Updating Old Methods: Is the Gain Worth the Pain?

Becoming a Better Chromatographer
HPLC educational webinar

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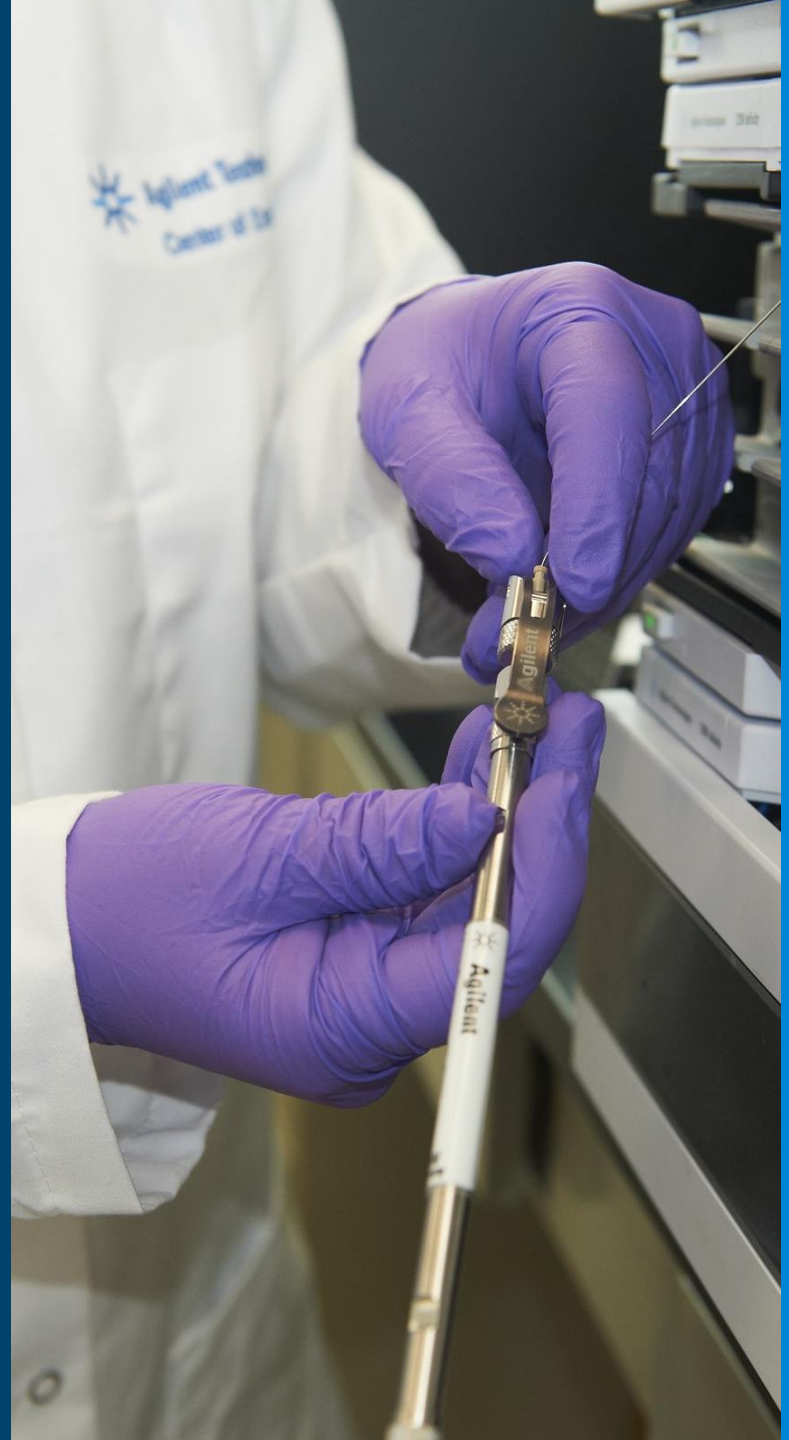
Applications Engineer
Columns and Supplies Technical Support



Agilent
Trusted Answers

Why Would You Consider Updating? What Is the Goal?

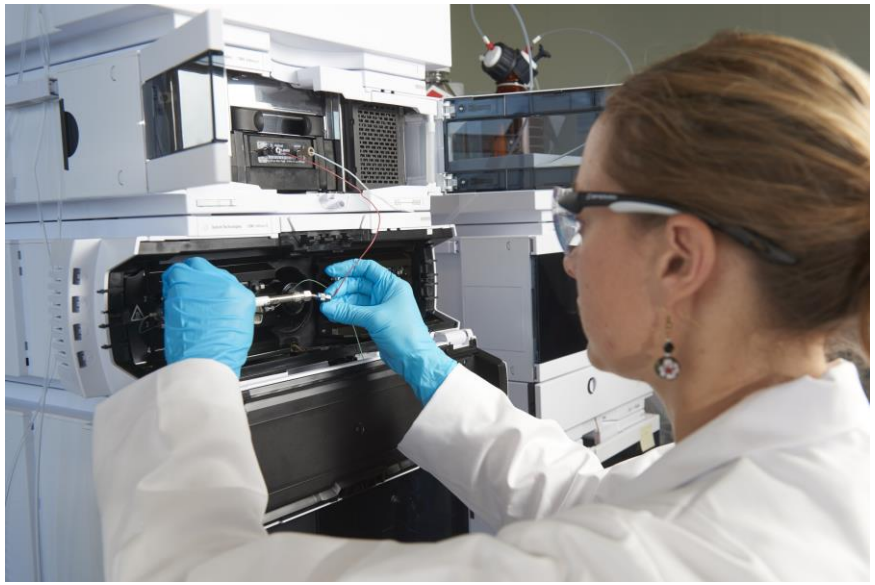
- Do I need to update?
- Reduce analysis time
  - Productivity (free up time for other tasks)
  - More analyses (free up instrument time)
  - More samples (higher throughput)
- Improve resolution
- Improve sensitivity
- Increase column life
- Save solvent
  - Cost
  - Disposal

What Can Be Changed? Summary of Allowable Adjustments: USP General Chapter <621>

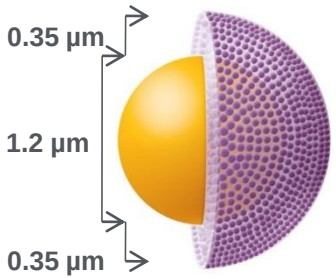
Parameters for System Suitability		
	Isocratic	Gradient
Particle Size Column Length	L/dp: -25% to +50% or N: -25% to +50%	No Changes allowed
Column Inner Diameter	Flexible, w/ constant linear velocity	No Changes allowed
Flow Rate	Based on dp: $F_2=F_1\times[(dc_2^2\times dp_1)/(dc_1^2\times dp_2)]$ Additional adjustments: ±50%, provided N decreases ≤20%	No Changes allowed
Injection volume	May be adjusted, as far as is consistent with precision and detection limits	May be adjusted, as far as is consistent with precision and detection limits
Column Temperature	±10°C	±10°C
Mobile phase pH	±0.2 units	±0.2 units
Salt Concentration	within ±10% if the permitted pH variation is met	within ±10% if the permitted pH variation is met
Ratio of Components in Mobile Phase	Minor component (≤50%): ±30% relative, but cannot exceed ±10% absolute; may only adjust 1 minor component in ternary mixtures	No Changes allowed * * Not specified in <621>, assume no changes are allowed
Wavelength of UV-Visible Detector	No changes allowed	No changes allowed

What Are the Column Options?

- Smaller particle size
  - Higher efficiency -> shorter column -> faster method
  - Increase resolution
  - Better sensitivity
  - Consider pressure limit of instrument
- Smaller diameter
  - Solvent savings
  - Depends on instrument configuration and plumbing
- Bonded phase
  - Match USP designation
  - More robust column life
  - Consider a different bonded phase?

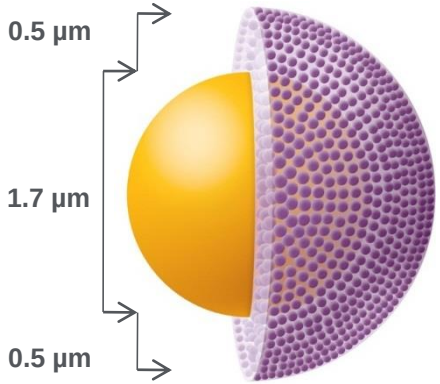


Agilent InfinityLab Poroshell 120 Particle Sizes



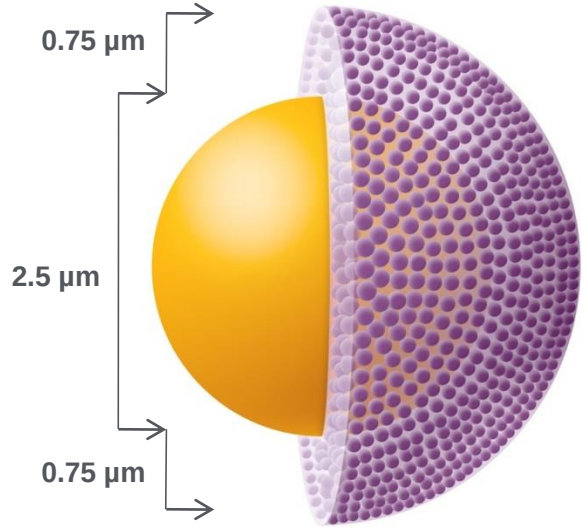
InfinityLab Poroshell 120
1.9 µm

Highest UHPLC performance



InfinityLab Poroshell 120
2.7 µm

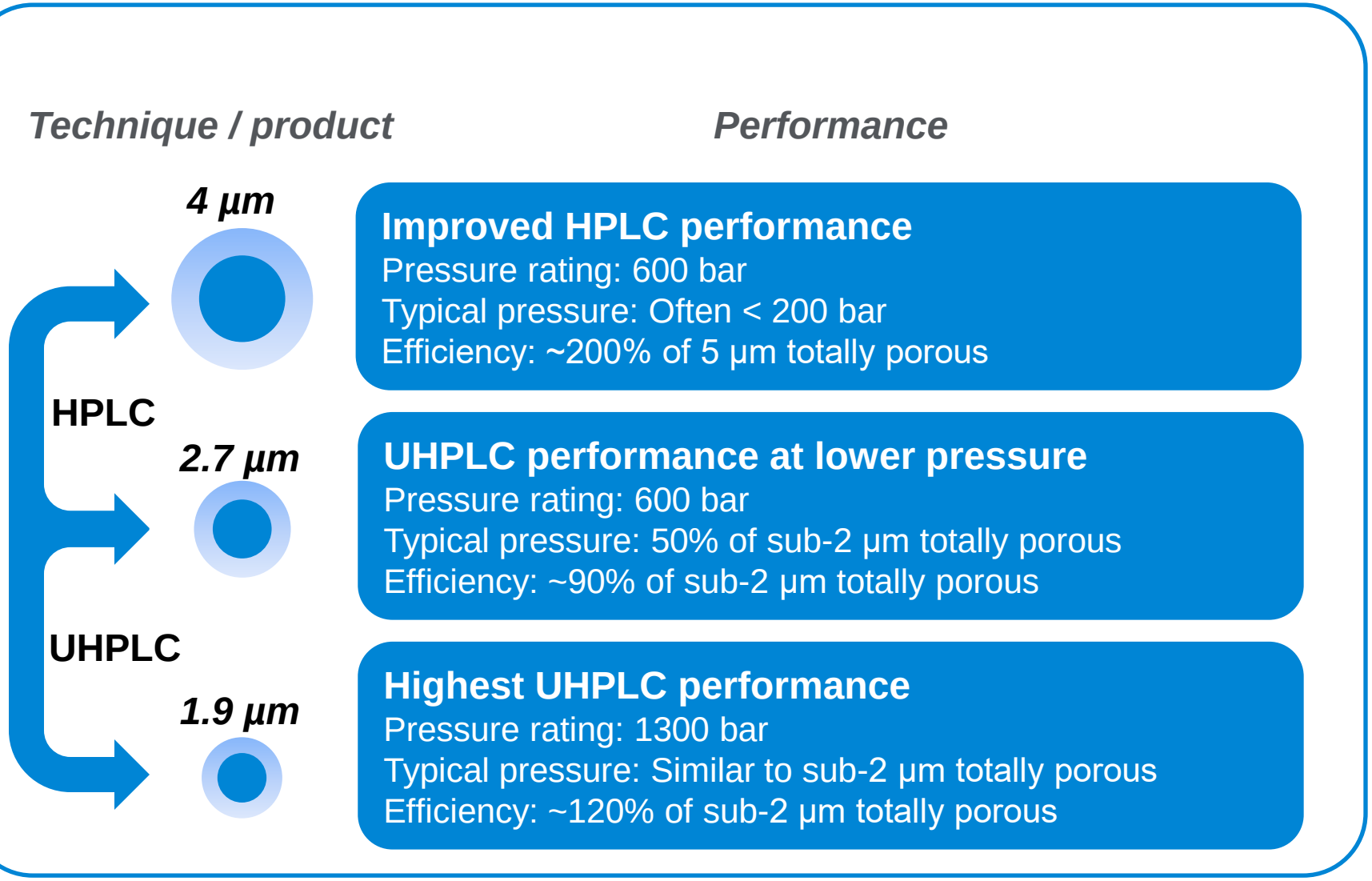
UHPLC performance at lower pressure



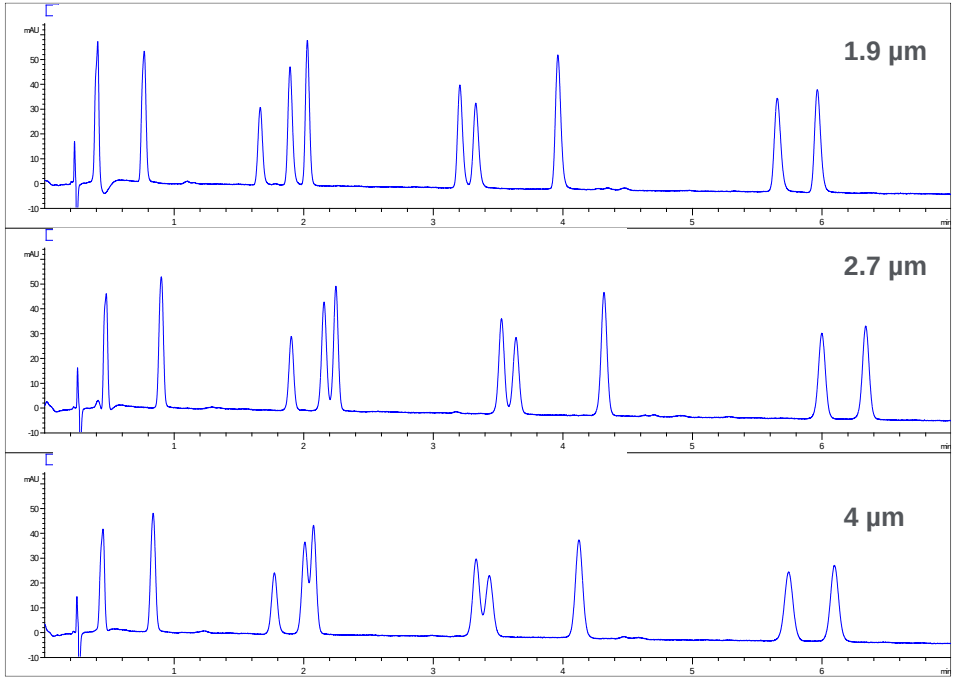
InfinityLab Poroshell 120
4 µm

Improved HPLC performance

Particle Size: When to Use What Size



Decreasing Particle Size Increases Efficiency



Columns:
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 1.9 um
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 2.7 um
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 4 um
Mobile phase A: 0.2% formic acid in water
Mobile phase B: Acetonitrile
Gradient: 5-16% B in 7 min
Flow rate: 0.5 mL/min
Detection: 240 nm @ 80 Hz
Sample: 1 µL of 0.06 mg/mL each of gallic acid, gallocatechin, epigallocatechin, catechin, caffeine, epicatechin, epigallocatechin gallate, gallocatechin gallate, epicatechin gallate, catechin gallate

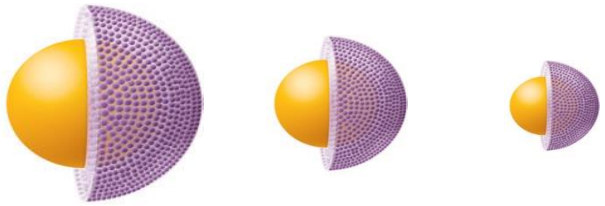
Particle	Pressure	R_{smin}
1.9 µm	226 bar	2.2
2.7 µm	131 bar	1.3
4 µm	53 bar	0.7

- Higher N improves resolution as particle size is decreased

$$N \propto \frac{L}{d_p}$$

Agilent InfinityLab Poroshell 120 phases

Best all around	Best for low pH mobile phases	Best for high and mid pH mobile phases	Best for alternative selectivity	Best for more polar compounds	HILIC for polar compounds	Chiral phases
Poroshell 120 **EC-C18 (L1)** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-C18 (L1)** 2.7 µm	Poroshell 120 **HPH-C18 (L1)** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **Phenyl-Hexyl (L11)** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-Aq (L96)** 2.7 µm	Poroshell 120 **HILIC (L43)** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **Chiral-CF** 2.7 µm
Poroshell 120 **EC-C8 (L7)** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **SB-C8 (L7)** 2.7 µm	Poroshell 120 **HPH-C8 (L7)** 2.7 µm, 4 µm	Poroshell 120 **Bonus-RP (L60)** 1.9 µm, 2.7 µm, 4 µm	Poroshell 120 **EC-CN (L10)** 2.7 µm	Poroshell 120 **HILIC-Z** 2.7 µm	Poroshell 120 **Chiral-CD** 2.7 µm
			Poroshell 120 **PFP (L43)** 2.7 µm		Poroshell 120 **HILIC-OH5** 2.7 µm	Poroshell 120 **Chiral-V** 2.7 µm
						Poroshell 120 **Chiral-T** 2.7 µm



4 µm **2.7 µm** **1.9 µm**

Benefits of Transferring to Agilent InfinityLab 120 Poroshell

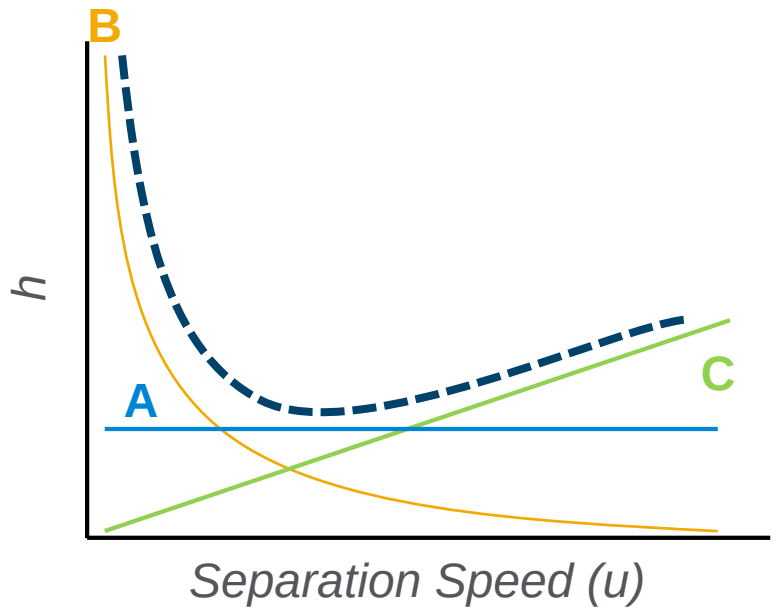
- Superficially porous particles
  - Can run at high speed without sacrificing resolution
  - Existing instruments can turn over more samples per hour
  - 30-50% increase in efficiency near the backpressure of a 3.5-5.0 µm particle
  - Boosts the sample throughput on low-pressure instruments

- Short column lengths (or narrow column diameters)
  - Cut solvent flow by 50-80%
  - Reducing solvent consumption
  - Cuts purchase and disposal costs

Van Deemter Equation

$$h = L/N$$

$$h = A + B/u + C \cdot u$$



- **A term: eddy diffusion and flow distribution**
  - Particle size & packing quality important
  - Narrow particle size distribution
- **B term: longitudinal diffusion**
  - Diffusion in the mobile phase
- **C term: mass transfer**
  - shorter diffusion paths
  - better with superficially porous particles
  - more effect on large molecules
- ***u*: linear velocity**
  - velocity of mobile phase through column
  - $u = L/t_0$ in cm/sec

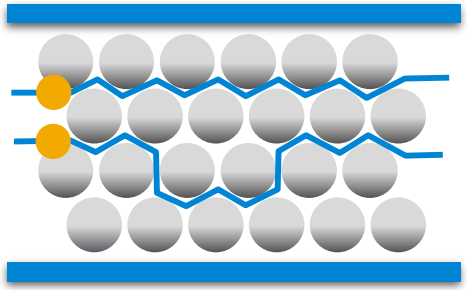
Lower *h (reduced plate height)* = higher efficiency

Van Deemter Equation

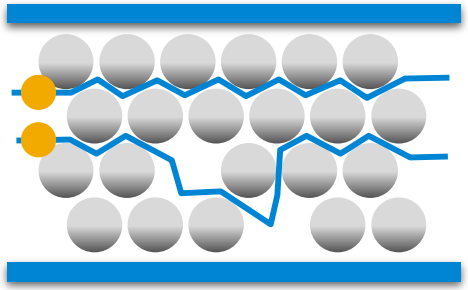
Eddy diffusion

$w_{eddy} \sim \lambda\, d_p$ λ: Quality of column packing

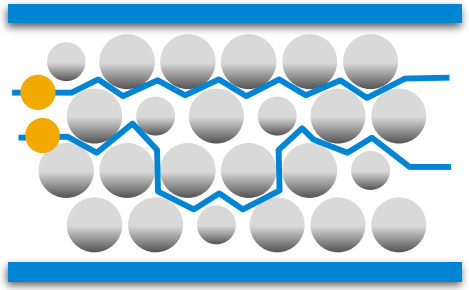
Differences in diffusion paths due to:



Different paths



Poor column packing



Broad particle size distribution

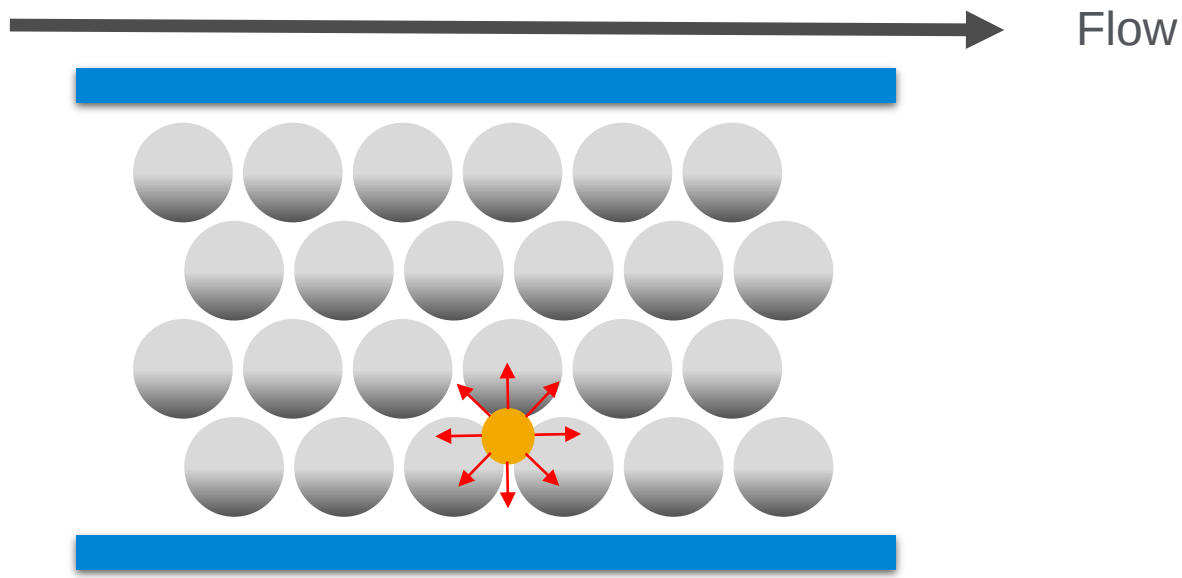
Van Deemter Equation

Axial or longitudinal diffusion

Increase in peak width due to self-diffusion of the analyte

At low flow the analyte remains in the mobile phase for a long time

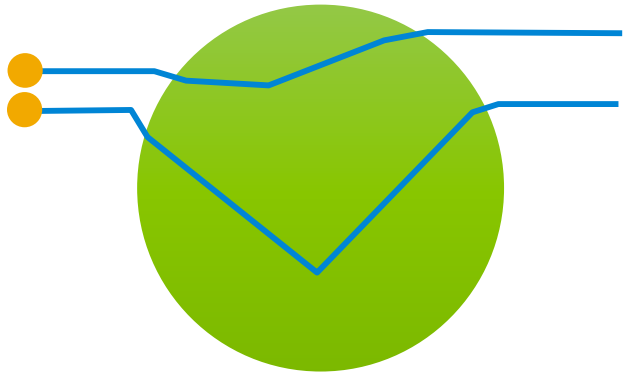
- High increase in peak width
- Increased height of a theoretical plate



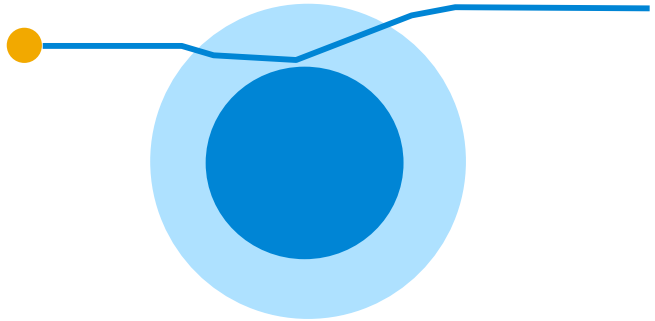
Van Deemter Equation

Resistance to mass transfer

$$w_C \sim d_p^2$$



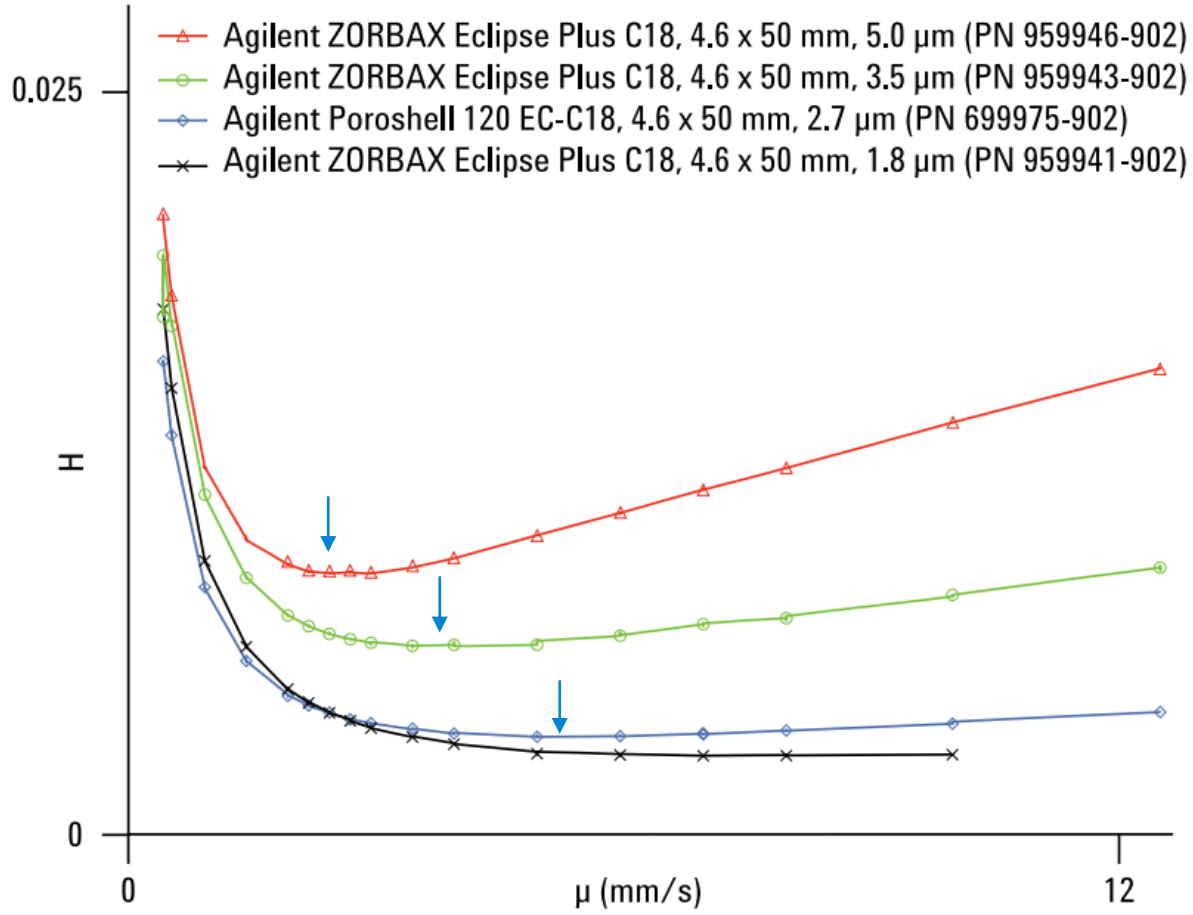
Different diffusion paths



Totally porous particle (TPP)

Superficially porous particle (SPP)

Van Deemter Curves

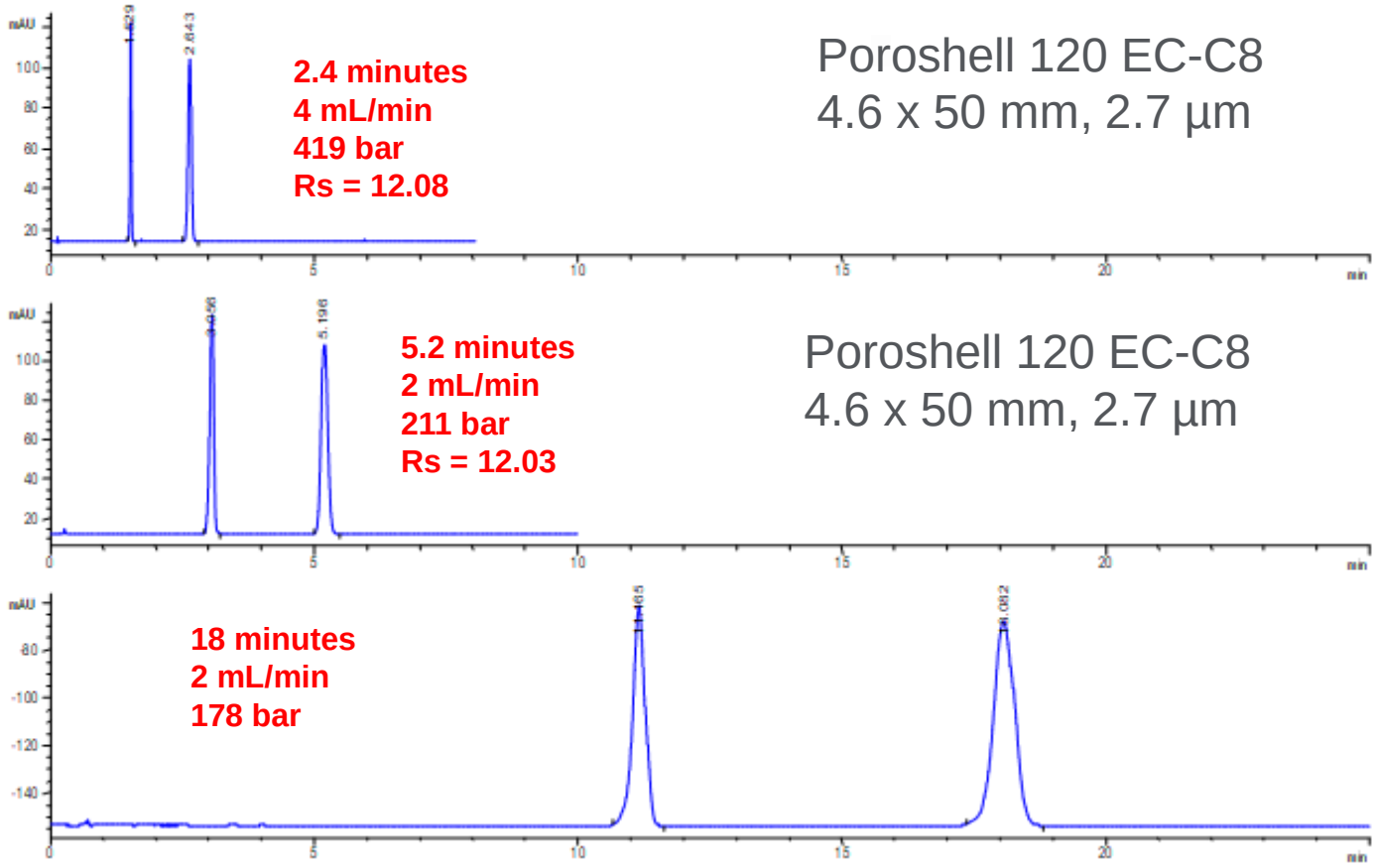


Optimize your flow rate for Agilent InfinityLab Poroshell 120:

- For 2.1 mm id, we suggest 0.42 mL/min
- For 3.0 mm id, we suggest 0.85 mL/min
- For 4.6 mm id, we suggest 2 mL/min

Overlay of van Deemter plots: the optimal flow rate for Agilent InfinityLab Poroshell 120 is faster than for 5 or 3.5 µm columns

Faster Ibuprofen Analysis on Agilent InfinityLab Poroshell 120 EC-C8 4.6 x 50 mm, 2.7 µm



USP Ibuprofen Oral Suspension (L7)
630 mL (0.7 mL/L phosphoric acid in water
370 mL acetonitrile
Benzophenone / ibuprofen (0.48 mg/mL)
Agilent 1260, 220 nm
1.7 µL on 4.6 x 50 mm
5 µL on 4.6 x 150 mm

"Transfer and Optimization of HPLC Methods to Superficially Porous UHPLC" Pittcon 2012, W. Long, A Brooks Oral Presentation 420-3.

Transfer of Isocratic USP Methods (Naproxen)

Parameters for System Suitability		
	Isocratic	Gradient
Particle Size	L/dp: -25% to +50% or N: -25% to +50%	No Changes allowed
Column Length		

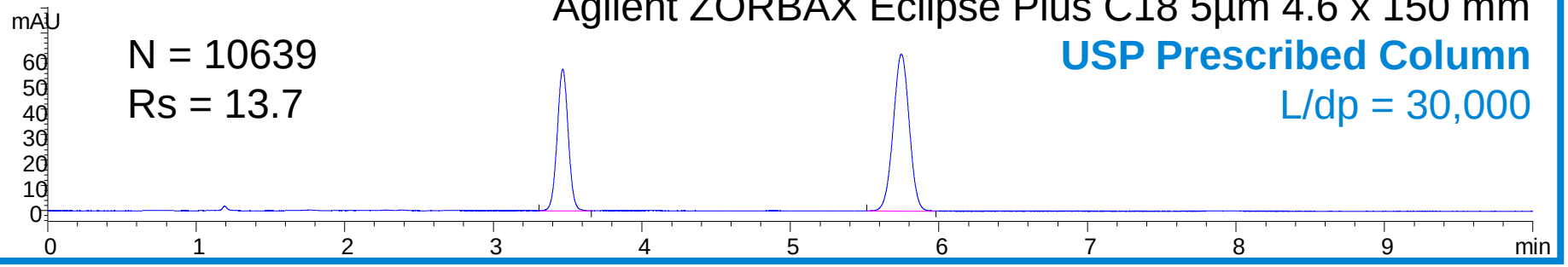
L (mm)	dp (µm)	L/dp	%	N	%	<621> compliant
150	5	30,000	100%	10,639	100%	Yes
150	4	37,500	125%	19,054	179%	Yes
100	4	25,000	83%	13,186	124%	Yes
100	2.7	37,037	123%	21,046	198%	Yes
50	2.7	18,519	62%	11,281	106%	Yes

Scaling USP Naproxen Method from a 5 µm TPP to 4 µm SPP

System Suitability Method Requirement: N > 4000, Rs > 11.5

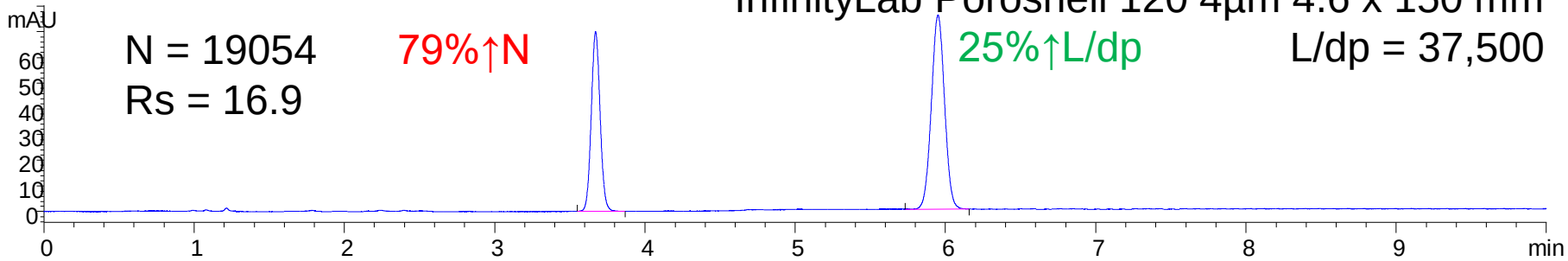
Agilent ZORBAX Eclipse Plus C18 5µm 4.6 x 150 mm
USP Prescribed Column
L/dp = 30,000

N = 10639
Rs = 13.7



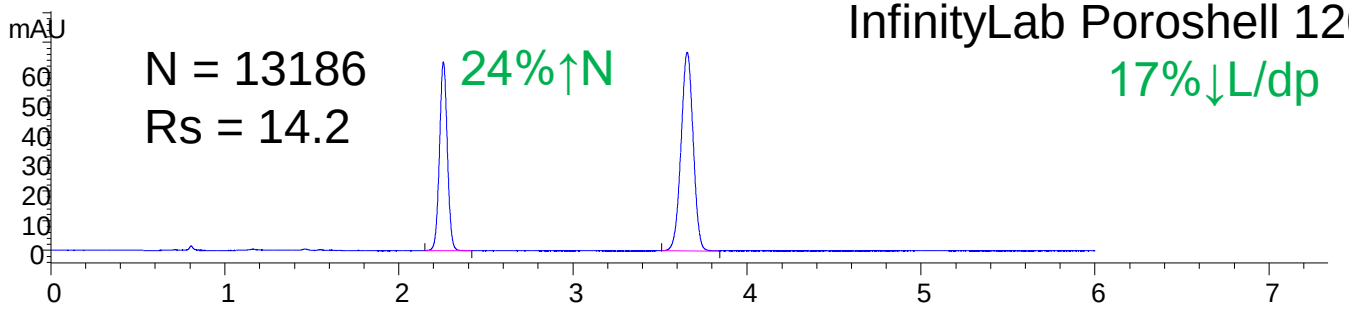
InfinityLab Poroshell 120 4µm 4.6 x 150 mm
L/dp = 37,500
25%↑L/dp

N = 19054
Rs = 16.9
79%↑N



InfinityLab Poroshell 120 4µm 4.6 x 100 mm
L/dp = 25,000
17%↓L/dp

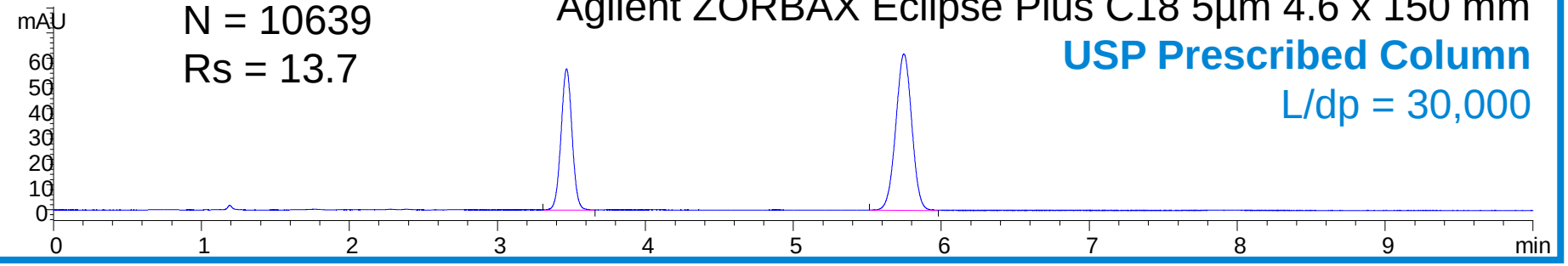
N = 13186
Rs = 14.2
24%↑N



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

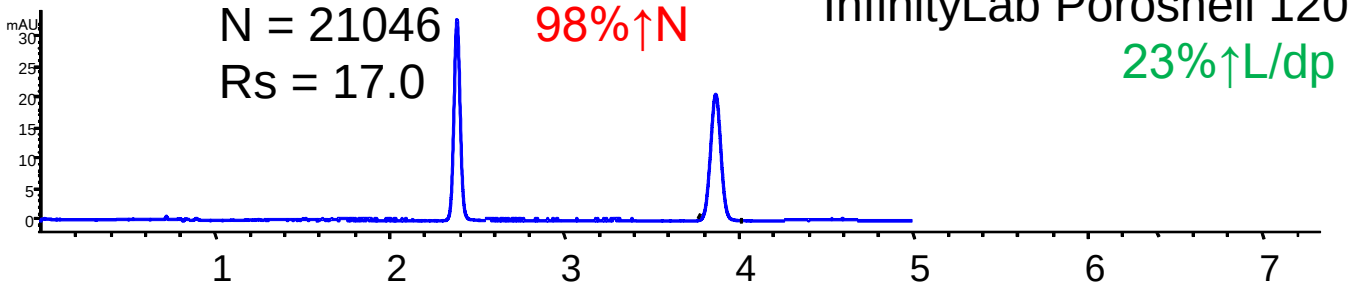
Scaling USP Naproxen Method from a 5 µm TPP to **2.7 µm SPP**

System Suitability Method Requirement: N > 4000, Rs > 11.5



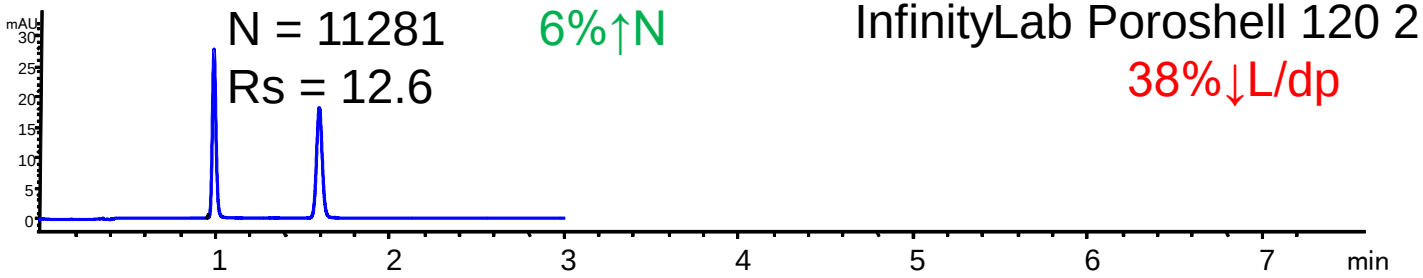
N = 10639
Rs = 13.7

Agilent ZORBAX Eclipse Plus C18 5µm 4.6 x 150 mm
USP Prescribed Column
L/dp = 30,000



N = 21046
Rs = 17.0
98%↑N

InfinityLab Poroshell 120 2.7µm 4.6 x 100 mm
23%↑L/dp
L/dp = 37,037



N = 11281
Rs = 12.6
6%↑N

InfinityLab Poroshell 120 2.7µm 4.6 x 50 mm
38%↓L/dp
L/dp = 18,518

Mobile phase: 50:49:1 MeCN:H2O Acetic Acid
Flow pate: 1.2 mL/min, **All Pressures < 300 bar**
Peak 1. Naproxen 2. Butyrophenone

Retention Factor - Gradients

$$k^* = \frac{t_G \cdot F}{S \cdot \Delta\Phi \cdot V_m}$$

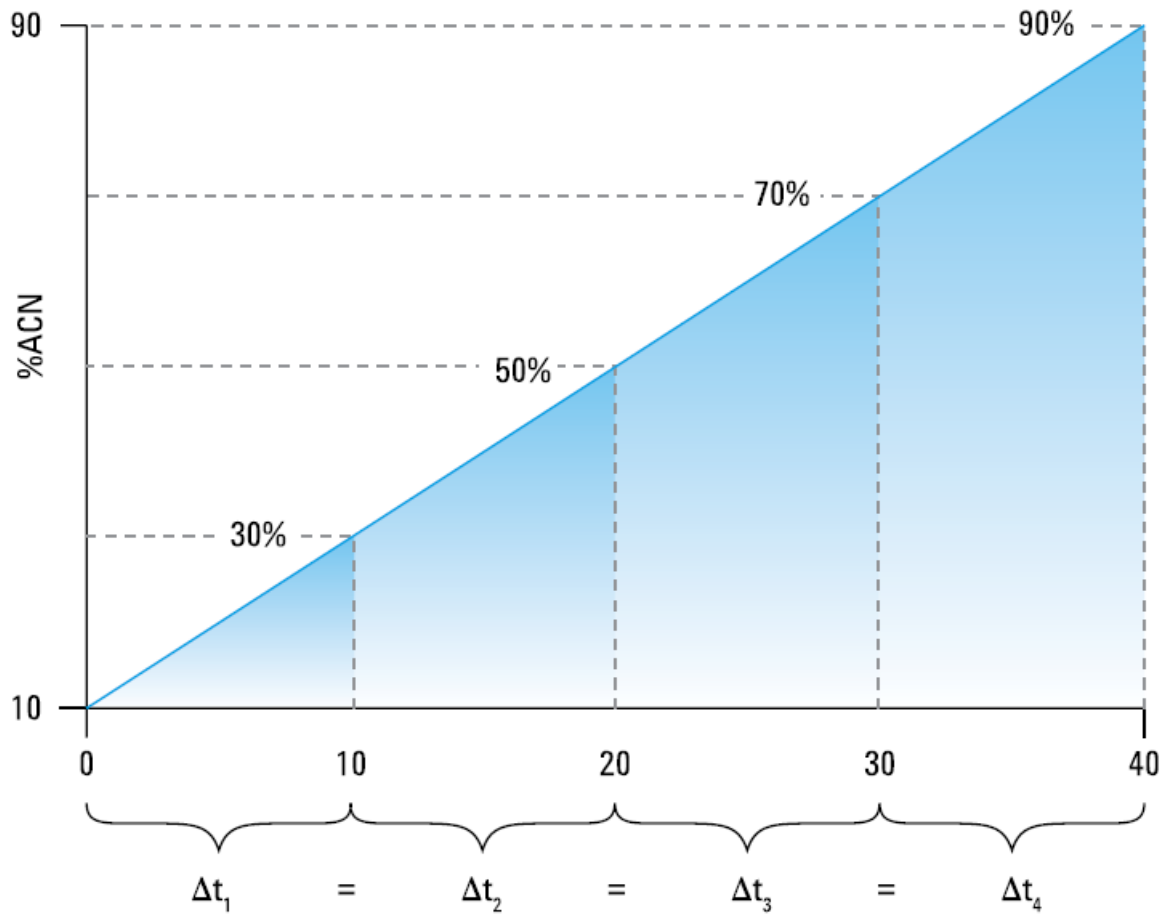
With gradient separations, the retention factor is influenced by

- F = flow rate
- t_G = gradient time (minutes)
- ΔΦ = change in volume fraction of B mobile phase
- V_m = column volume
- S = constant (4 - 6 for small molecules, 10 - 1000 for peptides and proteins)

To keep the retention factor constant, changes in the denominator need to be offset by proportional changes in the numerator, and vice versa.

Retention Factor - Gradients

$$k^* = \frac{t_G \cdot F}{S \cdot \Delta\Phi \cdot V_m}$$



Increasing the solvent strength = Increasing the % organic in the mobile phase

Linear solvent strength gradient = % per min is a constant

$\Delta\Phi = 80\%$

$t_G = 40$ min

$\frac{\Delta\Phi}{\Delta t_G} = 2\%/\text{min}$

Maintaining k*

Keep relative peak position and shorten analysis

Any decrease in	Can be offset by a proportional
• Column length	• Decrease in t_G or F • Increase in Δ%B
• Column volume (i.d.)	• Decrease in t_G or F • Increase in Δ%B
• Δ%B (same column)	• Decrease in t_G or F

$$k^* \propto \frac{t_G \bullet F}{S \bullet \Delta\Phi \bullet V_m}$$

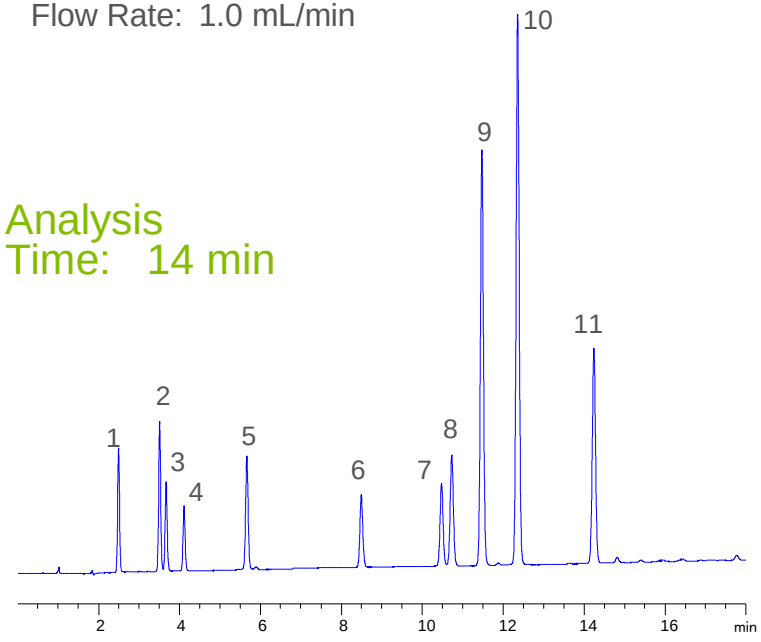
Reduce Analysis Time, Keep Gradient Steepness the Same

Sample: 1. Aldicarb sulfoxide, 2. Oxamyl, 3. Methomyl, 4. Aldicarb sulfone, 5. Carbofuran-3-hydroxy, 6. Aldicarb, 7. Propoxur, 8. Carbofuran, 9. Carbaryl, 10. Methiocarb, 11. 11. ISTD (BDMC)

Column: Agilent ZORBAX Eclipse Plus-C18
4.6 x 150 mm, 5 µm

Gradient Time: 20 min
Flow Rate: 1.0 mL/min

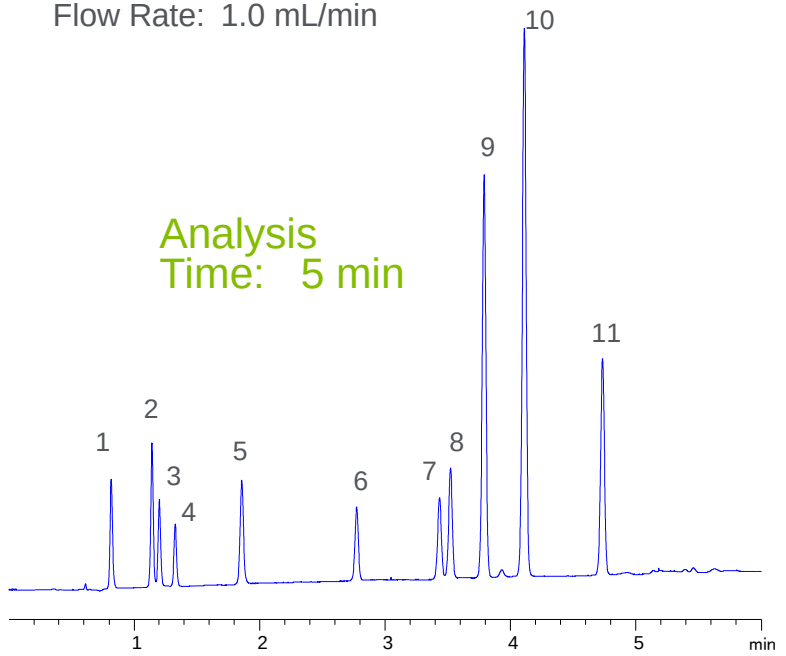
Analysis Time: 14 min



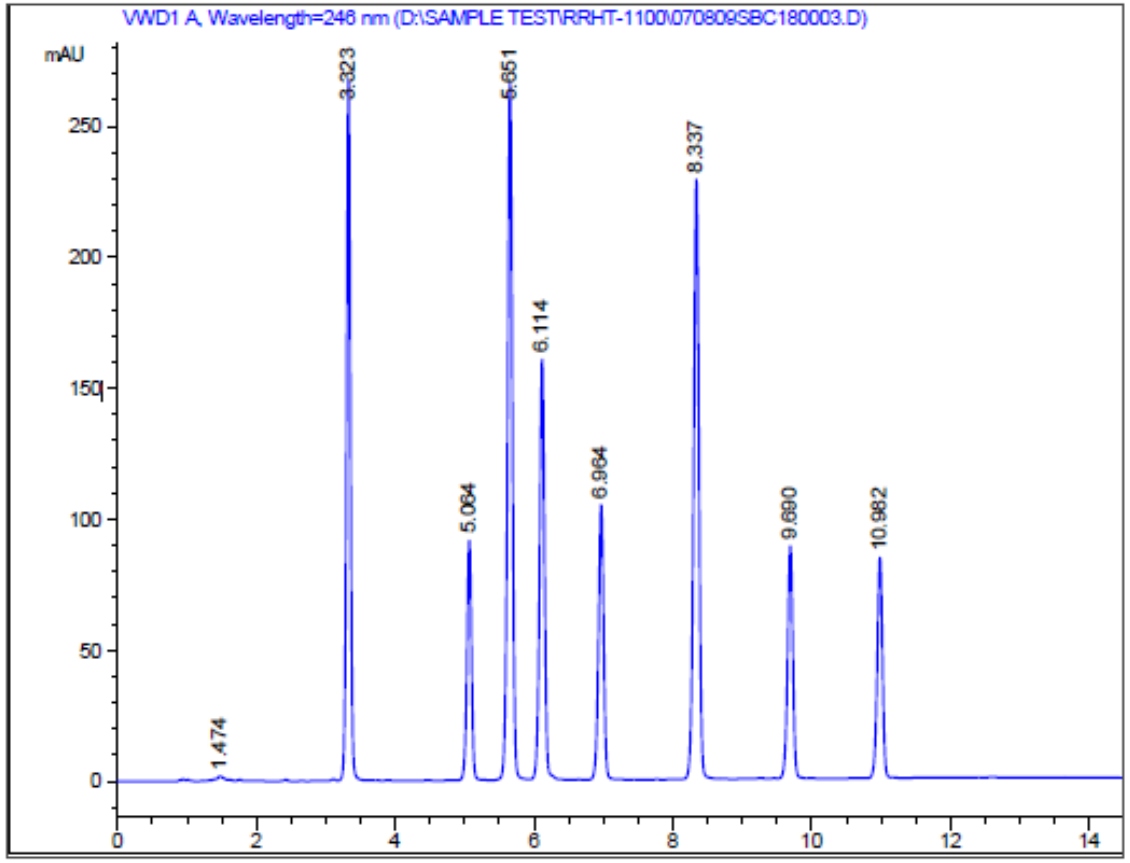
Column: Agilent InfinityLab Poroshell
120 EC-C18 4.6 x 50 mm, 2.7 µm

Gradient Time: 6.7 min
Flow Rate: 1.0 mL/min

Analysis Time: 5 min



Gradient Method Transfer– 4.6 x 150mm, 5 µm Agilent ZORBAX SB-C18

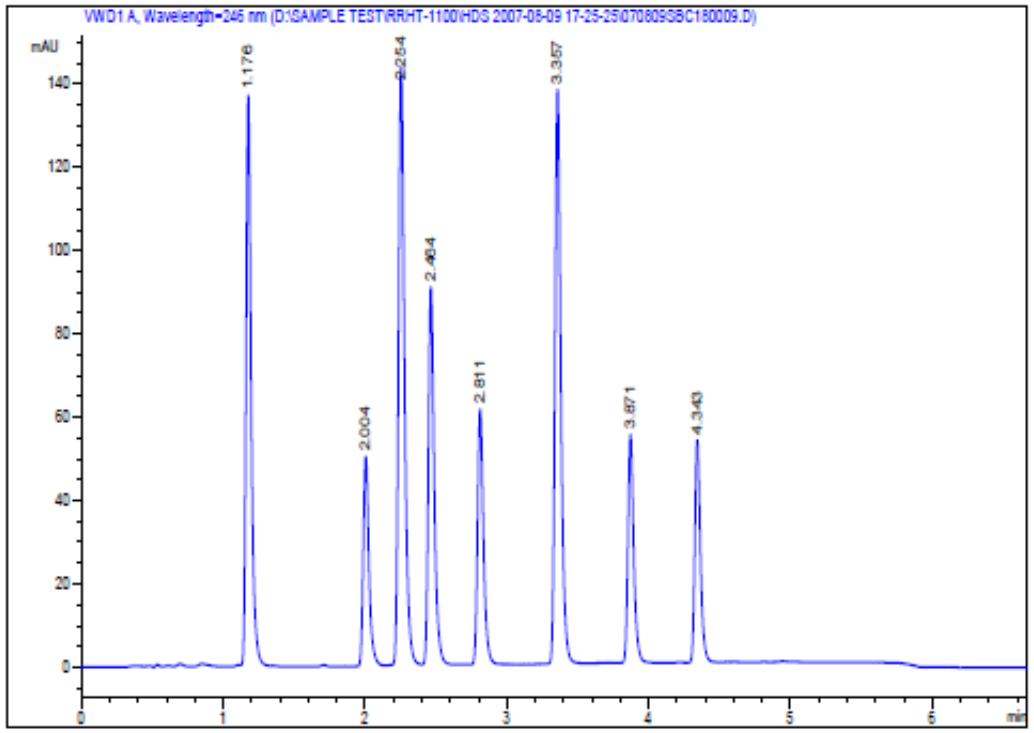


Flow Rate	1.0 ml/min
Injection Volume	15uL
Temperature	30° C
Wavelength	246nm
Sample rate	2.5 Hz

Time (min)	% Acetonitrile
0	50
10	90
13.5	90
13.6	50
15	50

Maintaining Peak Position & Resolution

Have shortened column & gradient time – need to do so by the SAME factor
1/3 column length – 1/3 gradient time
ex: RRHT column – 4.6 x 50 mm, 1.8µm, SB-C18



Flow Rate	1.0 ml/min
Injection Volume	5uL
Temperature	30° C
Wavelength	246nm
Sample rate	*13.74 Hz*

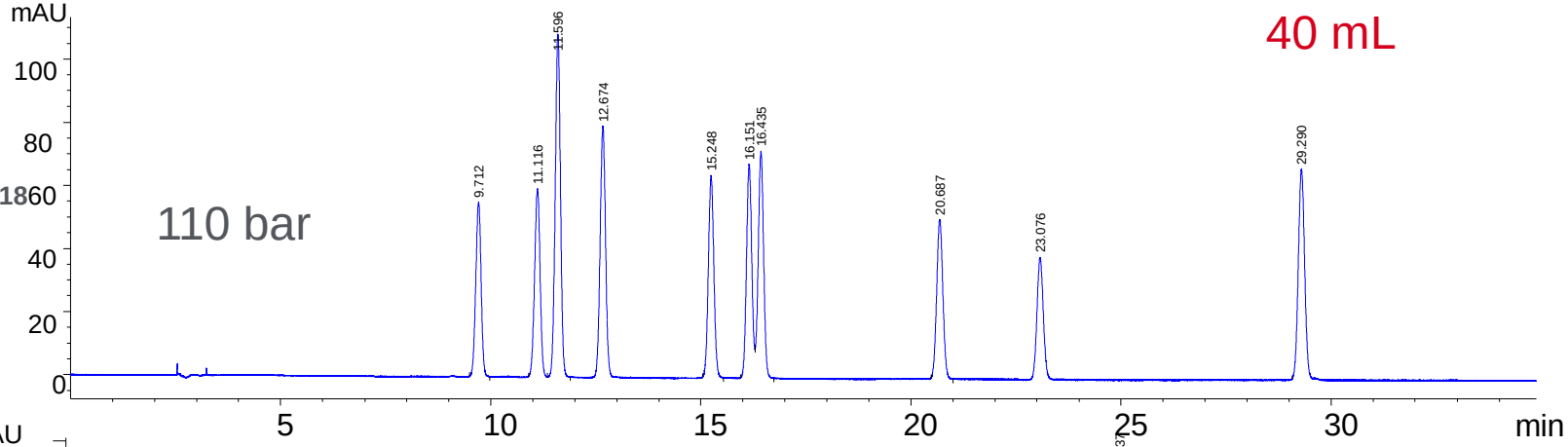
Time (min)	% Acetonitrile
0	50
3.33	90
4.5	90
4.53	50
5	50

Gradient Transfer: 4.6 x 250 mm, 5 µm to 4.6 x 100 mm, 2.7um

Mobile phase:
A: 0.1% formic acid in water
B: 0.1% formic acid in ACN

Time	%B
0	8
33	33
34	33

Column: **Agilent Eclipse Plus C18 4.6 x 250mm, 5 µm**
Flow rate: 1 mL/min

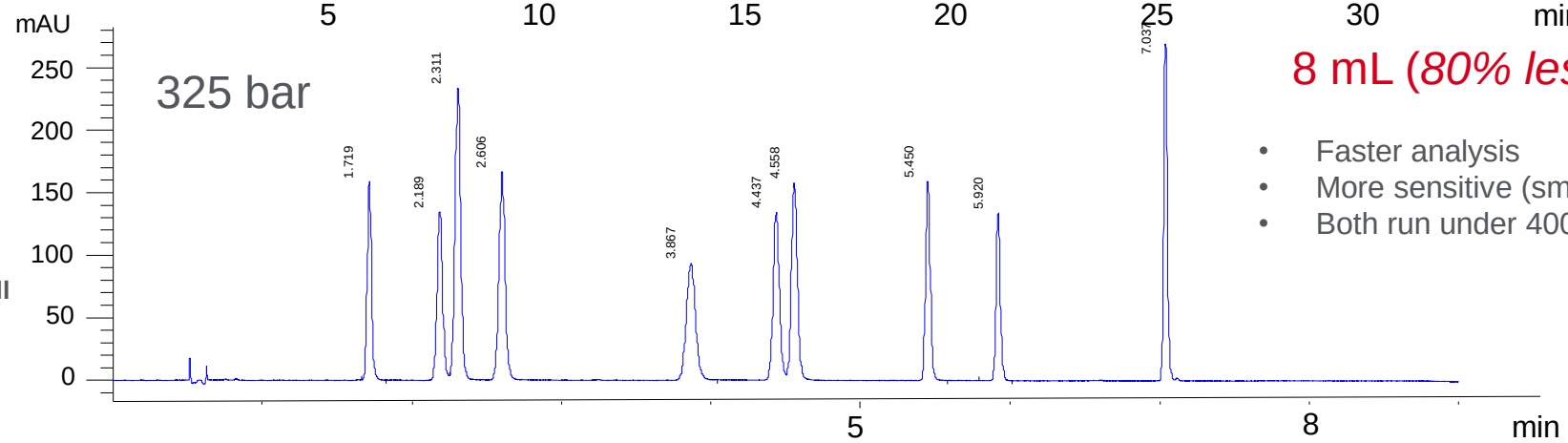


40 mL

Sulfadiazine,
Sulfathiazole
Sulfapyridine
Sulfamerazine,
Sulfamethazine,
Sulfamethazole,
Sulfamethoxypyridazine,
Sulfachloropyridazine
Sulfamethoxazole,
Sulfadimethoxine

Time	%B
0	8
12	33
13.2	33

Column: **4.6 x 100mm Agilent InfinityLab Poroshell 120 EC-C18, 2.7 µm**
Flow rate: 1 mL/min



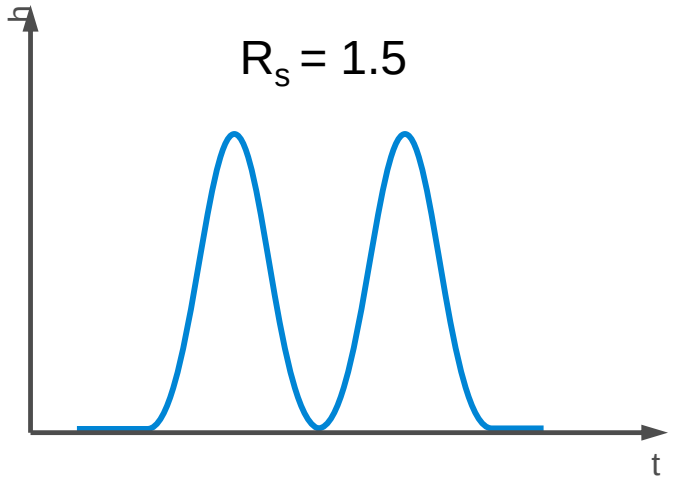
8 mL (*80% less*!)

- Faster analysis
- More sensitive (smaller particle sharpens peak)
- Both run under 400 bar

- Column length decreased from 250 to 100 mm (250 / 100 = 0.4)
- Gradient time points decreased proportionally: 33 * 0.4 = 13.2 (adjusted to ~12 minutes)

Resolution

Baseline separations for rugged methods

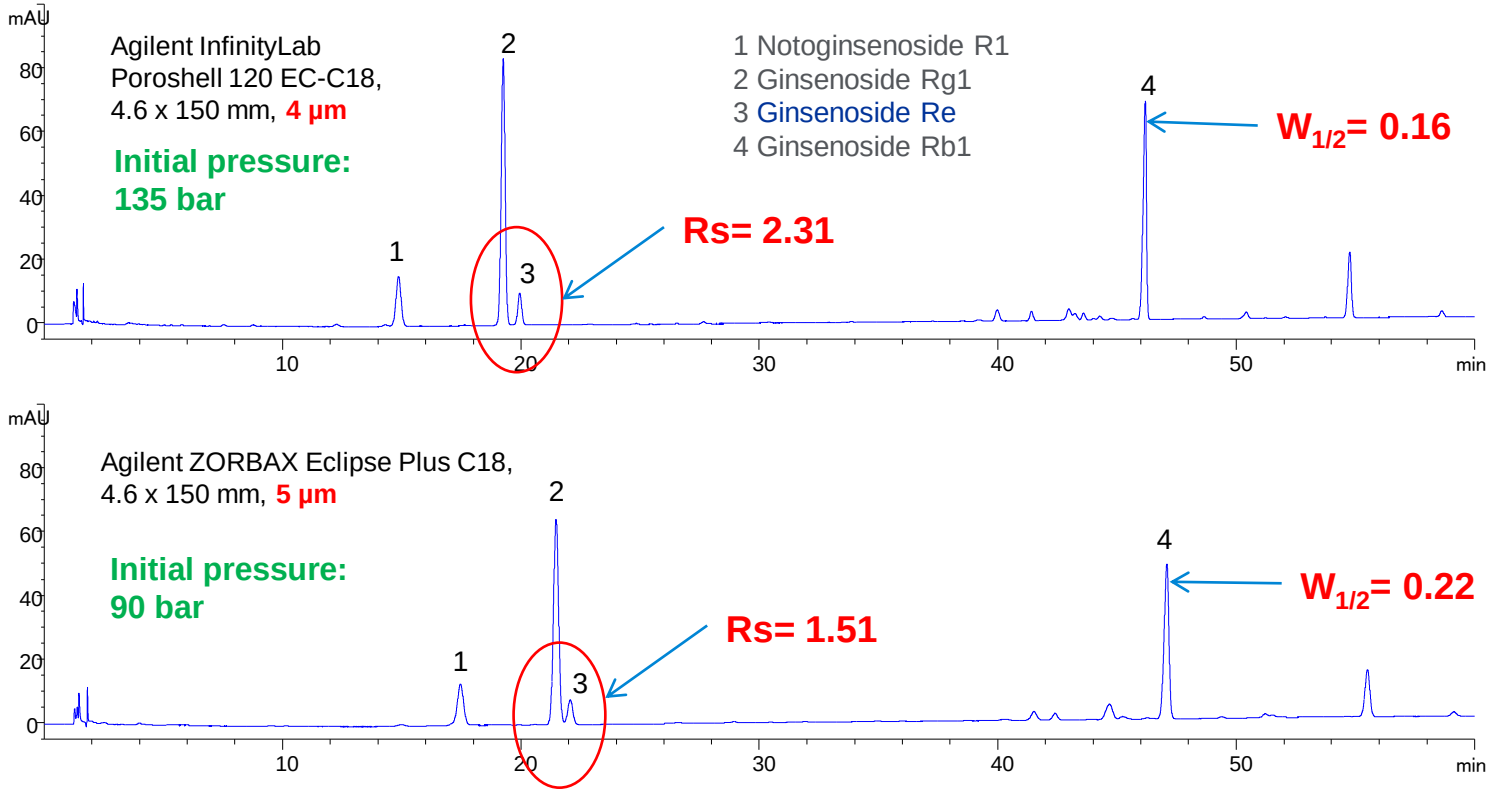


If we consider peaks of equal height:

- 1 - minimum for a measureable separation
- 0.6 - required to discern a valley between two equal-height peaks
- 1.5 - considered to be a baseline separation
- 1.7 or greater - desirable for rugged methods

Notoginseng Analysis: 4 µm Agilent Poroshell and 5 µm Totally Porous

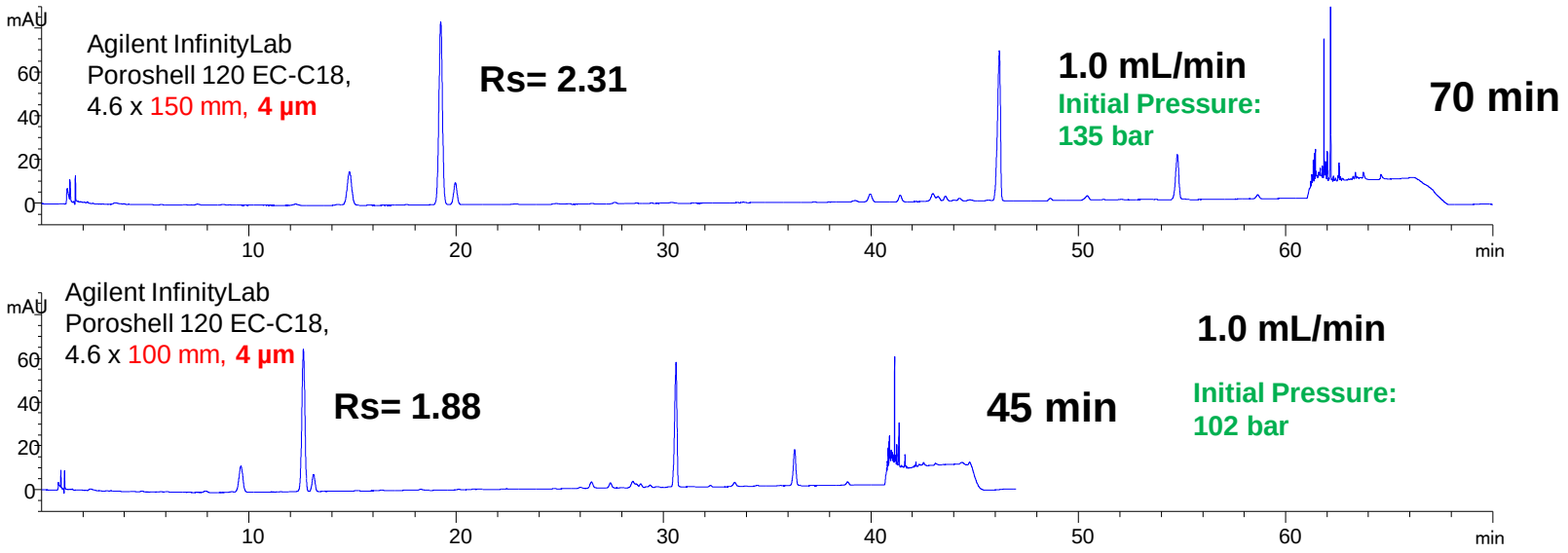
Same dimensions, Same conditions, higher resolution



Mobile phase:
A) water
B) acetonitrile
Gradient for 4.6 × 150 mm:

Time (min)	%A
0	81
12	81
60	64
61	10
65	10
66	81
70	81

Notoginseng Analysis: Speed Optimization



- Column length decreased from 150 to 100 mm (150 / 100 = 0.67)
- Gradient time points decreased proportionally
- 12 * 0.67 = 8 minutes
- 60 * 0.67 = 40 minutes

Gradient for 4.6 × 150 mm:

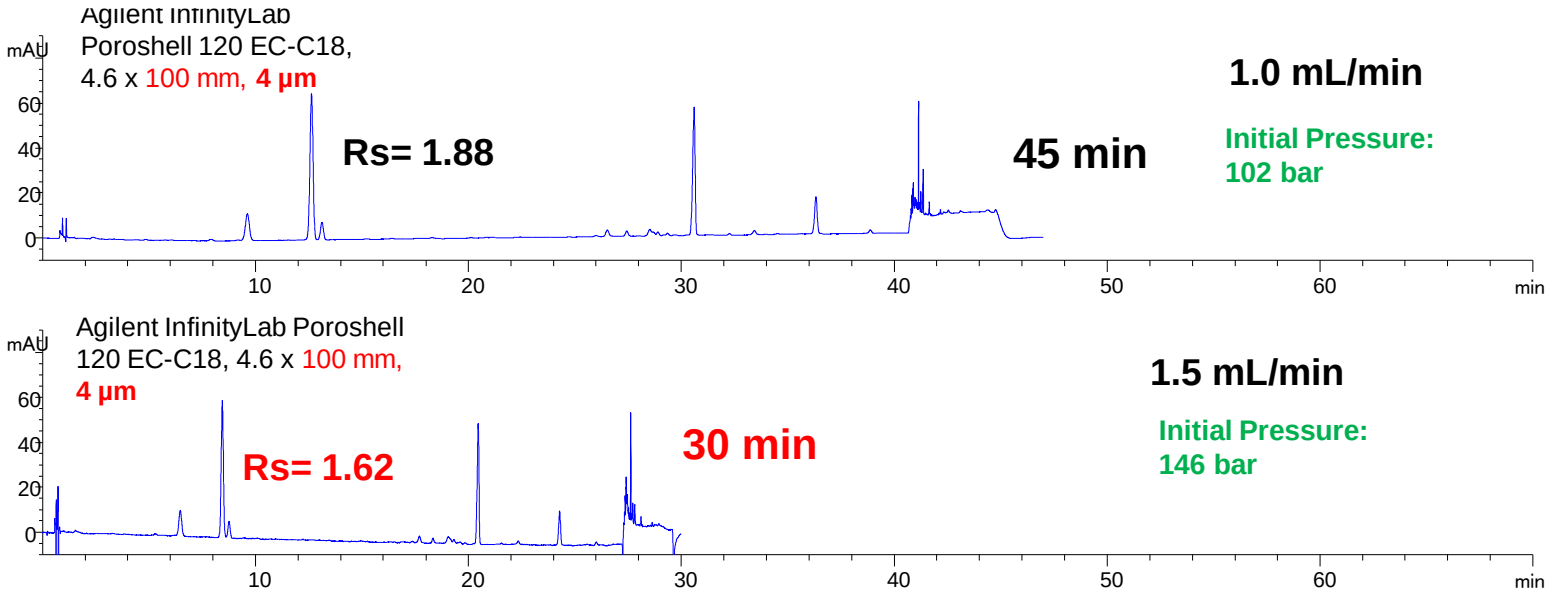
Time (min)	%A
0	81
12	81
60	64
61	10
65	10
66	81
70	81

Gradient for 4.6 × 100 mm:

Time (min)	%A
0	81
8	81
40	64
40.5	10
43.5	10
44	81
47	81

Notoginseng Analysis: Speed Optimization

- Flow rate increased from 1 mL/min to 1.5 mL/min (1.5 / 1.0 = 0.67)
- Gradient time points decreased proportionally
- 45 * 0.67 = 30 minutes

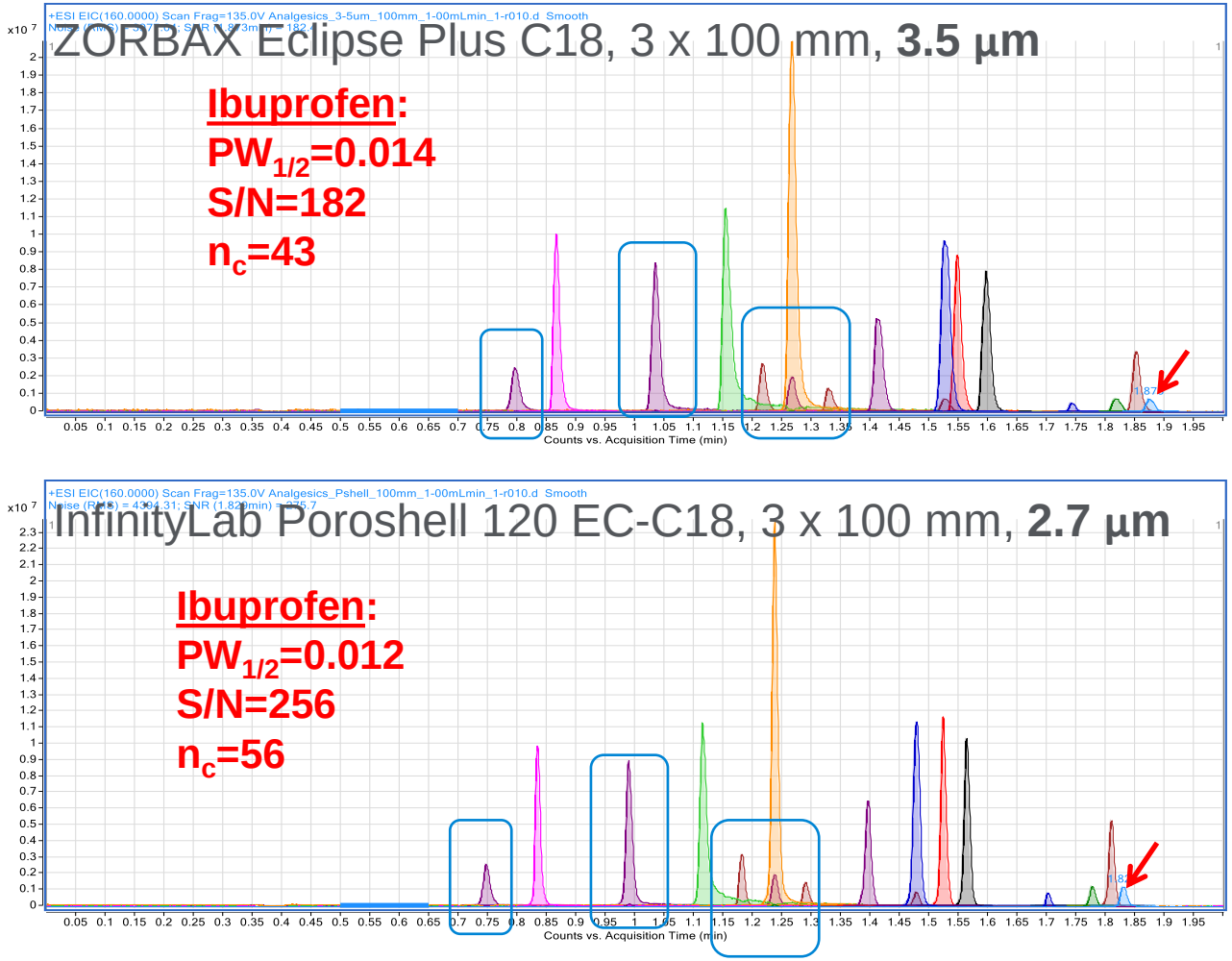


Gradient for 4.6 × 100 mm:

Time (min)	%A
0	81
8	81
40	64
40.5	10
43.5	10
44	81
47	81

Achieving Better Sensitivity

Totally porous 3.5 µm to 2.7 µm superficially porous

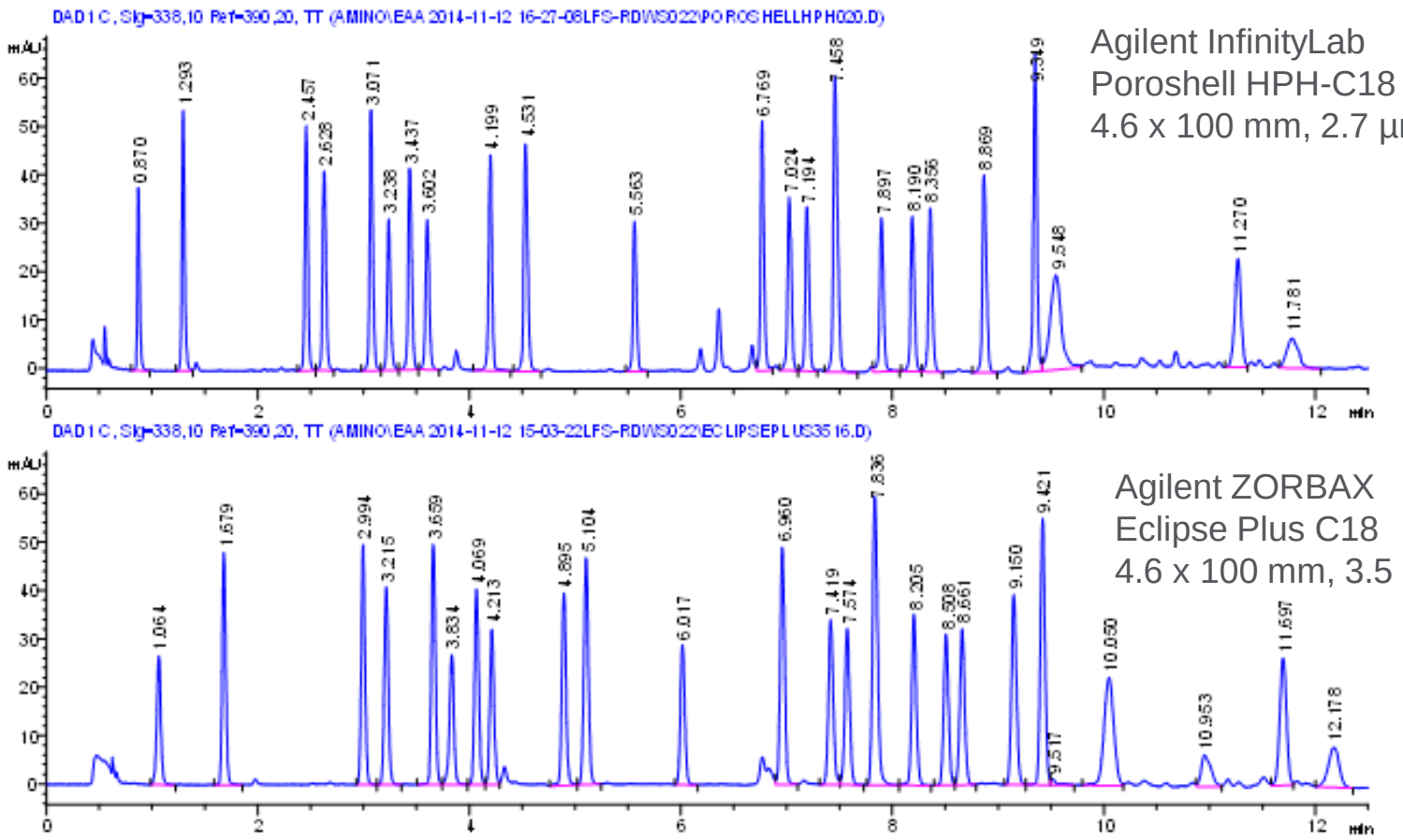


Same method can be used with either column due to **similar selectivity**

2.7 µm columns:

- **Taller, narrower peaks**
- **>40% more sensitivity**, as noted by the S/N of ibuprofen
- Conditional **peak capacity >20% higher** than the 3.5 µm column
- 15 compounds, 2 min

Amino Acid Analysis on Agilent InfinityLab Poroshell 120 HPH-C18



Agilent InfinityLab Poroshell HPH-C18
4.6 x 100 mm, 2.7 µm

Agilent ZORBAX Eclipse Plus C18
4.6 x 100 mm, 3.5 µm

Column: Agilent InfinityLab Poroshell HPH-C18 or Agilent ZORBAX Eclipse Plus C18

Column Temperature: 40 °C

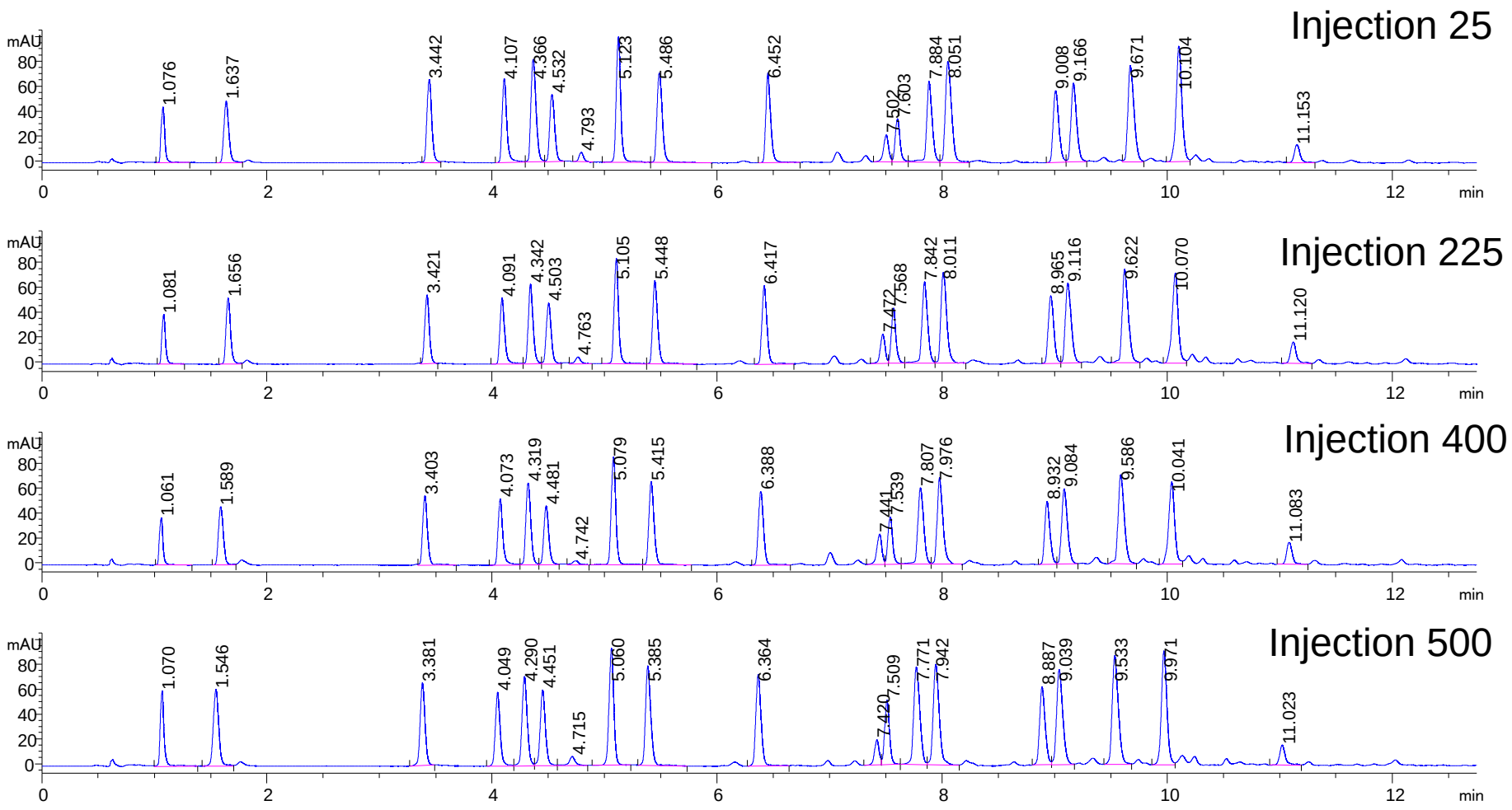
Mobile Phase A: 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 8.2: 5 mM NaN_3

Mobile Phase B: Acetonitrile: Methanol: Water (45:45:10, v: v: v)

Injection Diluent: (0.25 mL H_3PO_4 + 100 mL H_2O)

- Poroshell HPH uses a special coating process
- Particle resists attack at high pH
- Extends column lifetime in alkaline mobile phase

Amino Acid Analysis on HPH-C18 in pH 8.3 Phosphate Buffer

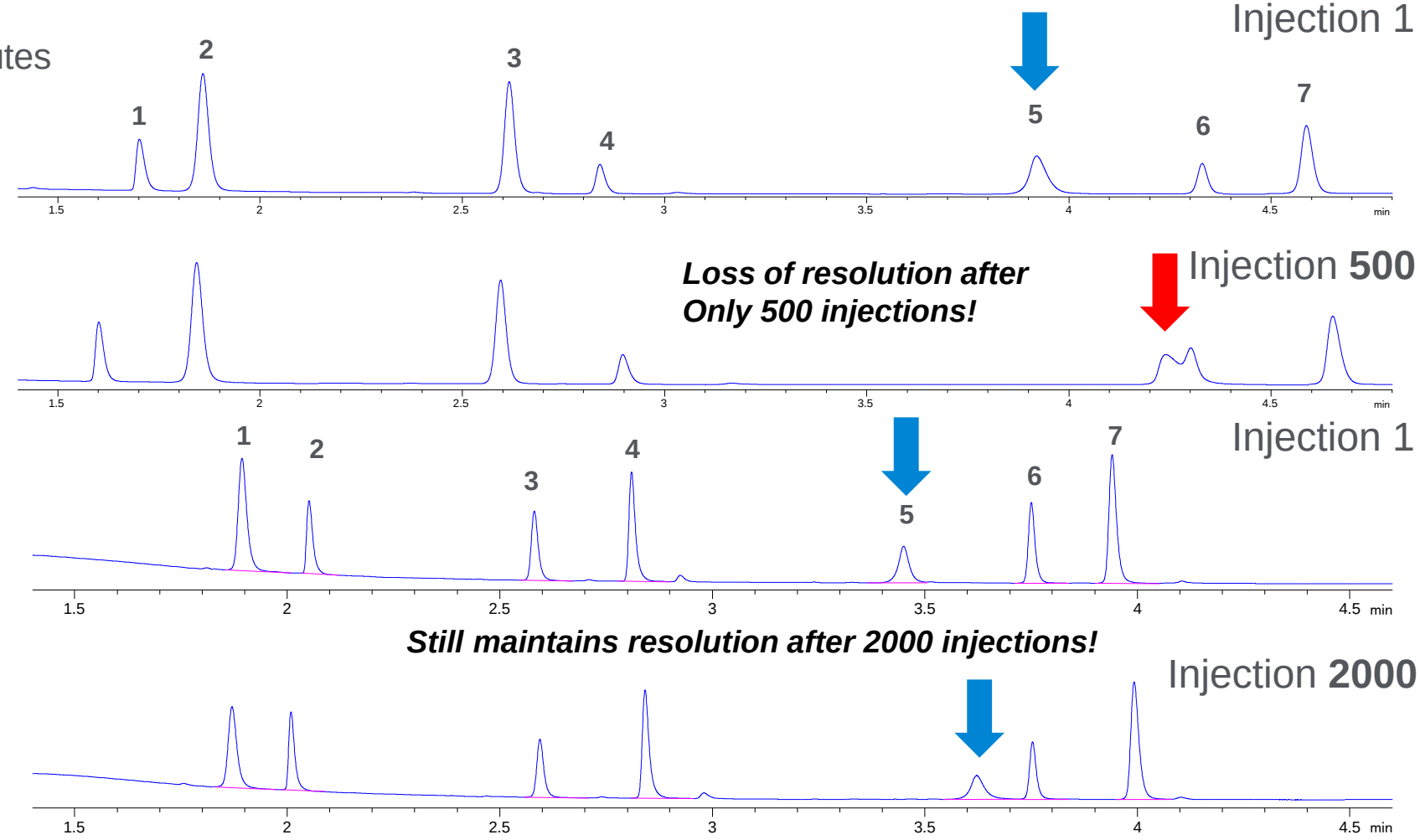


Competitor High pH column vs Agilent InfinityLab Poroshell 120 HPH-C18: Stress Test

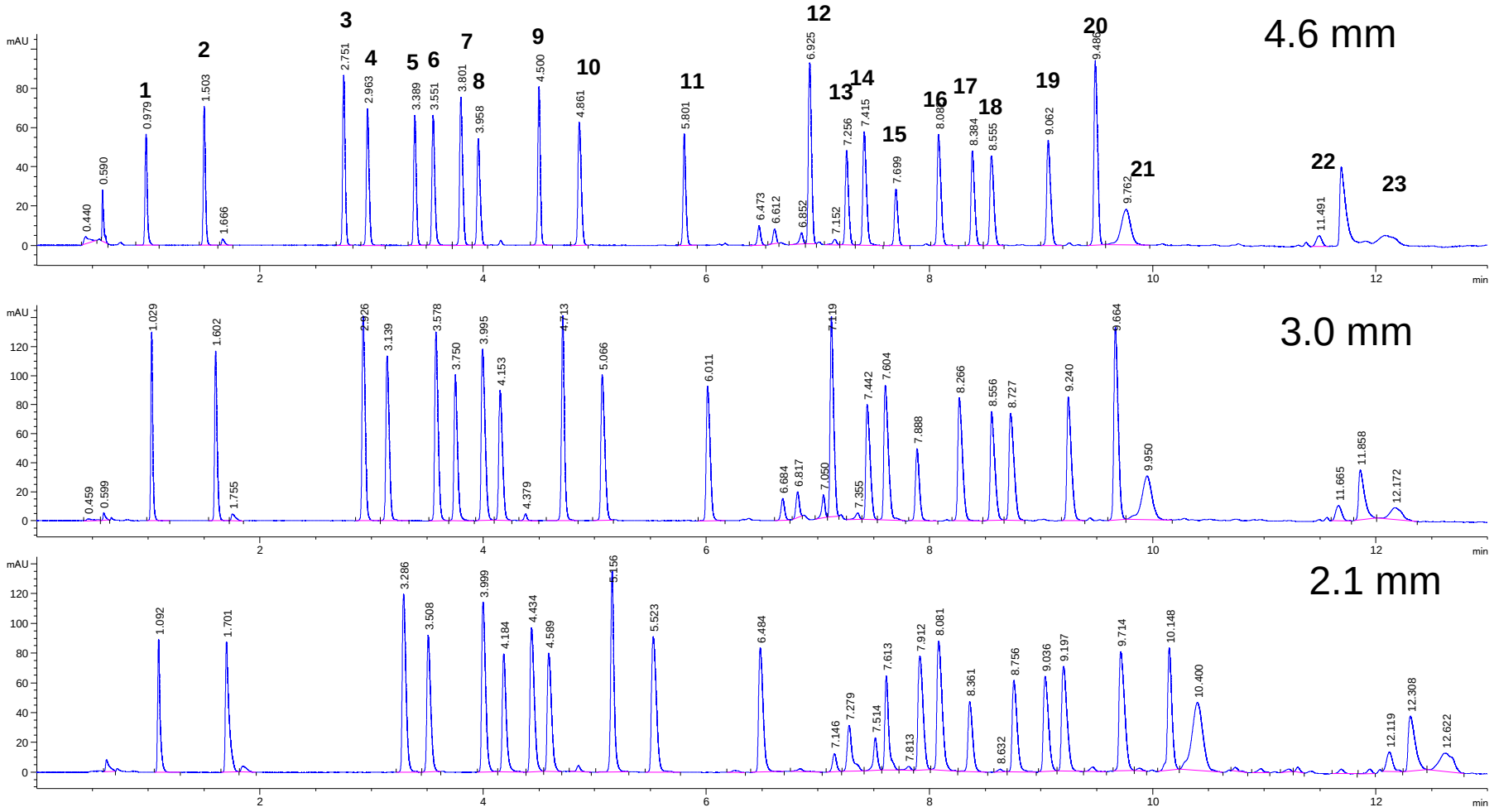
A: 10 mM ammonium bicarbonate, pH 10
B: acetonitrile
5-95%B over 5 minutes

Other column

Agilent InfinityLab
Poroshell
120 HPH-C18
2.1 x 50 mm, 2.7 µm

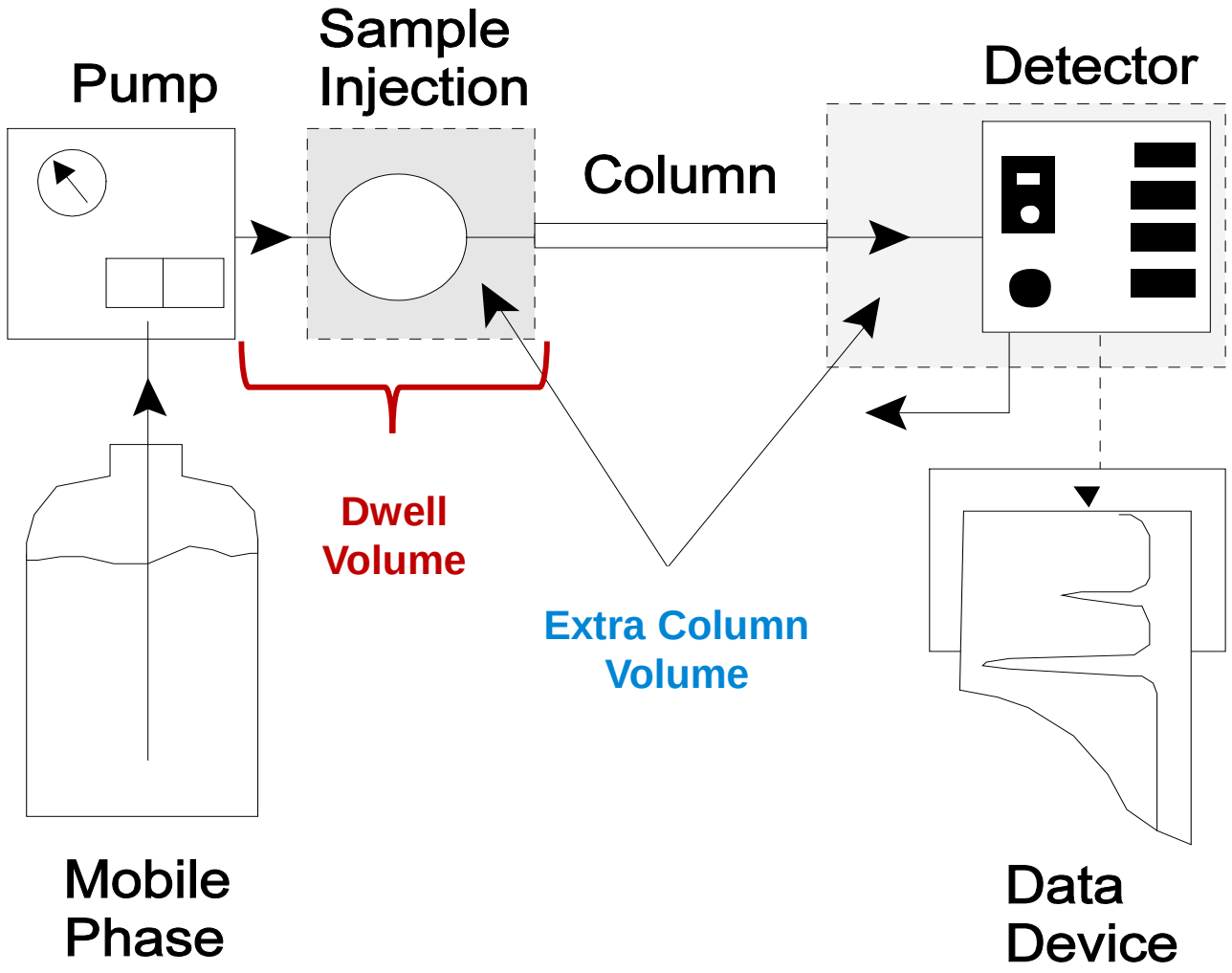


Solvent Use



Amino Acid Analysis on Agilent InfinityLab Poroshell 120, 100 mm, 2.7µm HPH-C18

System Considerations



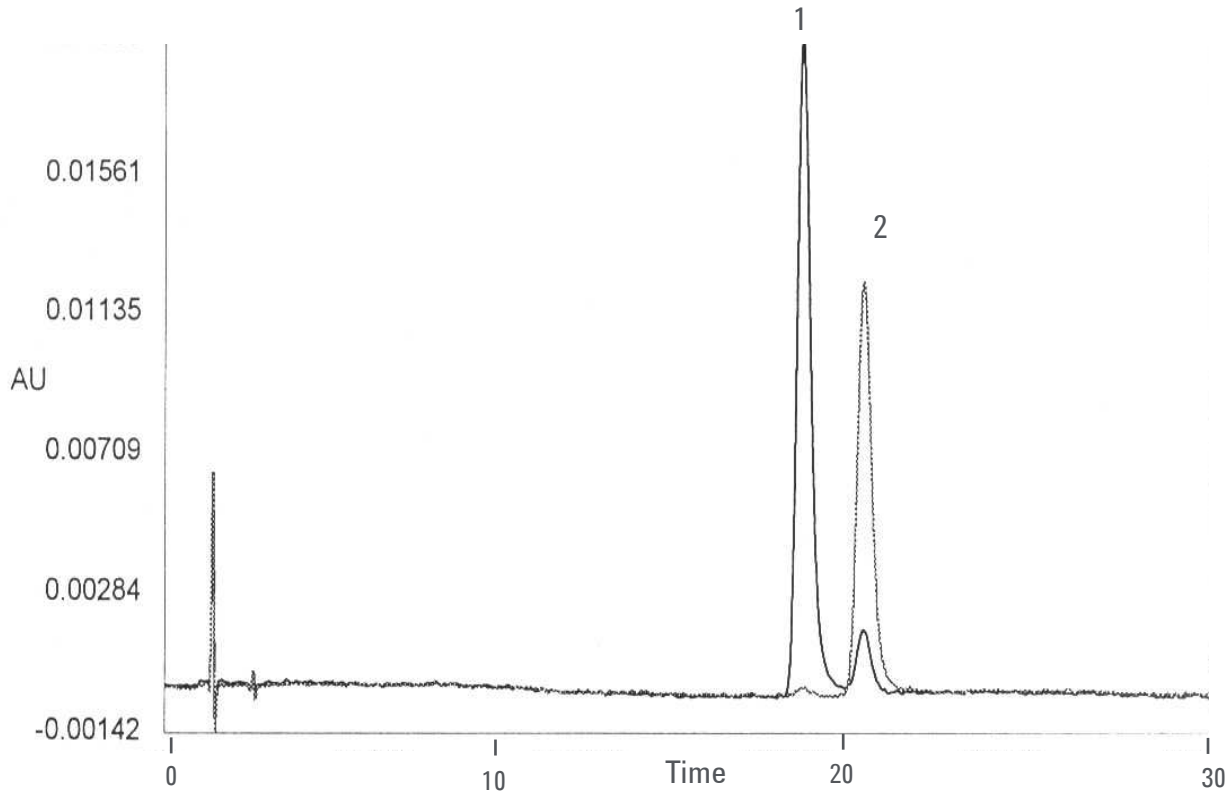
Dwell Volume: from formation of gradient to top of column

-minimize for faster equilibration and more efficient gradient formation

Extra Column Volume from injection to detector (flow cell) outside of the column

-minimize to reduce band broadening, for sharper peaks and better resolution

Paraquat and Diquat on ZORBAX Eclipse XDB-C8 Ion Pairing Conditions



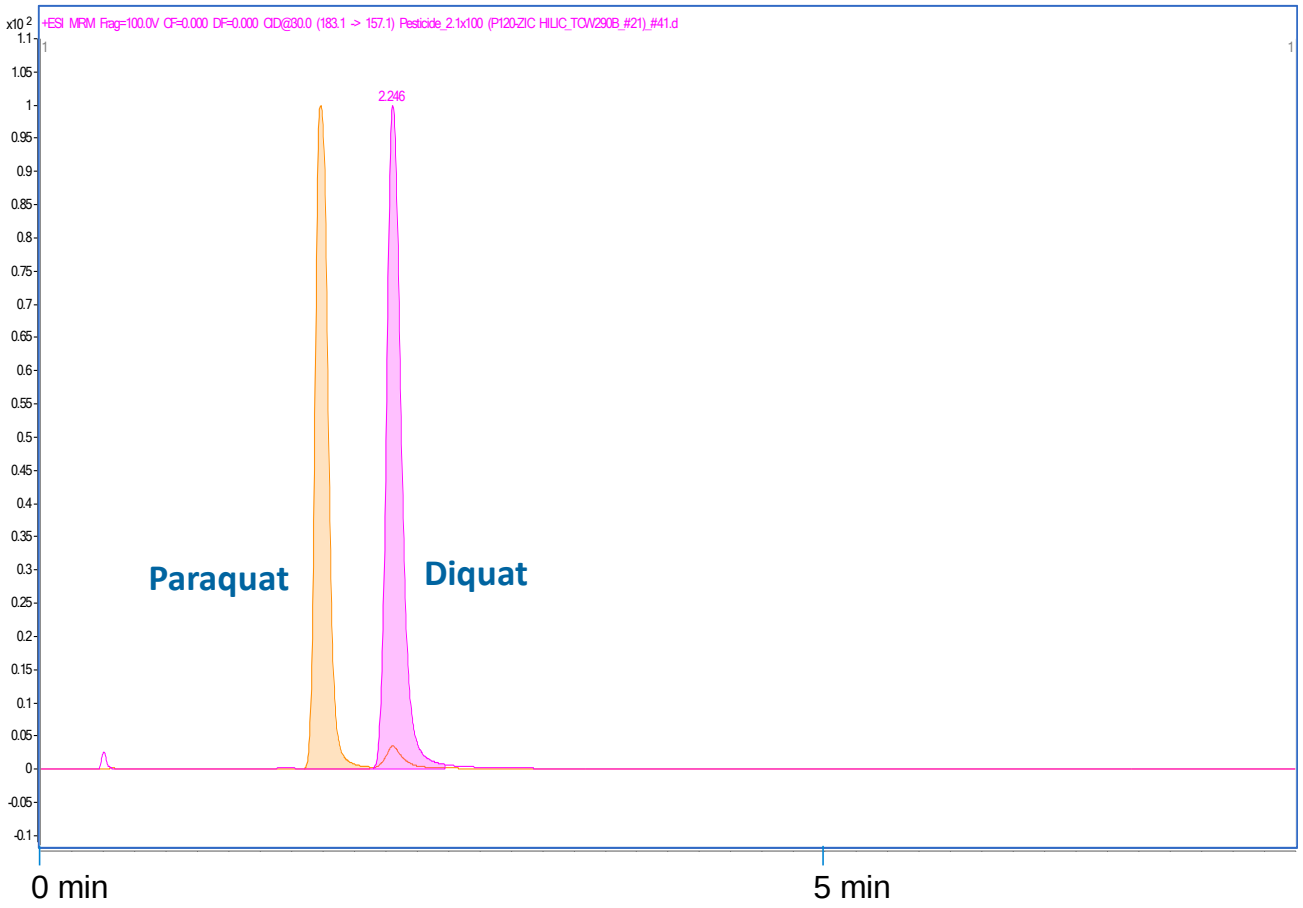
Conditions:
Column: ZORBAX Eclipse XDB-C18
4.6 x 150 mm, 5 mm
Mobile Phase: 70% water + heptanesulfonic acid phosphoric acid to pH = 3.0 :30% methanol
Temperature: 30ºC
Flow Rate: 1 mL/min
Detection: UV 257 nm (Paraquat)
UV 308 nm (Diquat)
Sample: 50 ng on column
1. Paraquat
2. Diquat

Ion Pairing methods

- Advantage: Uses standard system and C18 columns
- Disadvantage: Permanently contaminates system, lengthy method development, restricted to positive or negative mode MS

Agilent InfinityLab Poroshell 120 HILIC-Z Approach

- **No loss in throughput:** InfinityLab Poroshell HILIC columns operates as quickly and reliably as reverse phase
- **Easy to implement:** Uses the same solvents and buffers as reverse phase
- Positive or Negative ESI



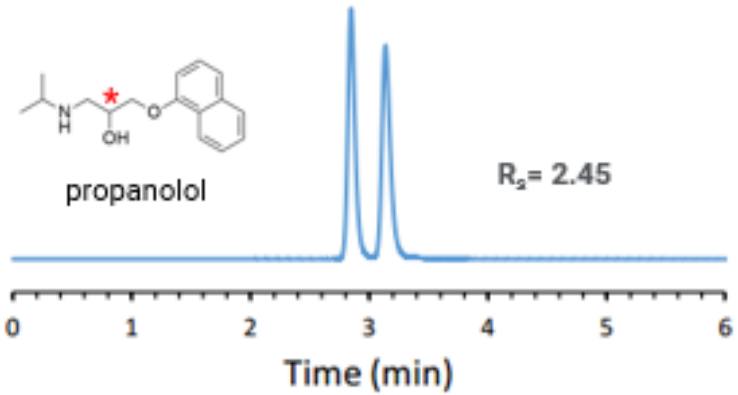
InfinityLab Poroshell HILIC-Z 2.1 x 100 2.7 µm
Mobile phase A: 20 mM ammonium formate, pH 3
Mobile phase B: MP A in 90% acetonitrile

Min	%B
0	80
1	80
5	73
6	80
8	80

ESI Positive

Updating Chiral Separations

- 4 chiral stationary phases on 2.7µm Agilent Poroshell particles
- Fast, efficient analysis using superficially porous particles (<5 min!)
- High efficiency provides superior peak shape and resolution compared to totally porous chiral phases
- Use common reversed-phase solvents for maximum method flexibility - even LCMS



Column: 4.6x100mm, 2.7µm
Mobile phase: 100/0.2/0.05: Methanol/Acetic Acid/Amm Hydroxide
Flow Rate: 1.0 mL/min Detection: UV 230 nm

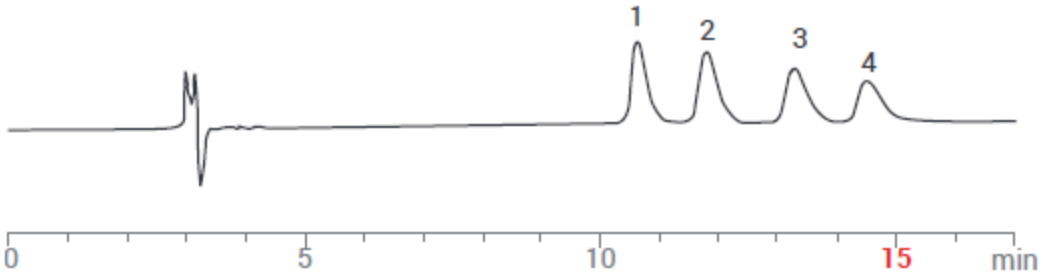
Best for Chiral
InfinityLab Poroshell **Chiral-V** 2.7 µm
InfinityLab Poroshell **Chiral-T** 2.7 µm
InfinityLab Poroshell **Chiral-CD** 2.7 µm
InfinityLab Poroshell **Chiral-CF** 2.7 µm

Method guidance available: Chiral Application Notebook 5991-8450EN

Fast, High Efficiency Chiral Separations

Traditional Chiral Separation—totally porous particle

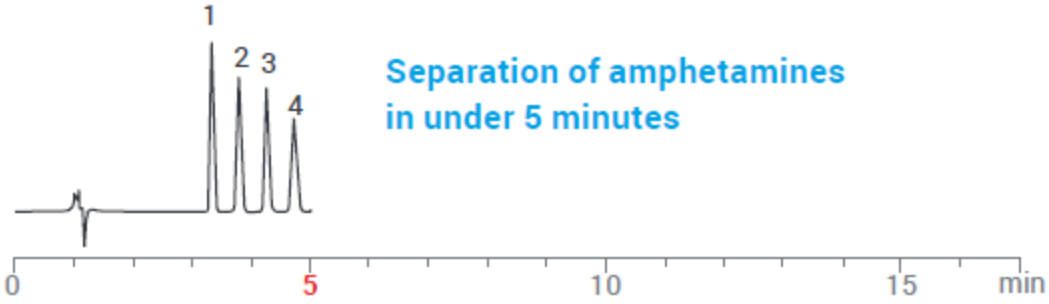
Chirobiotic V2 (250 x 4.6 mm, 5 µm)



1. D-(+)-Amphetamine, 2. L-(-)-Amphetamine, 3. D-(+)-Methamphetamine
4. L-(-)-Methamphetamine 100/0.1/0.02 MeOH/HOAc/NH_4OH with a
1.0 mL/min flow rate at room temperature and UV at 220 nm

Agilent InfinityLab Poroshell 120 Chiral Separation—superficially porous particle

InfinityLab Poroshell 120 Chiral-V (100 x 4.6 mm, 2.7 µm)



Separation of amphetamines in under 5 minutes

1. D-(+)-Amphetamine, 2. L-(-)-Amphetamine, 3. D-(+)-Methamphetamine
4. L-(-)-Methamphetamine 100/0.1/0.02 MeOH/HOAc/NH_4OH with a
1.0 mL/min flow rate at room temperature and UV at 220 nm

Tips for Method Transfer

Adjusting flow for different column diameters

$$Flow_{column\ 1} \times \left(\frac{diameter_{column\ 2}}{diameter_{column\ 1}}\right)^2 = Flow_{column\ 2}$$

$$1\ mL/min \times \left(\frac{2.1\ mm}{4.6\ mm}\right)^2 = 0.21\ mL/min$$

Tips for Method Transfer

Adjusting injection volume

$$V_m = \pi \times r^2 \times L \times \sim 0.6$$

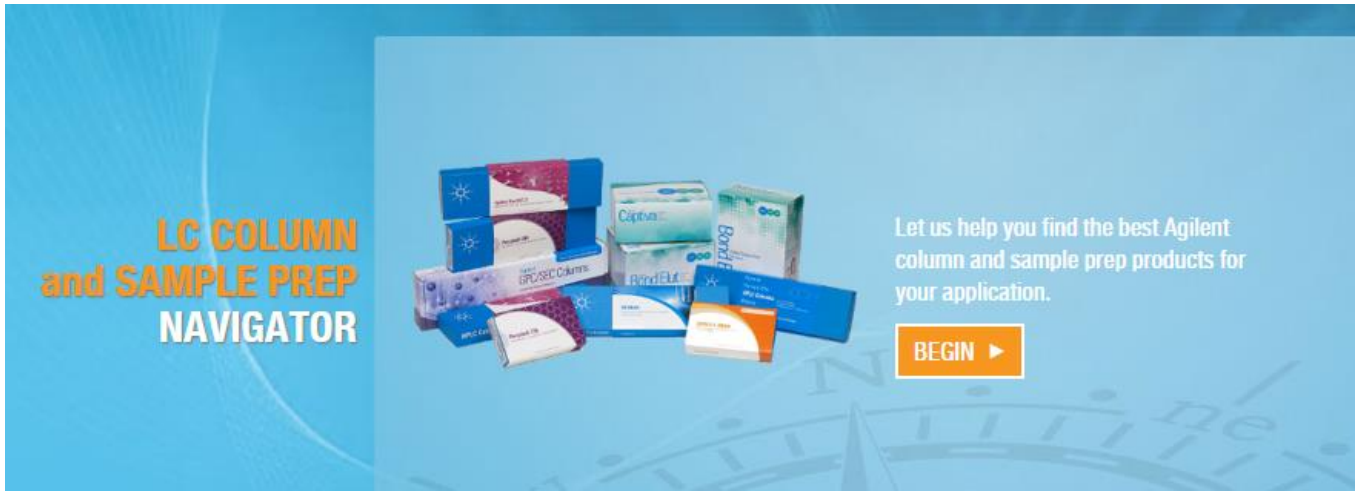
$$Injection_{column\ 1} \times \left(\frac{Volume_{column\ 2}}{Volume_{column\ 1}}\right) = Injection_{column\ 2}$$

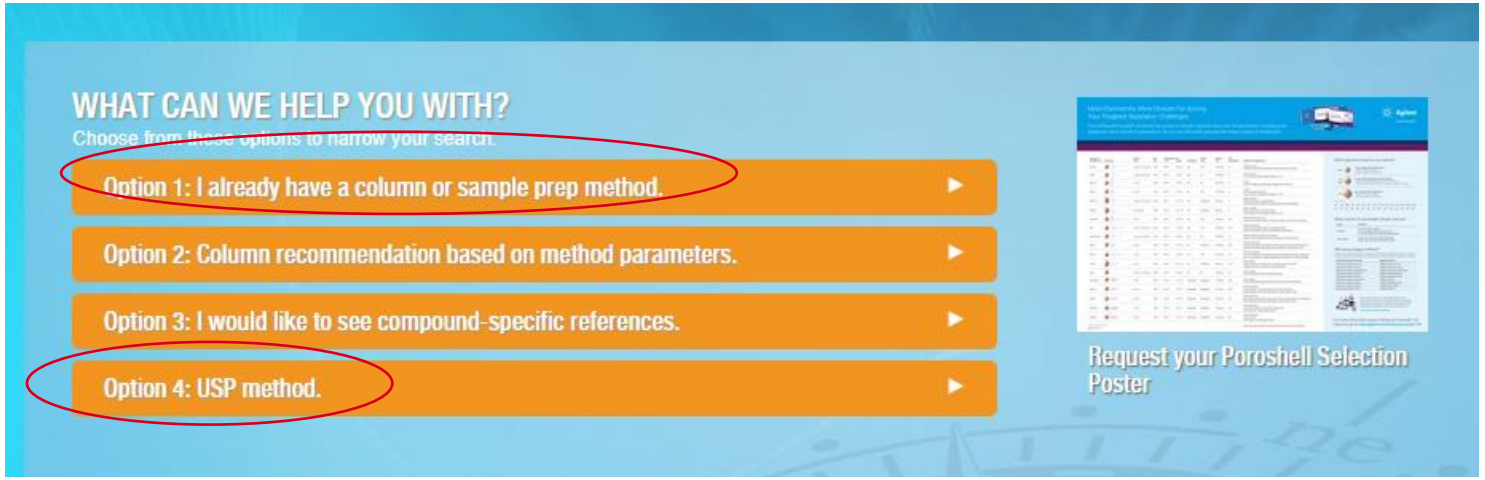
Original 4.6 x 250 mm: ~2.5 mL
Transferred to 2.1 x 100 mm: ~0.21 mL

$$30\ \mu L \times \left(\frac{0.21\ mL}{2.5\ mL}\right) = 2.5\ \mu L$$

Agilent LC Column Navigator

http://navigator.chem.agilent.com/





Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 Option 3, Option 3:

Option 1 for GC and GC/MS columns and supplies

Option 2 for LC and LC/MS columns and supplies

Option 3 for Sample preparation, filtration and QuEChERS

Option 4 for Spectroscopy supplies

Available in the USA & Canada 8-5 all time zones

gc-column-support@agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com