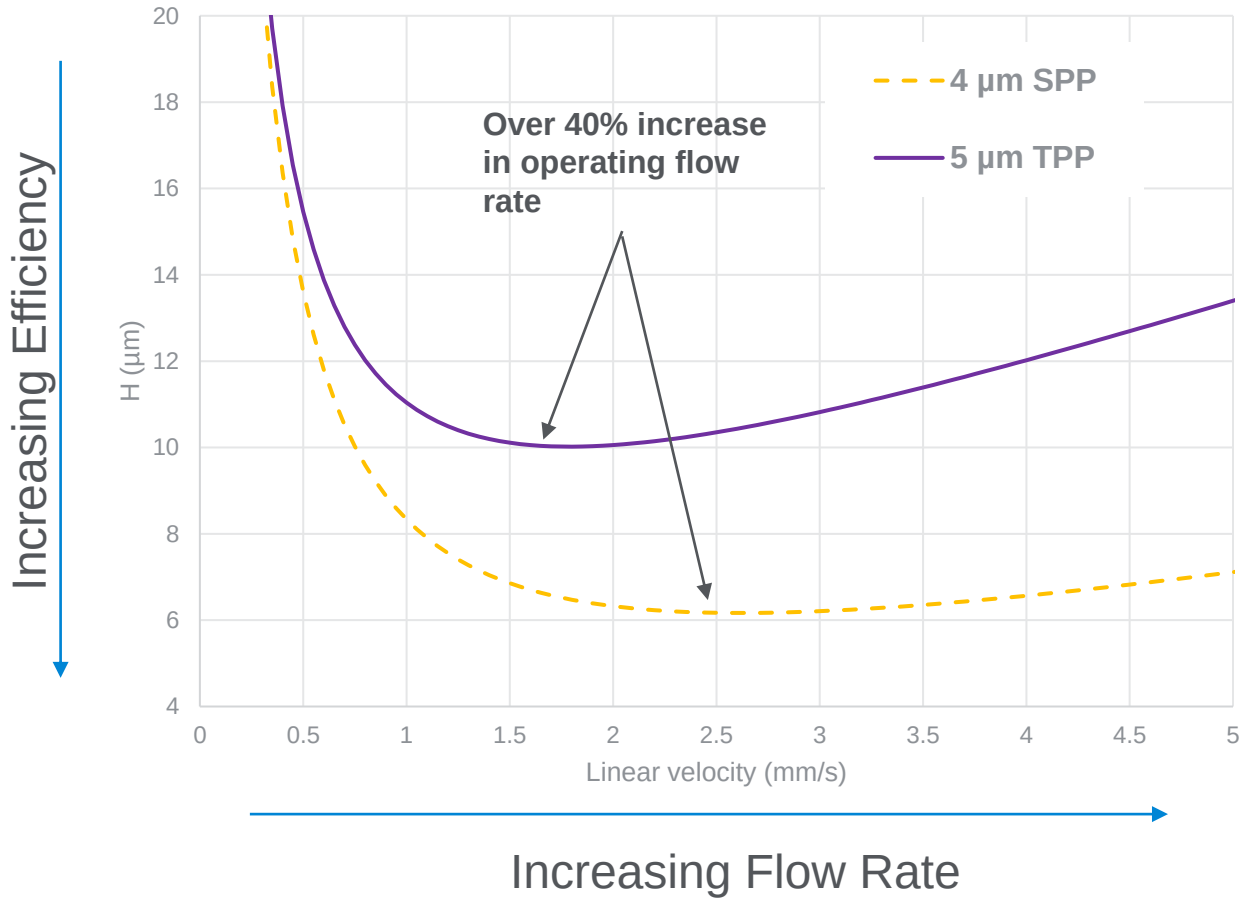
## Proper particle size is key




- 4 µm SPPs provide speed and efficiency advantage over 5 µm TPPs
- SPP benefits without the high backpressure generated from a smaller particle size

## Van Deemter plots of 4 µm SPP and 5 µm TPP columns



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# Addressing challenges – system volume

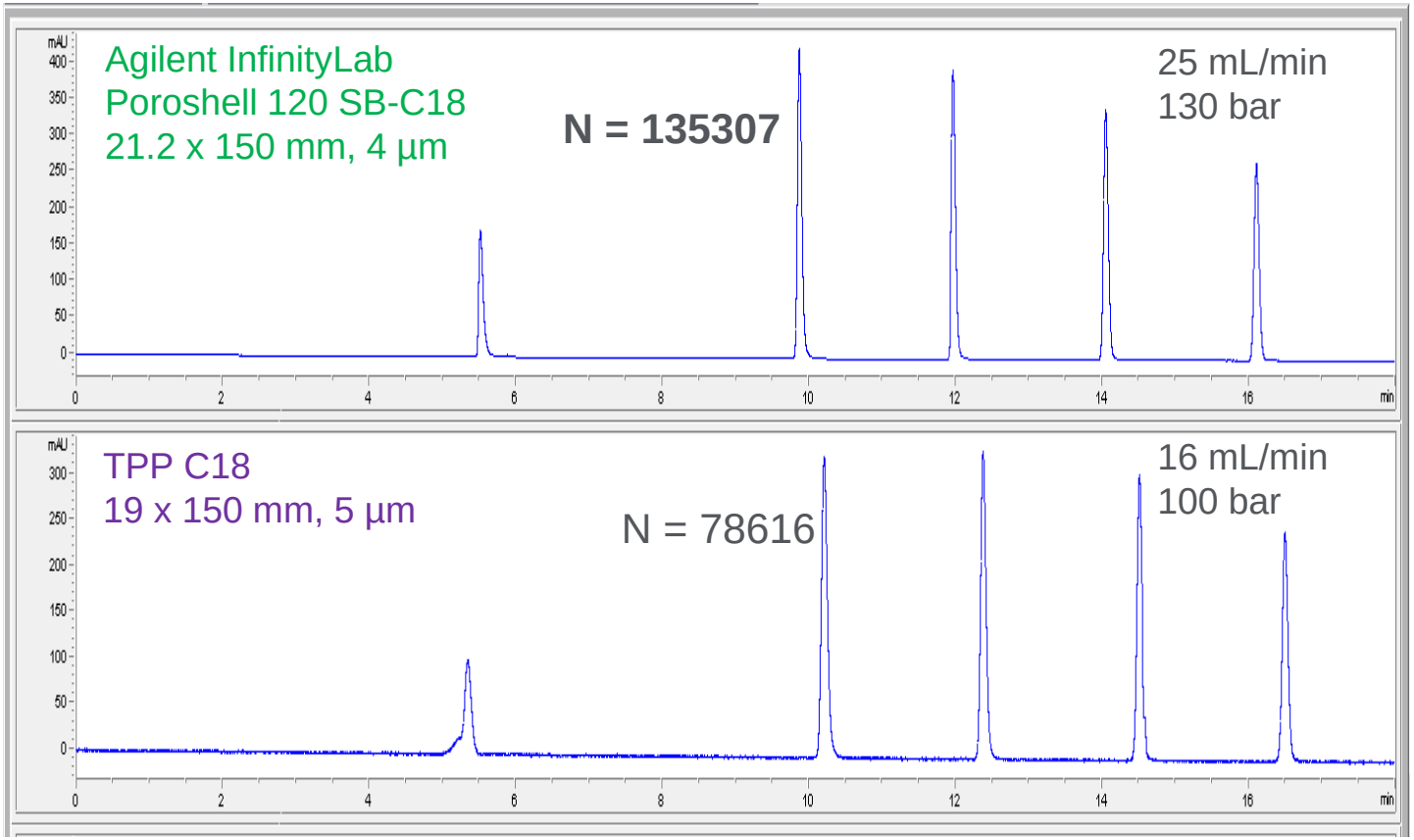
- Efficiency benefits of 4 µm SPP preparative columns can be seen on systems with standard configuration/plumbing
- **Note**: regardless of particle type, proper consideration should be given to tubing size, based on the diameter of the column

**Paraben Mix (in DMSO):**

- Caffeine
- Methyl Paraben
- Ethyl Paraben
- Propyl Paraben
- Benzyl Paraben

Agilent 1290 Infinity II autoscale preparative LC system

5-65% ACN gradient

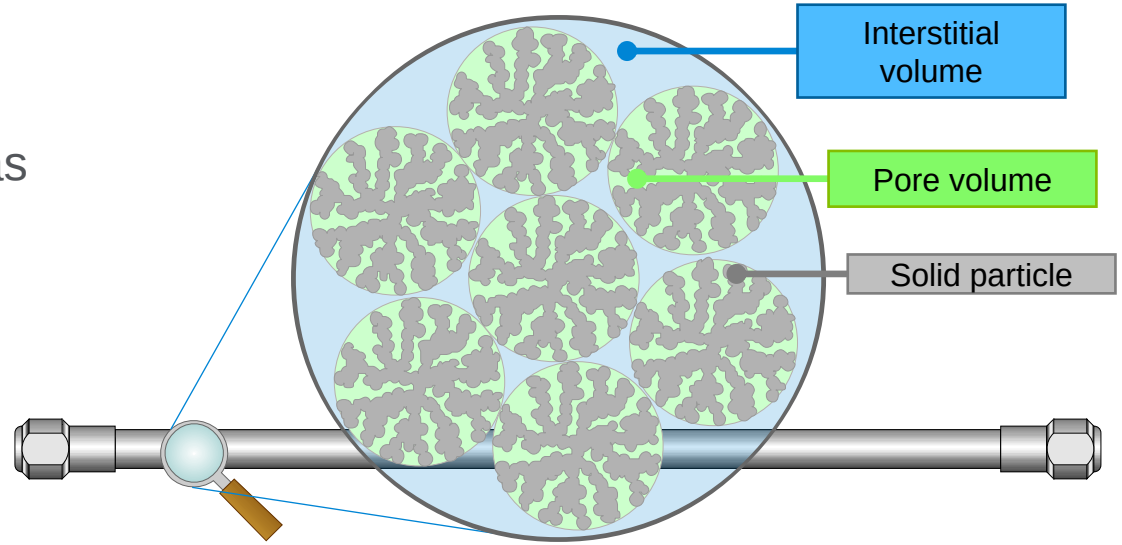



Flow rate: Agilent – 25 mL/min; TPP – 16 mL/min; Mobile phase: A – water + 0.1% formic acid; B – acetonitrile + 0.1% formic acid; gradient: 5 – 65% B in 18 min; injection volume: Agilent – 80 µL; TPP – 64 µL; Detection: UV 254 nm.

# Addressing challenges – sample loading

- Stationary phases with higher surface areas will generally have more retention (and more separation between peaks) than columns with lower surface areas
- Higher surface areas (~400 $m^2/g$) are used for additional loading

| Phase | Surface Area ($m^2/g$) | Pore Size (Å) |
|---|---|---|
| Poroshell SB-C18 (4 µm SPP) | 130 | 120 |
| Vendor X C18 (5 µm TPP) | 185 | 135 |
| Vendor Y C18 (5 µm SPP) | 200 | 100 |
| Vendor Y C18 | **400** | 100 |
| Pursuit XRs C 18 | **440** | 100 |





Higher surface area phases are ideal for loading


- Lower surface area phases offer fast separations
- Smaller particle sizes offer higher efficiency to resolve complicated matrices

# Loading on InfinityLab Poroshell 120 SB-C18

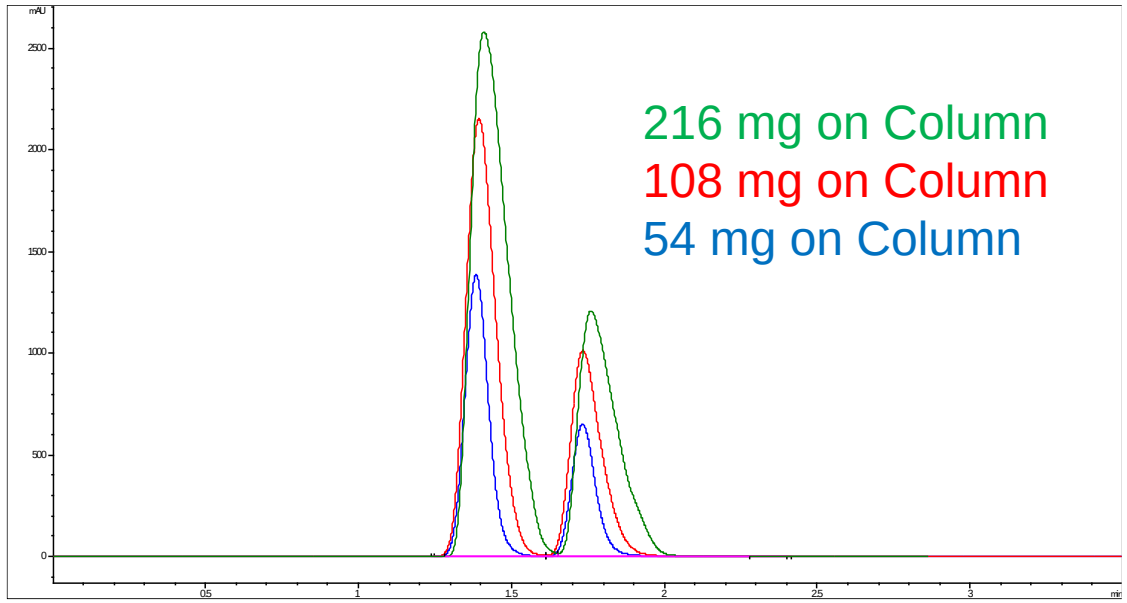
**Sample:** Sulfanilamide (A) + Sulfamethoxazole (B) in 50/50 acetonitrile/water

- Sample 1: 30 mg/mL A + 30 mg/mL B
- Sample 2: 60 mg/mL A + 60 mg/mL B
- Sample 3: 120 mg/mL A + 120 mg/mL B

| | |
|---|---|
| **Preparative instrument** | Agilent 1290 Infinity II preparative LC system |
| **Preparative column** | InfinityLab Poroshell 120 SB-C18, 21.2 x 150 mm, 4 µm |
| **Flow rate** | 25 mL/min |
| **Mobile phase** | 55/45 acetonitrile+ 0.1 formic acid/water + 0.1% formic acid |
| **Injection volume** | 900 µL |
| **Wavelength** | 238 nm |

**High Throughput**

Discovery labs typically purify between 10 – 100 mg at a time

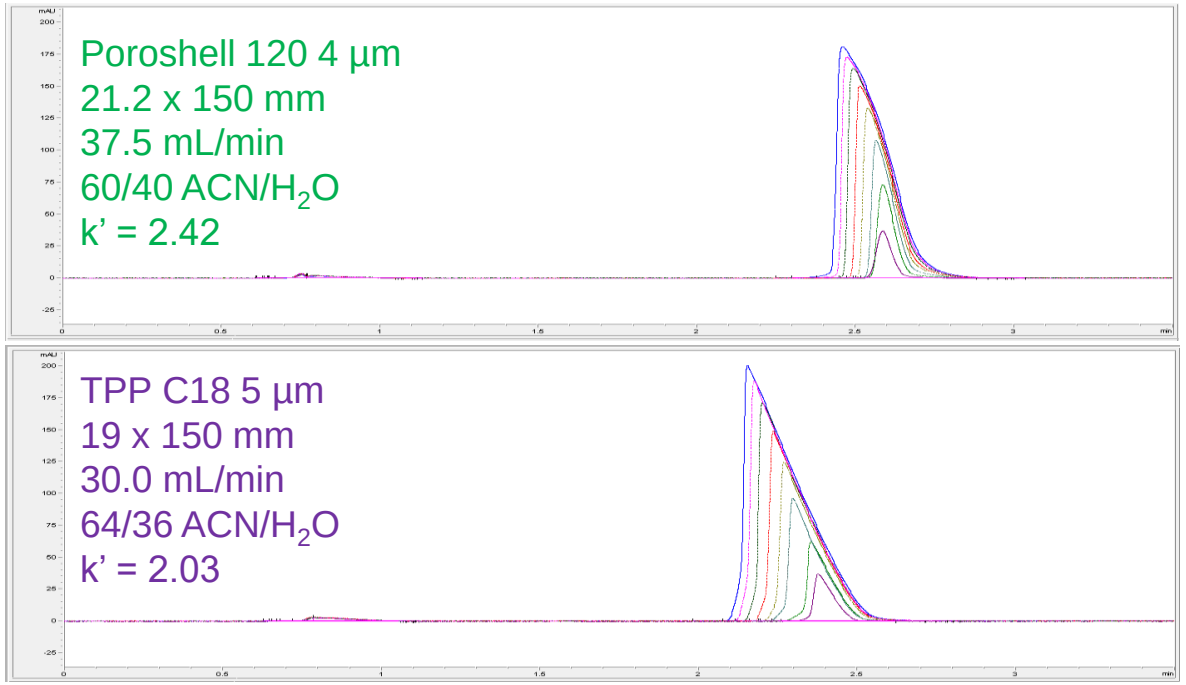



**Peaks still retain shape with over 200 mg on column**

# Mass loading comparison at fast flow rates

## Analyte - toluene

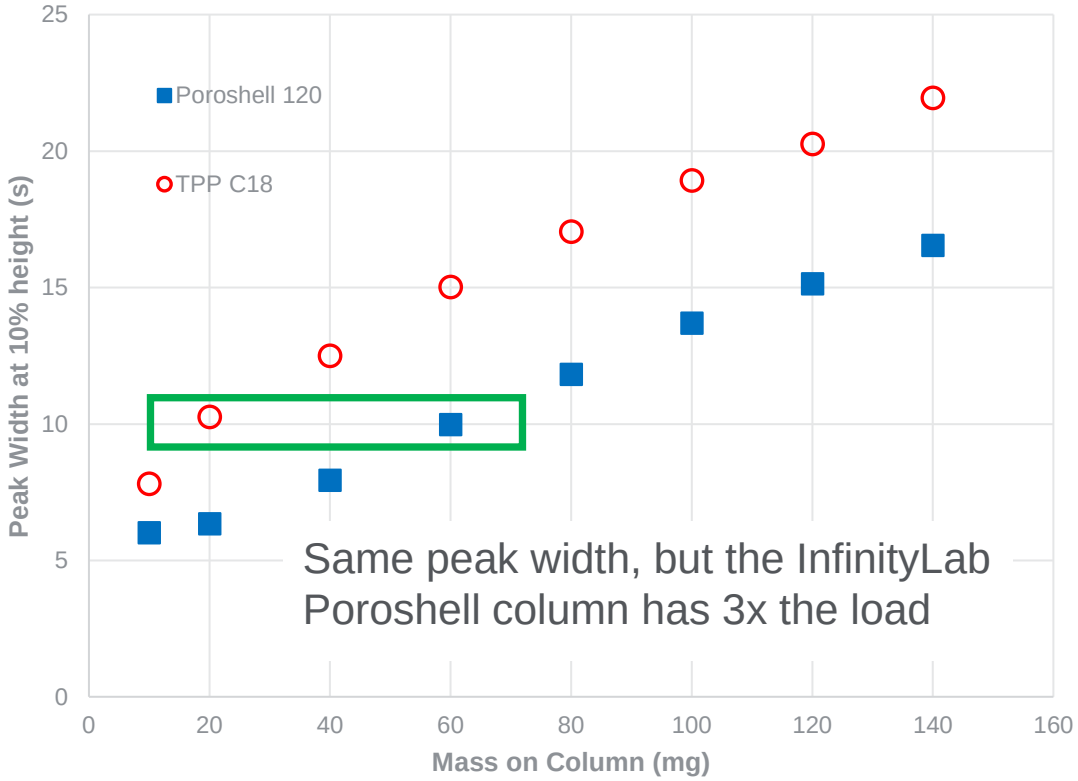
- Comparing peak widths at 10% peak height
- Adjusted flow rate and mobile phase composition on TPP C18 to match $t_0$ and and k' of toluene on Poroshell column
- Injection volume – 400 µL
  - Determined by volume loading study (not shown) on Poroshell 120 column
- Toluene sample concentrations in 50/50 ACN/DMSO (mg/mL)
  - 25, 50, 100, 150, 200, 250, 300, 350




**Mass on column – 4 mg to 140 mg**

# Mass loading results and ROI

## Peak width at 10% height vs mass on column




**InfinityLab Poroshell column delivers a 64% savings and 728% ROI over TPP column**

## For 1,000 sample campaign:

| | TPP Column | InfinityLab Poroshell 120 |
|---|---|---|
| Number of samples | 1,000 | 1,000 |
| Number of injections per sample | 3 | 1 |
| Number of injections per campaign | 3,000 | 1,000 |
| Total time (analysis time x number of injections) | 175 hours | 58 hours |
| Total lab overhead (lab overhead x total time) | $21,875 | $7,292 |
| Total scientist cost (scientist cost x total time) | $26,250 | $8,750 |
| Total consumables cost (consumables cost x no of injections) | $62,621 | $23,361 |
| Total cost per campaign | $110,746 | $39,402 |
| Purification cost per sample | $111/sample | $39/sample |
| **Cost savings** | **$71,344 or $71/sample** | |
| **% savings** | **64%** | |

| | | TPP Column | InfinityLab Poroshell 120 |
|---|---|---|---|
| Return on Investment | Total cost per campaign + net cost of change | $110,746 | $48,022 |
| | Agilent column saves | | $62,724 |
| | **ROI% (savings/net cost to change)** | | **728%** |

# Integrating SPP columns in purification workflow

## Driving throughput

# Needs for a high throughput environment

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**High Throughput**

**What's important?**

- Collecting enough material (10-100 mg)
- High purity
- Minimal method development

**Ways to improve throughput:**

- Screening (step 2) – analytical columns
- Size (step 5) - preparative columns

# Step 2: Improving screening by leveraging $L/d_p$

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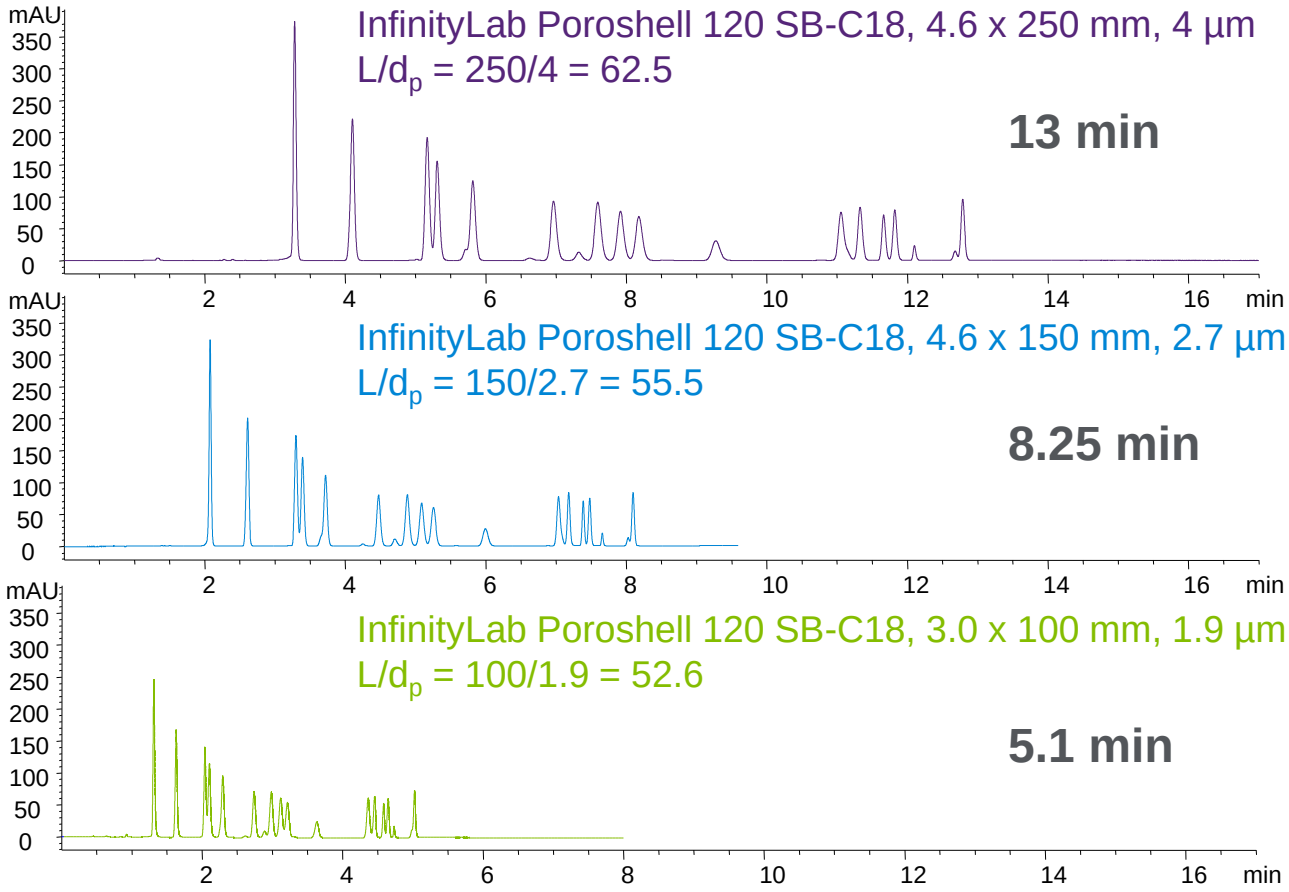
1. Check sample formulation | **2. Screen column phases** | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | 6. Scale conditions to prep column | 7. Purify the compound(s)

- Preferred columns: 2.1 x 50 mm, sub-2 µm or smallest available to further improve throughput
- Small columns and fast gradients (2-3 min) are combined to minimize method development time
- Methods can be aggressive - The goal is to identify a column/mobile phase combination that will have the highest chance of separation, not a final separation
- Columns within 10 $L/d_p$ units have similar resolving power

**High Throughput**

Screen with sub-2 µm columns and then scale with L/dp (Length/particle diameter)



# Step 5: high throughput purification

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1. Check sample formulation | 2. Screen column phases | 3. Optimize separation | 4. Determine max sample load | **5. Determine prep column size** | 6. Scale conditions to prep column | 7. Purify the compound(s)

## 5. Determine prep column size

- Instead, a column is chosen based on anticipated load. The most popular column IDs for discovery work are 21.2 mm and 30 mm ID. If you have sufficient loading on a smaller column, do you really need a large column?

| Column ID | Flow Rate | Difficult Separation (1.2 < α < 1.5) |
|---|---|---|
| 21.2 mm | 20 mL/min | 20 – 70 mg |
| 30 mm | 40 mL/min | 40 – 140 mg |

**Using a smaller column:**

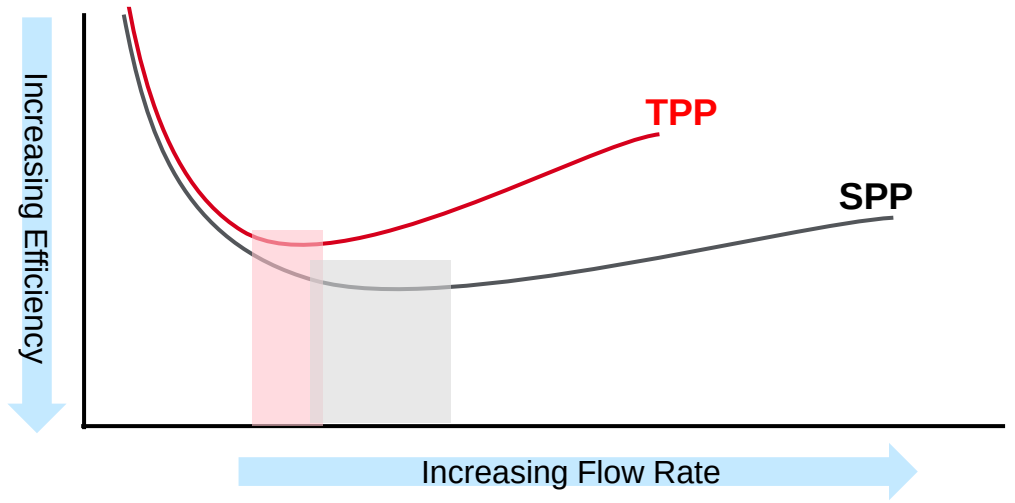
- **Reduces column costs (smaller columns are less expensive)**
- **Reduces solvent consumption and waste disposal**
- **Reduces fraction dry down time**

# Summary

## High throughput environments can benefit from analytical and preparative columns that:

- can resolve samples in complex matrices
- can be run aggressively while still maintaining performance
- have the loading capability to purify required amounts of sample

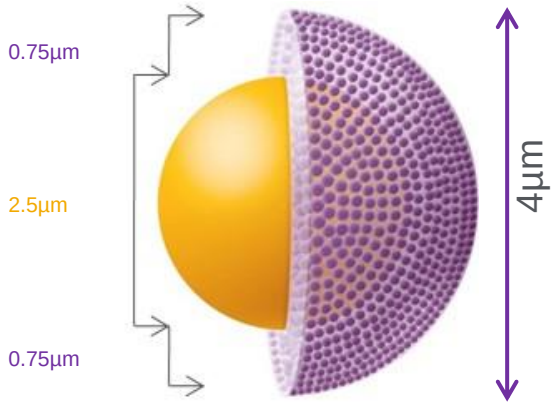
With little to no change to instrumentation




## Columns packed with superficially porous particles:

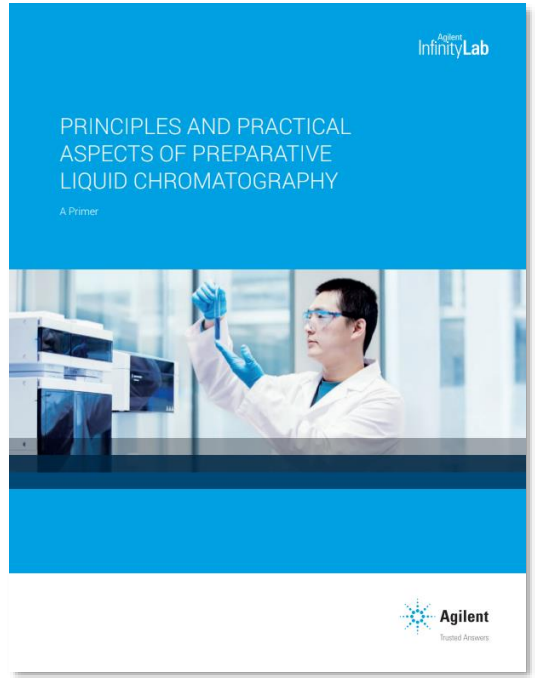
- Have higher efficiency than their TPP counterparts
- Maintain efficiency at high flow rates
- Meet the loading needs of discovery and high throughput environments

Using conventional analytical and preparative hardware



# Resources for support

- **Product webpage:** www.agilent.com/chem/prepcolumns
- **Product brochure:** InfinityLab Poroshell120 Preparative LC Columns 5994-3601EN
- **Primer:** Principles and Practical Aspects of Preparative Liquid Chromatography 5994-1016EN
  - Great overview that covers fundamentals and best practices
- **Application note:** Developing Fast Purification Methods 5994-3518EN
- **Part numbers:**
  - 670050-702: InfinityLab Poroshell 120 HPH-C18, 21.2 x 50 mm, 4 µm
  - 670050-902: InfinityLab Poroshell 120 SB-C18, 21.2 x 50 mm, 4 µm
  - 670150-702: InfinityLab Poroshell 120 HPH-C18, 21.2 x 150 mm, 4 µm
  - 670150-902: InfinityLab Poroshell 120 SB-C18, 21.2 x 150 mm, 4 µm

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