Where Do I Go from Here: Updating Old HPLC Methods

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Columns and Supplies Technical Support
February 17, 2021

Agilent InfinityLab

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Is Now the Time to Update a Method?

- Improved technology available
  - E.g., Poroshell 120
  - UHPLC
- Save time
- Save solvent
- Improve results
  - Peak shape
  - Resolution
  - Reproducibility or accuracy
- Accommodate new applications or formulations
- Difficulty duplicating results on a method you've "inherited"

Ask Yourself

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Is my method isocratic or gradient?

Does it make sense to update my current method?

- Is it going to be used many times?
  - Cost effective
  - Improved productivity
- Will it be used over a long period of time?
- Is my method rugged?
  - Improved performance

Does the reference method meet my needs?

Am I ready to finalize my new method?

Common Sources of Reference Methods

USP – Pharmaceutical compounds

NIOSH – Industrial pollutants

AOAC – Food components

EPA – Environmental methods

Productivity and Performance Improvements Considerations

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Faster results

- More efficient sample preparation
- More samples per instrument/time
- More samples per operator
- Preserving uptime and faster data review

Minimize change

- Protect current investments (upgrade path)
- Don’t need to revalidate
- Don’t need to retrain

More information

- Higher resolution
- Increased peak capacity
- Greater sensitivity
- Increased dynamic range
- Fast scan MS and MS/MS
- Dual source ESI and APCI

Reliability and ease-of-use

- Increased uptime
- Ease of transfer

What's Your Goal?

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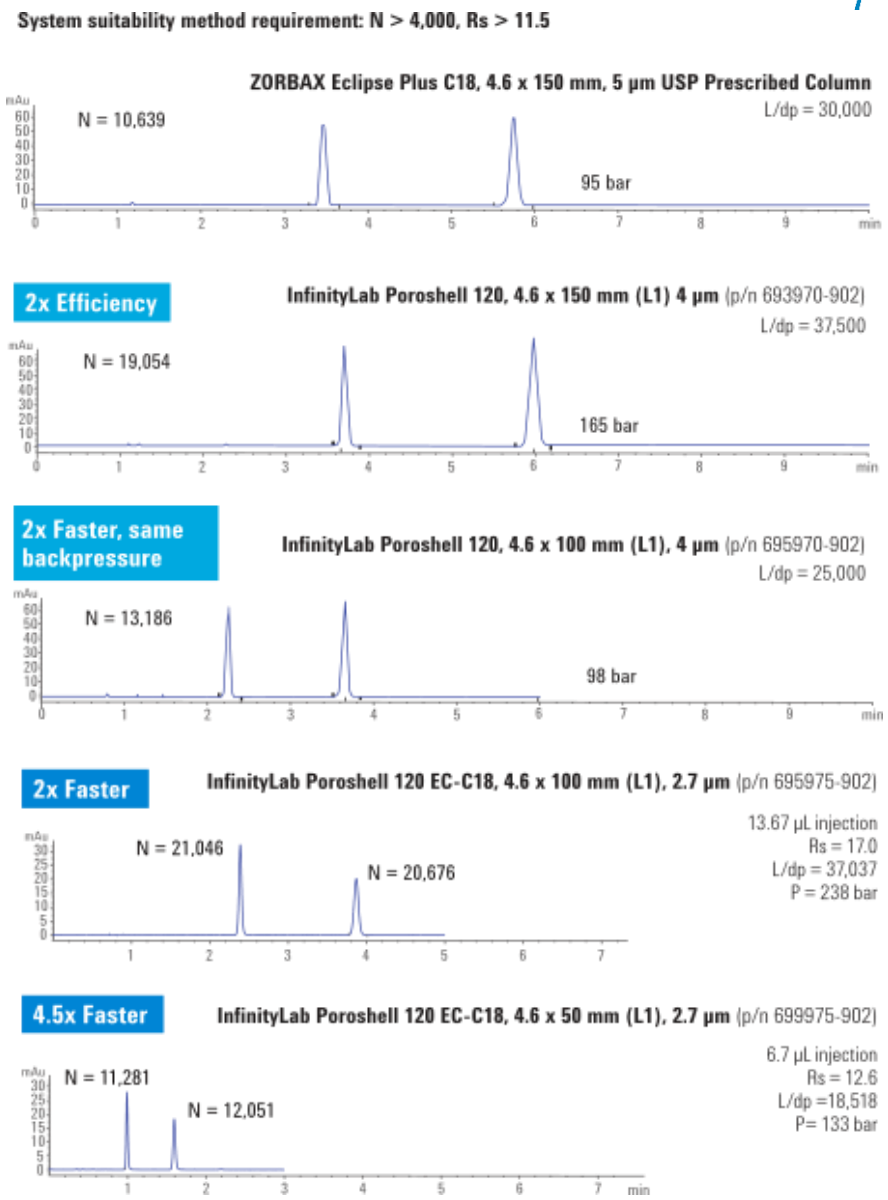
Faster separations, same backpressure

- Increase throughput
- Save solvent
- Increase resolution and throughput but keep your workflow and sample preparation

Or

Improved resolution at reduced backpressure

- Run "UHPLC"-like applications on HPLCs
- Run applications similar to "sub-2 µm" on general UHPLC instruments



Changes in Instrument and Column Technology

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What's the best choice for your method?

SPP Particle	Used For	Maximum Pressure	Typical Pressure	Efficiency	Target System
1.9 µm	Highest UHPLC performance	1300 bar	Similar to sub-2 µm totally porous	~120% of sub-2 µm totally porous	1290 Infinity II
2.7 µm	UHPLC performance at lower pressures	600 bar/ 1000 bar	50% of sub-2 µm totally porous	~90% of sub-2 µm totally porous	1290 Infinity II 1260 Infinity II
4 µm	Improved HPLC performance	600 bar	Typically <200 bar	~200% of 5 µm totally porous	1260 Infinity II VL 1220 Infinity II (VL)

Transfer to a New Column

Method transferability across product families

Traditional ZORBAX chemistries are aligned with InfinityLab Poroshell chemistries to offer simplified method transfer from fully porous particles to superficially porous particle columns.

InfinityLab Poroshell Chemistries	Aligned Chemistry
InfinityLab Poroshell 120 EC-C18	ZORBAX Eclipse Plus C18
InfinityLab Poroshell 120 EC-C8	ZORBAX Eclipse Plus C8
InfinityLab Poroshell 120 Phenyl-Hexyl	ZORBAX Eclipse Plus Phenyl-Hexyl
InfinityLab Poroshell 120 SB-C18	ZORBAX StableBond SB-C18
InfinityLab Poroshell 120 SB-C8	ZORBAX StableBond SB-C8
InfinityLab Poroshell 120 SB-Aq	ZORBAX StableBond SB-Aq
InfinityLab Poroshell 120 Bonus-RP	ZORBAX Bonus-RP
InfinityLab Poroshell 120 EC-CN	ZORBAX Eclipse XDB-CN
InfinityLab Poroshell 120 HILIC	ZORBAX HILIC-Plus

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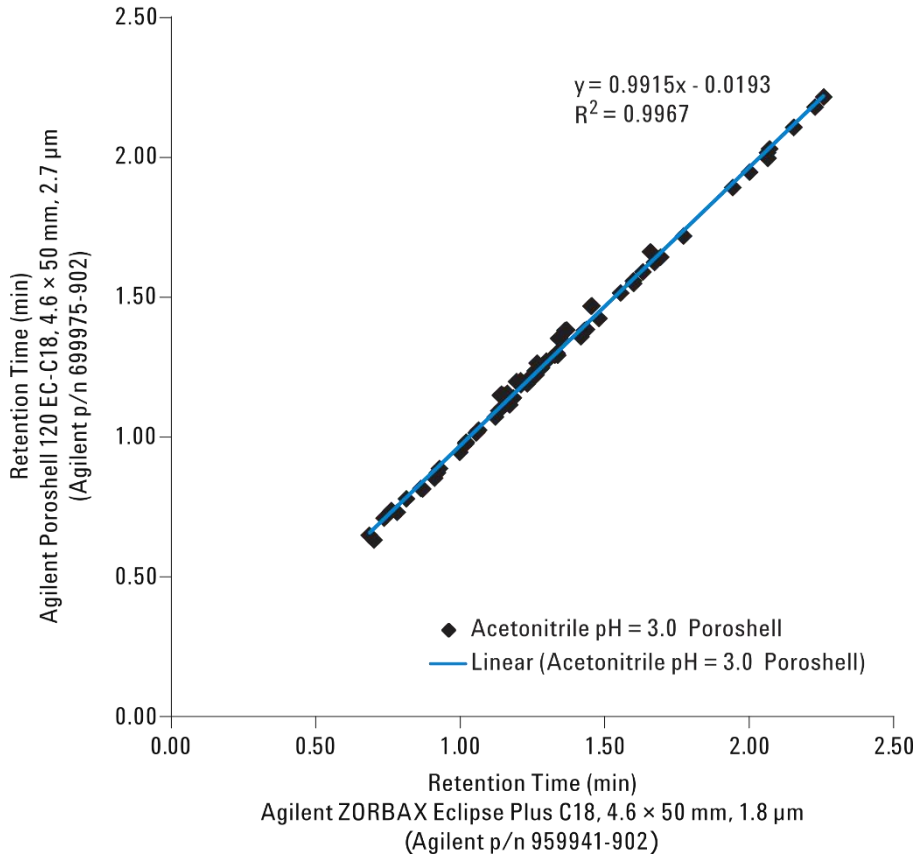
Acetonitrile pH 3.0, Agilent Poroshell 120 EC-C18 versus Agilent ZORBAX Eclipse Plus C18



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InfinityLab Poroshell 120 HILIC	ZORBAX HILIC-Plus

For more information on method transfer, see technical overview 5990-6588EN

66 compounds

two solvents (MeOH, ACN)

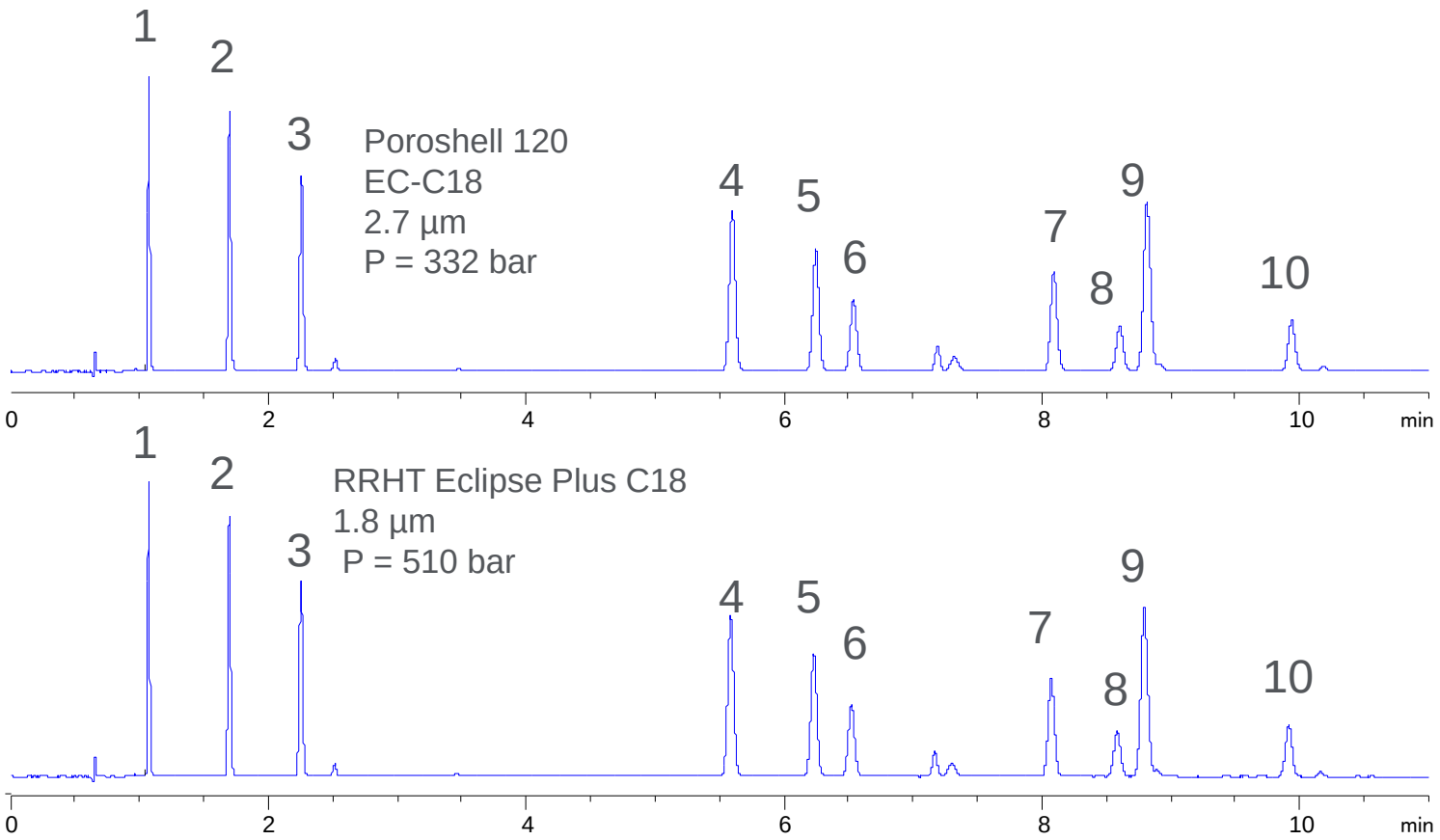
at 3 pH values each

pressure vs. linear velocity

Poroshell 120 Compared with Sub-2 µm

Very similar performance

Columns: 4.6 x 100 mm
A: Water 0.1% formic acid
B: Acetonitrile 0.1% formic acid
Gradient 2 mL/min
Initial 8 % B
10 min 30% B
275 nm 2 mm flow cell
Injection: 10 µL
40 °C
Agilent 1200



1. Hydroquinone
2. Resorcinol
3. Catechol
4. 4-Nitrophenol
5. p-cresol
6. o-cresol
7. 2-Nitrophenol
8. 2,3 Dimethyl phenol
9. 2,5 Dimethyl phenol
10. 1-Naphthol

Transfer Isocratic Method to a Shorter Column with the Same Internal Diameter

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Questions to ask

- What is the resolution for the critical pair(s) in the current method?
- What is the mobile phase composition and backpressure for the current method on the current instrument?
- What flow rate is currently used and will be used for the smaller column?
- Will you need smaller flow cell and smaller id tubing for the shorter column to get maximum efficiency?

Calculate the following for the shorter column:

- Theoretical plates: current column and new column f (length, d_p)
- Resolution: current Rs x SQRT($N_{smaller}/N_{larger}$)
- Injection Volume: adjust by column volume ratio
- Flow rate: adjust by square of id ratio
- Expected pressure: f (temperature, solvent comparison, viscosity, flow rate, length, d_p)
- Expected RT for last peak: k values should match first

Update Isocratic Methods
Smaller Particle Size

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Connect and Go

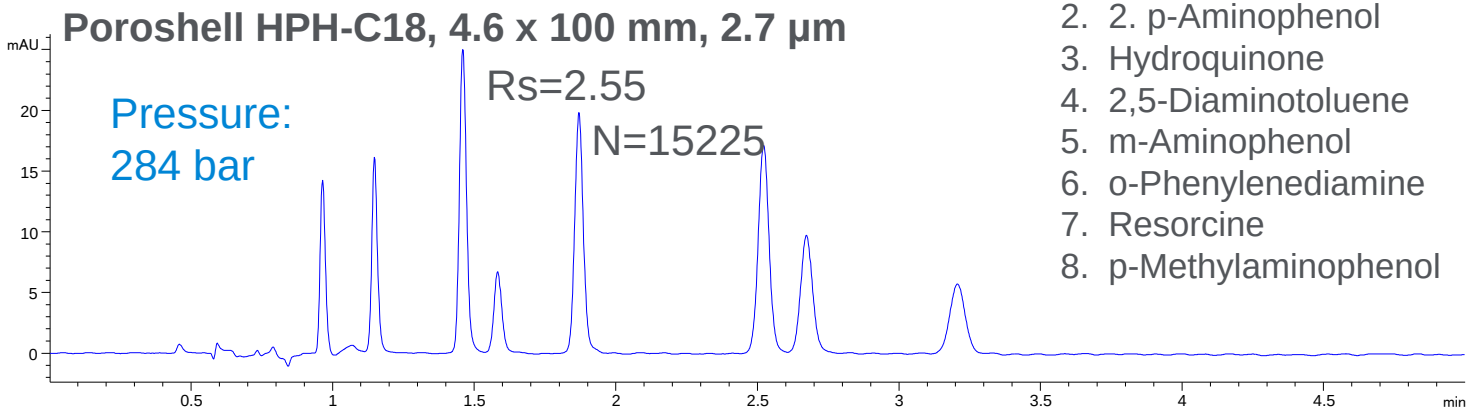
Step 1. Replace column, e.g., 4.6 x 150 mm, 5 µm column with a RR (3.5 µm) or RRHT (1.8 µm) column.

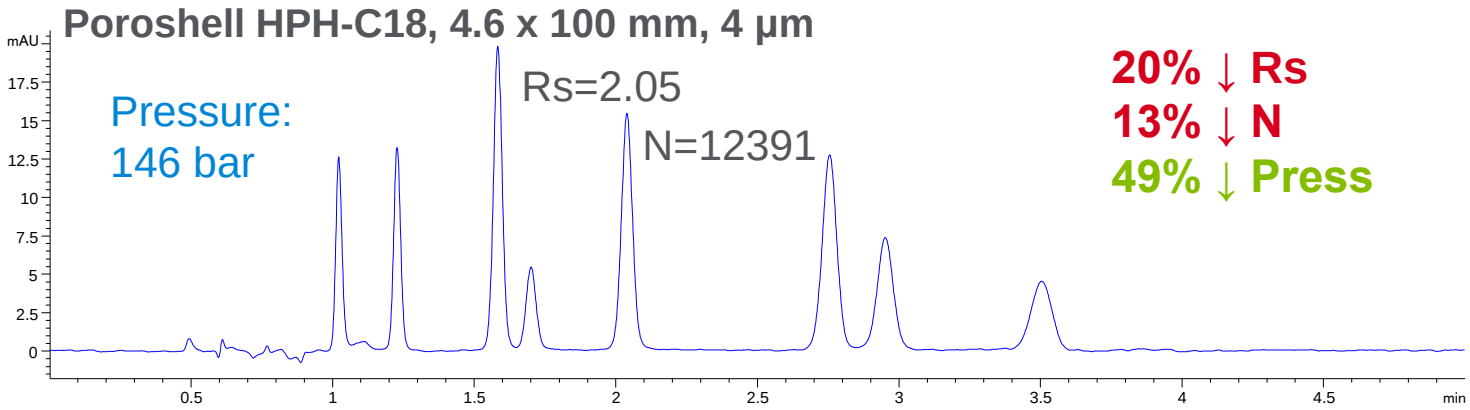
Step 2. Run pre-existing method.

Choosing a Particle Size

Mobile phase: 10mL/L triethanolamine in ultra pure water (pH 7.7 by phosphoric acid)/ ACN (96:4)
Flow rate: 1.5 mL/min
Injection : 2 µL
Detector: UV 280 nm

1. p-Phenylenediamine
2. 2. p-Aminophenol
3. Hydroquinone
4. 2,5-Diaminotoluene
5. m-Aminophenol
6. o-Phenylenediamine
7. Resorcine
8. p-Methylaminophenol





20% ↓ Rs
13% ↓ N
49% ↓ Press

Some Things Change, Some Things Stay the Same

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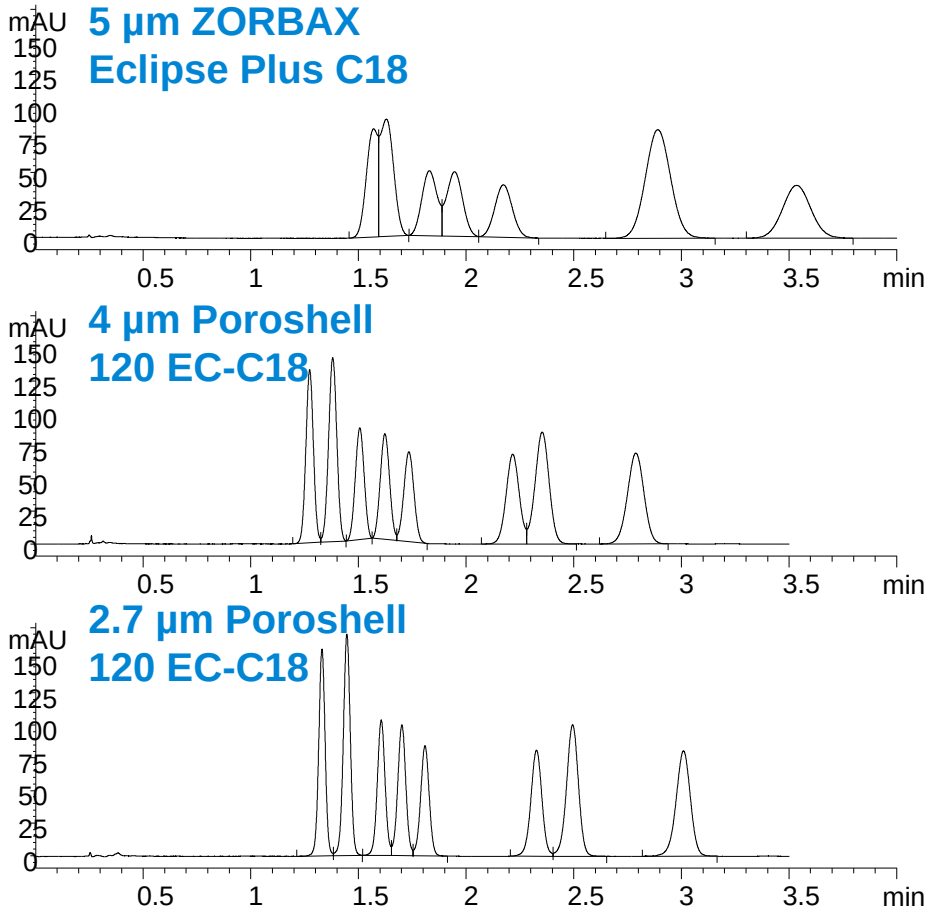
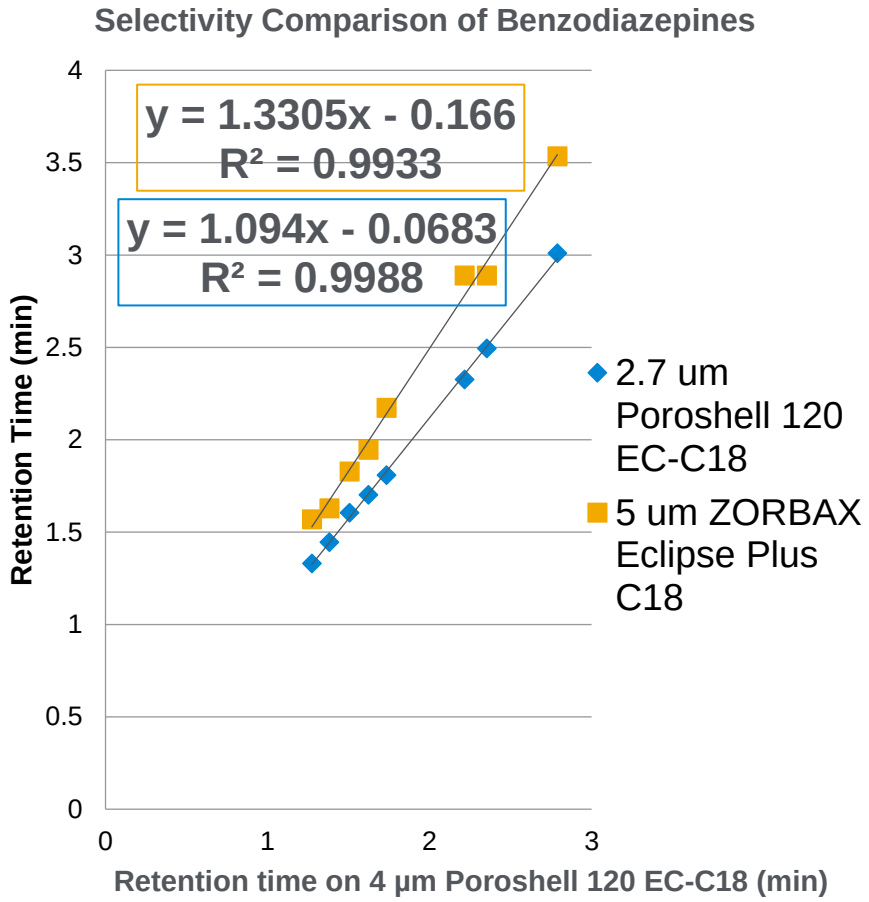
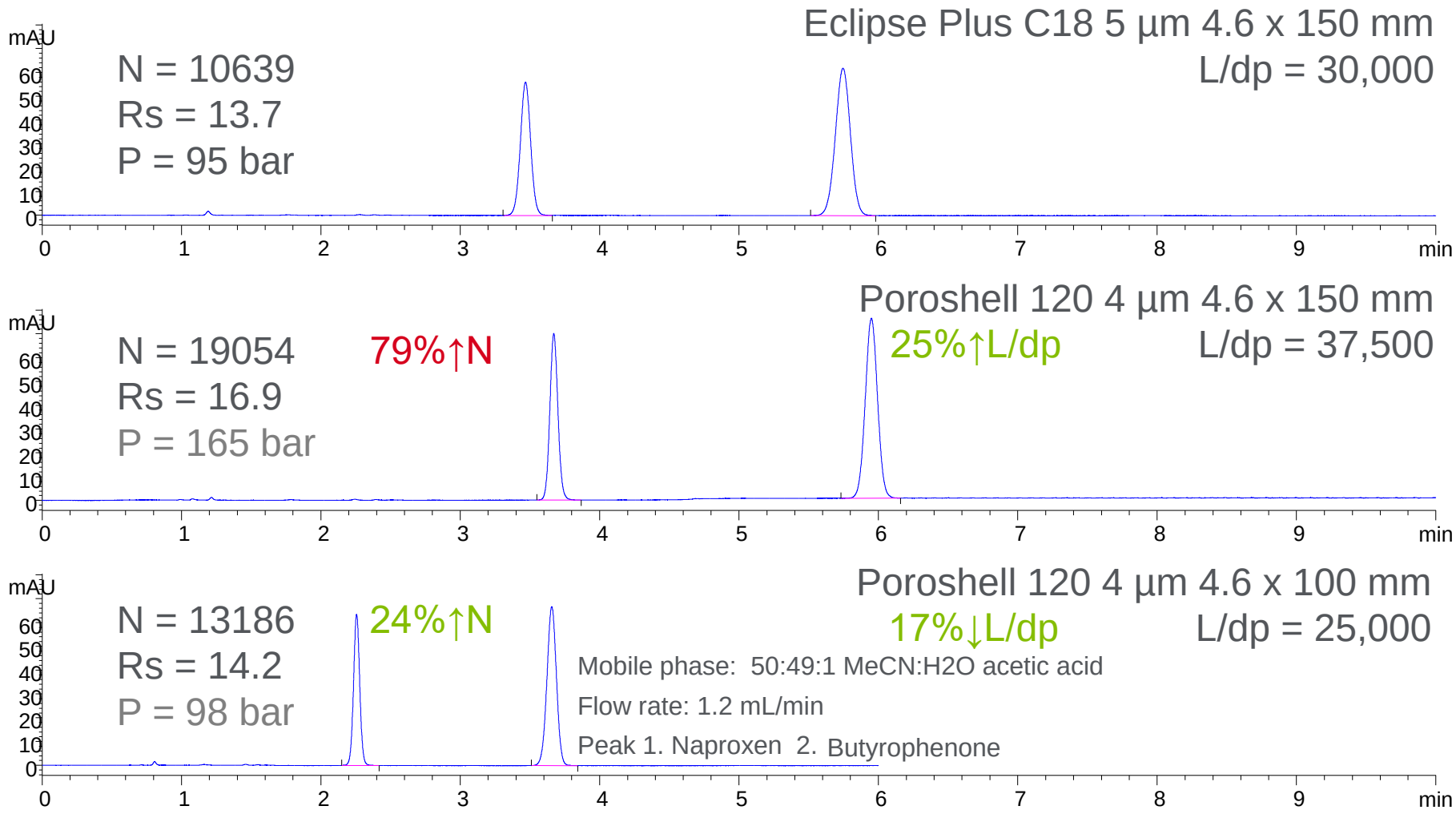
Same dimensions, similar column, different particle size, similar selectivity = improved resolution

A: 0.1% formic acid in water
B: 0.1% formic acid in ACN
2 mL/min, 32%B isocratic 25 °C
254 nm, ref 360 nm 80 Hz,
1 µL inj., Cerilliant Benzodiazepine 8 Component Mixture
4.6 x 50 mm columns





Scaling From a 5 µm TPP to 4 µm SPP



Increase Sensitivity (Signal/Noise)
Same Injection Volume on Different Size Columns

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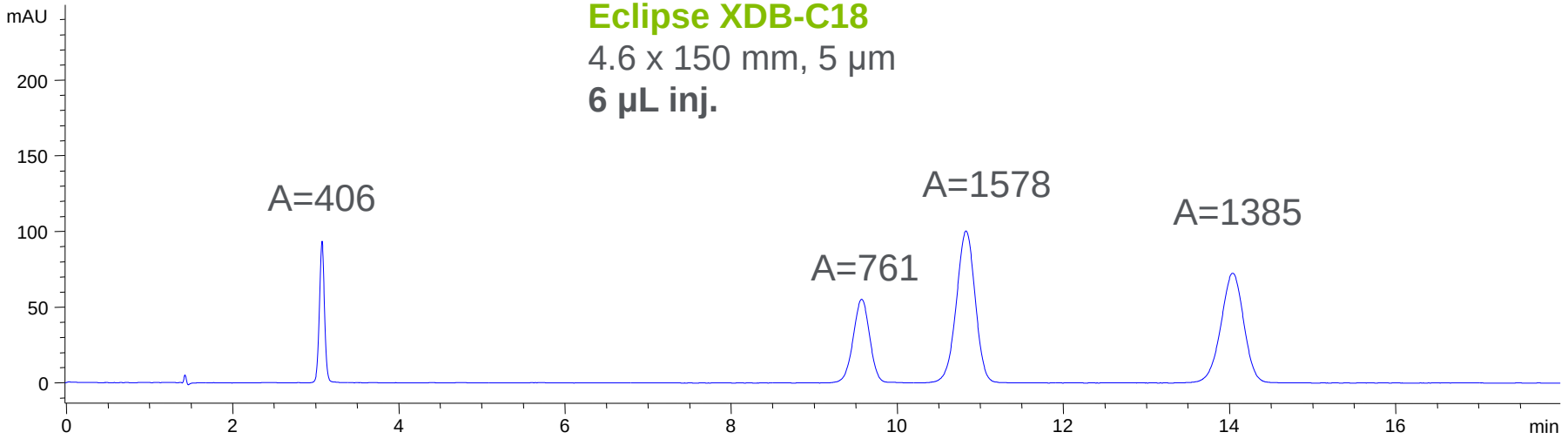
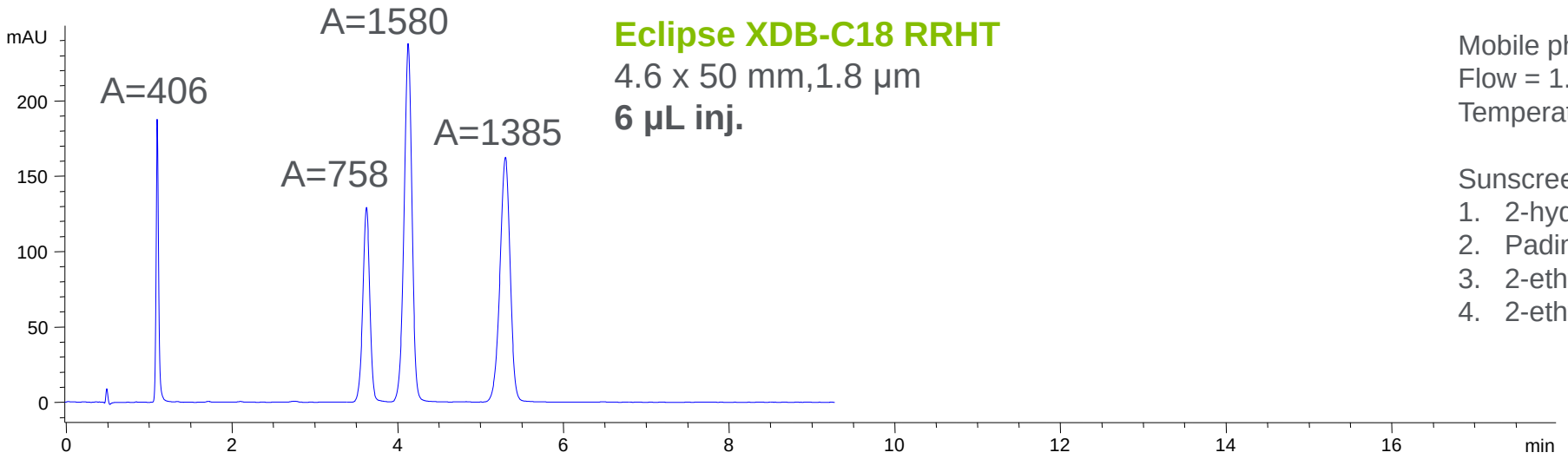


Eclipse XDB-C18
4.6 x 150 mm, 5 µm
6 µL inj.



Eclipse XDB-C18 RRHT
4.6 x 50 mm,1.8 µm
6 µL inj.

Mobile phase: (85:15) MeOH: water
Flow = 1.0 mL/min
Temperature: ambient

Sunscreens:
1. 2-hydroxy-4-methoxybenzophenone
2. Padimate-O
3. 2-ethylhexyl trans-4-methoxycinnamate
4. 2-ethylhexyl salicylate

Changing Column Diameter

Maintain equivalent linear velocity for different column ids

Column Type	Column ID	Flow Rate
Analytical	4.6 mm	1.0 mL/min
Solvent Saver	3.0 mm	0.42 mL/min
NarrowBore	2.1 mm	0.21 mL/min
MicroBore	1.0 mm	47 µL/min
Capillary	0.5 mm	12 µL/min
Capillary	0.3 mm	4.2 µL/min
Nano	0.1 mm	472 nL/min
Nano	0.075 mm	266 nL/min

Maintain equivalent mobile phase linear velocity when changing column diameter.

Convert Gradient Method to Smaller Particle Size Column

Step 1. Do the Math

Calculate a new gradient time proportional to the change in column length: $t_{G2} = t_{G1} \times (L_2/L_1)$

- t_{G1} = Gradient time with original column
- t_{G2} = Gradient time with column 2
- L_1 = Length of original column
- L_2 = Length of column 2

Step 2. Change original column to e.g., RR (3.5 µm) or RRHT (1.8 µm) column.

Step 3. Run new method.

A Closer Look at the Math
How Conversion Works for Time

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Run time or gradient segment time adjustment

$$\text{Time}_{\text{col1}} \times \left(\frac{\text{Length}_{\text{column2}}}{\text{Length}_{\text{column1}}}\right) = \text{Time}_{\text{col2}}$$

$$\text{For example, } 25 \text{ min} \times \left(\frac{150 \text{ mm}}{250 \text{ mm}}\right) = 15 \text{ min}$$

Assumes flow is proportional for columns 1 and 2.

How Conversion Works for Flow

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Flow modification, for columns of different diameters

$$\text{Flow}_{\text{col 1}} \times \left(\frac{\text{Diam.column2}}{\text{Diam.column1}} \right)^2 = \text{Flow}_{\text{col 2}}$$

Column Type	Column ID	Flow Rate
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For example, 1.0 mL/min x $\left(\frac{2.1\ \text{mm}}{4.6\ \text{mm}} \right)^2$ = 0.21 mL/min

Injection Volume Conversion

Keep injection volume proportional to column volume.*

$$\text{Inj.Vol.}_{\text{col 1}} \times \left(\frac{\text{Volume}_{\text{column2}}}{\text{Volume}_{\text{column1}}}\right) = \text{Inj.Vol.}_{\text{col 2}}$$

*~*ZORBAX column volume = 3.14 x r^2 x L x 0.6 (r and L in cm)*

$$\text{For example, } 20\ \mu\text{L}_{\text{col 1}} \times \left(\frac{0.4\ \text{mL}}{2.0\ \text{mL}}\ \frac{\text{column2}}{\text{column1}}\right) = 4\mu\text{L}_{\text{col 2}}$$

Gradient Slope vs. Time and Flow

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Gradient slope effects k* (“k star”) – the gradient term for the isocratic capacity factor.

Increasing k’ or k* generally increases resolution. This can be done by decreasing solvent strength or decreasing gradient slope, respectively.

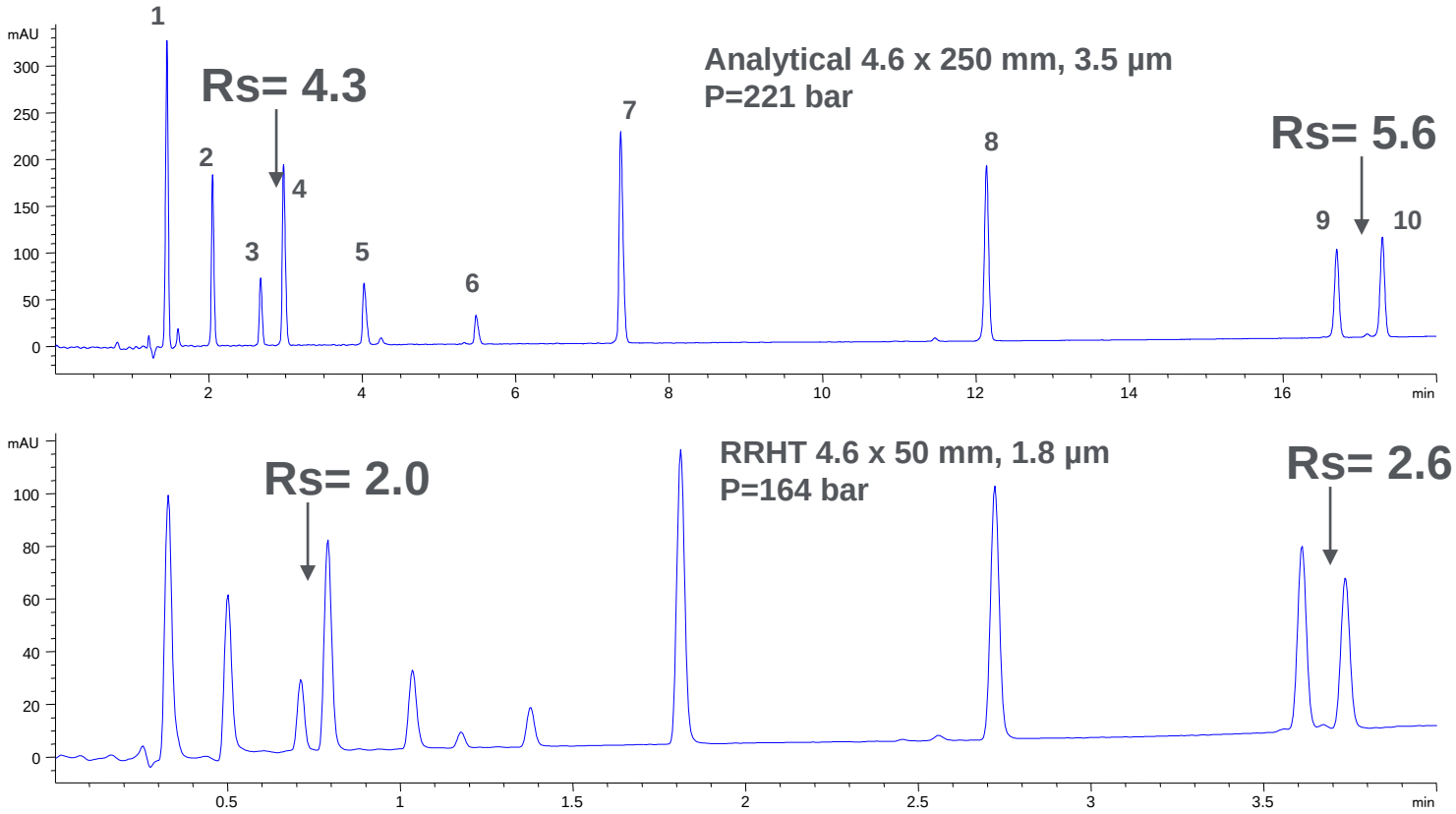
$$\%\ \text{Gradient slope} = \left(\frac{(\text{End}\% - \text{Start}\%)}{\#\ \text{Column volumes}}\right)$$

$$\text{For example, } 8\% = \left(\frac{(100\% - 20\%)}{10\ \text{col. volumes}}\right)$$

column volumes = (flow x gradient time)/column volume

~ZORBAX column volume = 3.14 x r^2 x L x 0.6 (r and L in cm)

Gradient Scalability 3.5 µm to 1.8 µm



1. Procainamide
2. Procaine
3. Nadolol
4. Pindolol
5. Lidocaine
6. Disopyramide
7. Propranolol
8. Nifedipine
9. Nimodipine
10. Nisoldipine

Columns:	SB-C18
Mobile phase:	A: 0.1% TFA, 5% MeCN, (v/v)
	B: 0.08% TFA, 95% MeCN (v/v)
Sample:	0.1 mg/mL each x 0.5 µL injection
Temperature:	60 °C
Flow:	1 mL/min gradient
Detection:	230,16 nm, ref=360,100
	response time: 0.5 s
LC:	Agilent 1100

RRHT Column	
min	%B
0	12.5
3.5	60
4	60
4.01	12.5

Analytical Column	
min	%B
0	12.5
17.5	60
20	60
20.01	12.5

Speed up the Analysis: Gradient
Similar Selectivity with Improved Resolution

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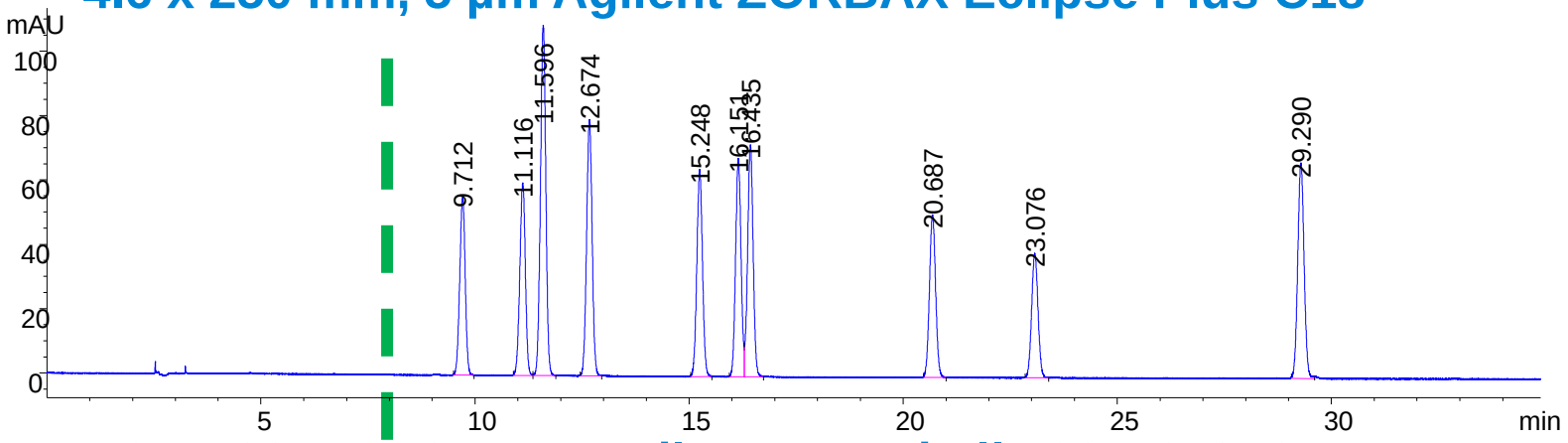
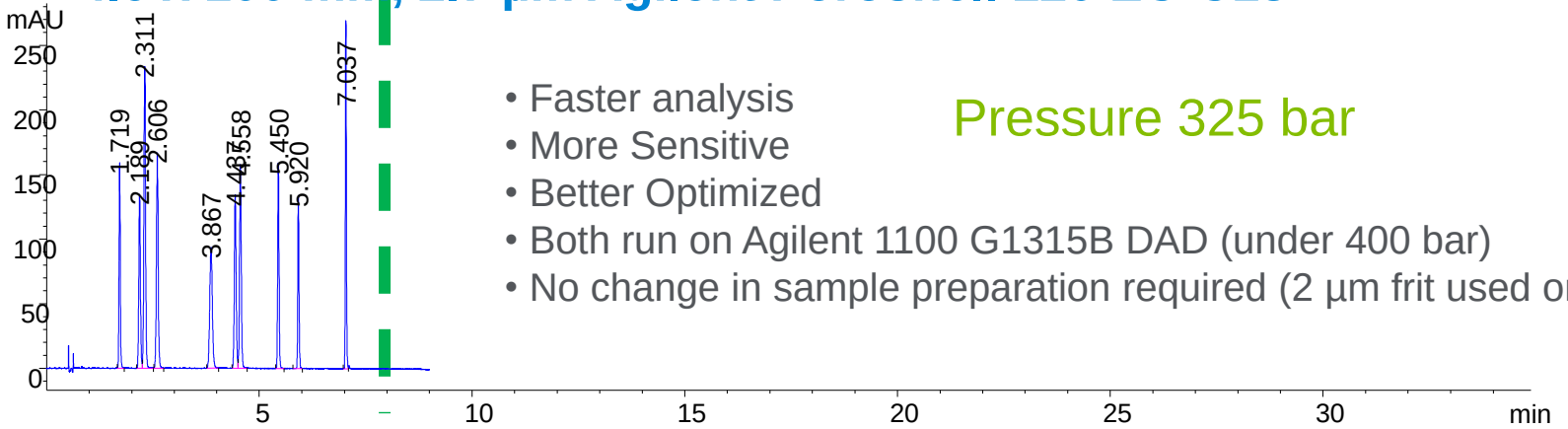
4.6 x 250 mm, 5 µm Agilent ZORBAX Eclipse Plus C18



4.6 x 100 mm, 2.7 µm Agilent Poroshell 120 EC-C18



- Faster analysis
- More Sensitive
- Better Optimized
- Both run on Agilent 1100 G1315B DAD (under 400 bar)
- No change in sample preparation required (2 µm frit used on both columns)

Pressure 325 bar

0.1% formic acid in water with 8-33% CH_3CN in 30 or 8 min, 1 or 2 mL/min, 25 °C, 254 nm

Agilent application note: 5990-5572EN

Optimizing Gradient Separations

With 1.8 µm RRHT columns: 10x faster analysis

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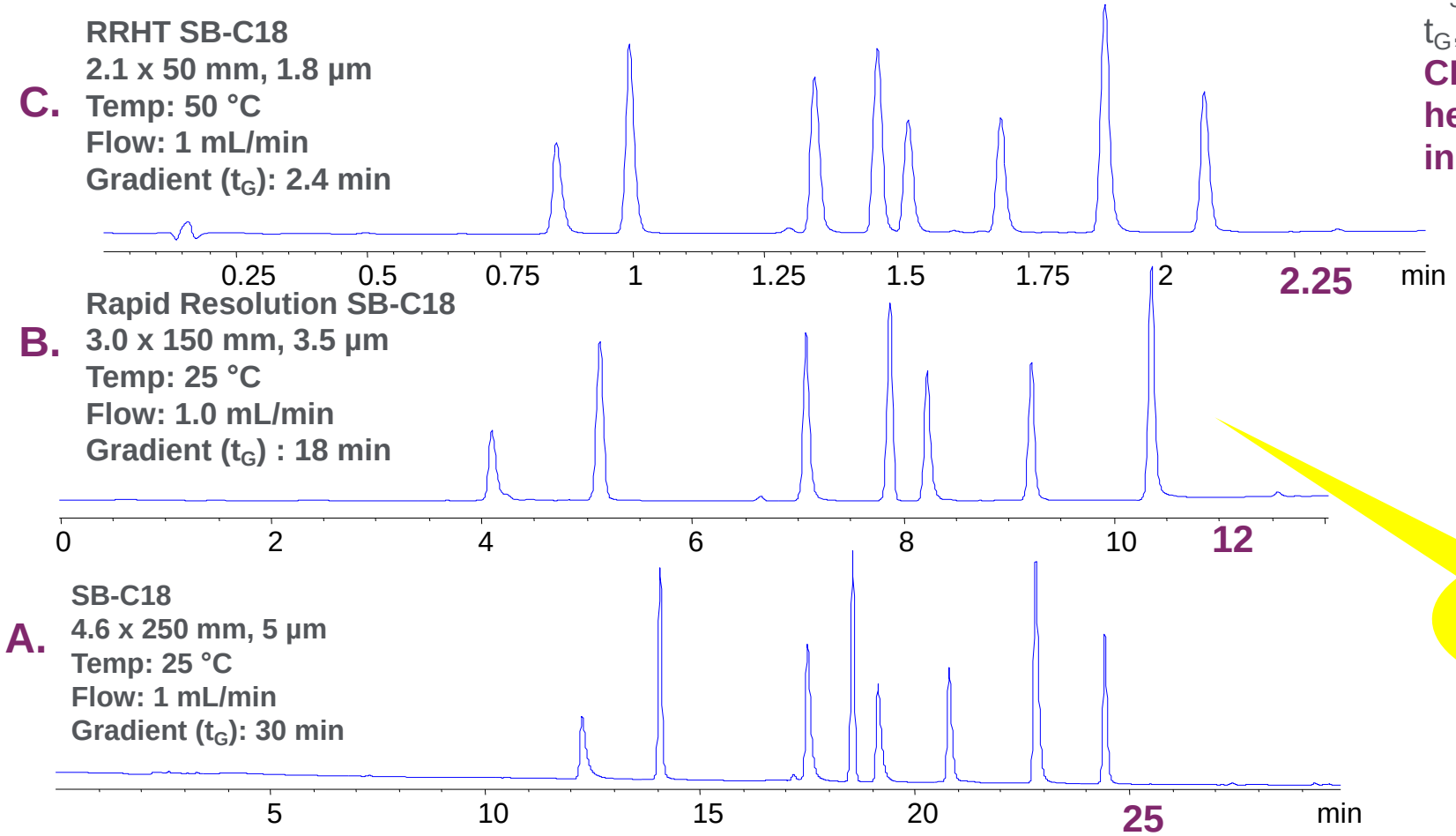
Column: SB-C18
Gradient: 10 – 90% ACN/25 mM H_3PO_4, Gradient time: t_G, as noted
CPAHs = Chlorphenoxyacid herbicides – environmental sample in water



R_s optimized

Key parameters

- Particle size
- Flow rate
- Linear velocity
- Gradient time
- Column length
- Column id
- Temperature

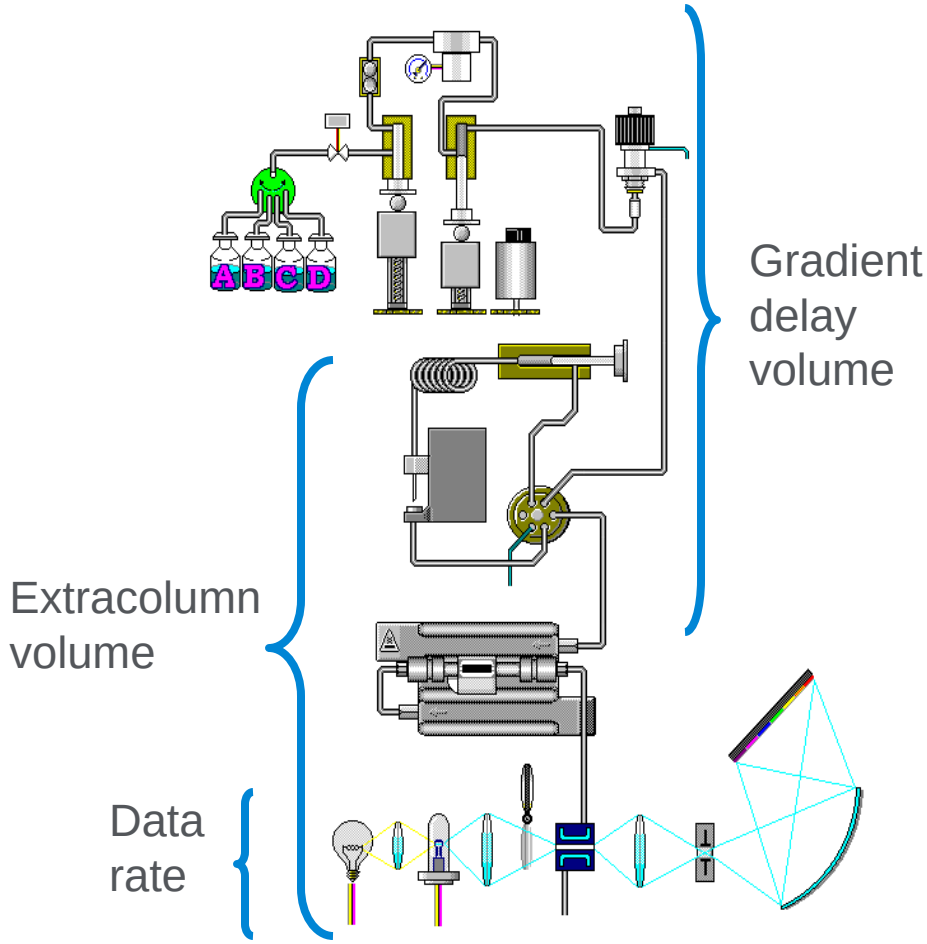
Sample: CPAH= Chlorophenoxy herbicides : Picloram, Chloramben, Dicamba, Bentazon, 2,4-D, Dichlorprop, 2,4,5-TP, Acifluorfen.

Instrument Considerations

What Can You Do With the Instrument You Have?

Things to consider on your system

- **Gradient delay volume** – affects column re-equilibration and gradient profile
- **Extracolumn volume** – affects peak dispersion and peak width
- **Data rate** – match to expected peak widths. If rate is too slow sensitivity and peak detection suffer in Iso and Gradient separations.



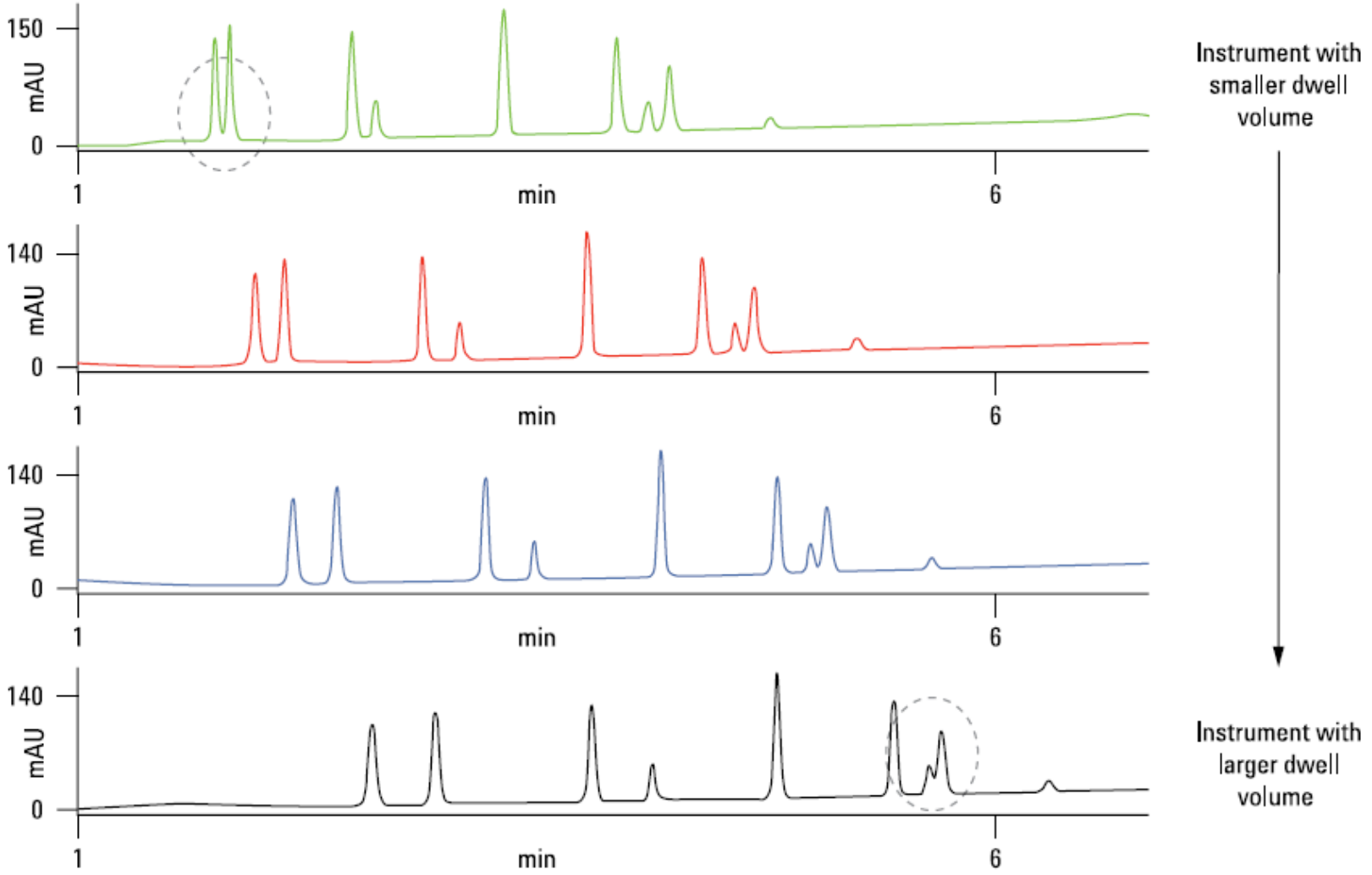
Gradient Delay Volume

A key parameter that can cause differences in chromatography between systems

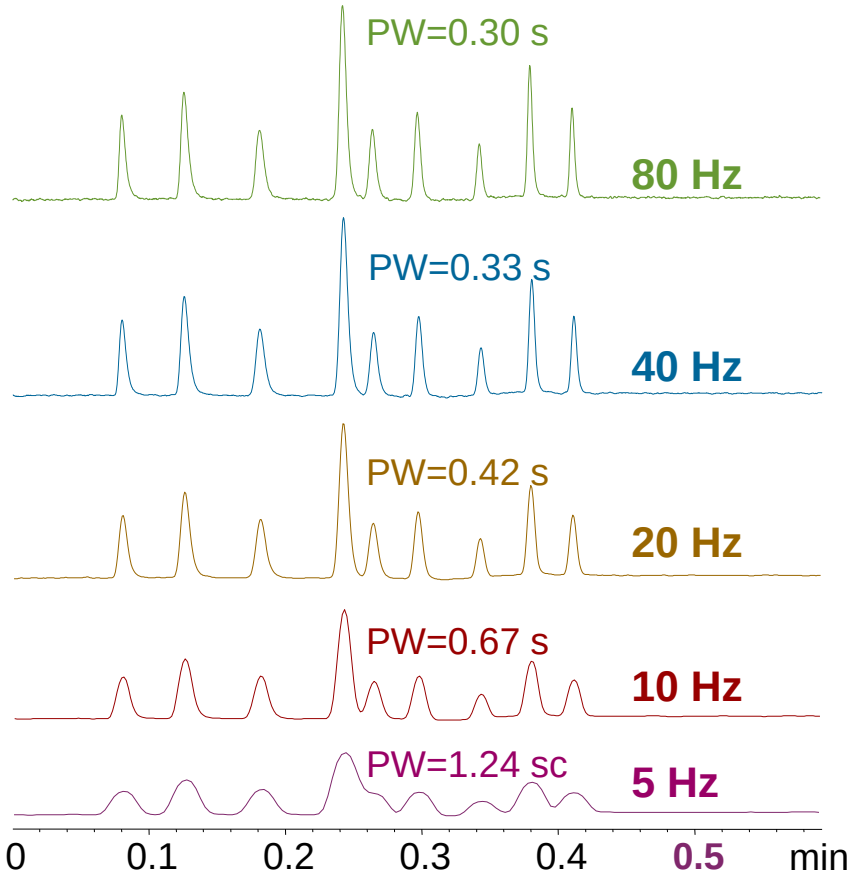
Why is delay/dwell volume important?

1. Different dwell volumes result in RT shifts
   (Definition: the time (or volume) for the mobile phase from the point of first mixing to reach the column head)
2. Different dwell volume could affect resolution
   (Peaks spend different amounts of time under isocratic/gradient conditions)
3. Additionally, the dwell volume affects the gradient shape
   (Dispersion effects, flush out behavior => the programmed gradient becomes deteriorated)
4. Therefore, even with the same “geometrical” delay volume, the chromatograms could look different on different systems
5. The dwell volume has a big impact on narrow bore applications.

Chromatographic Test Result: Different Delay Volumes



Match Detector Speed to Peak Width for Optimum Detection



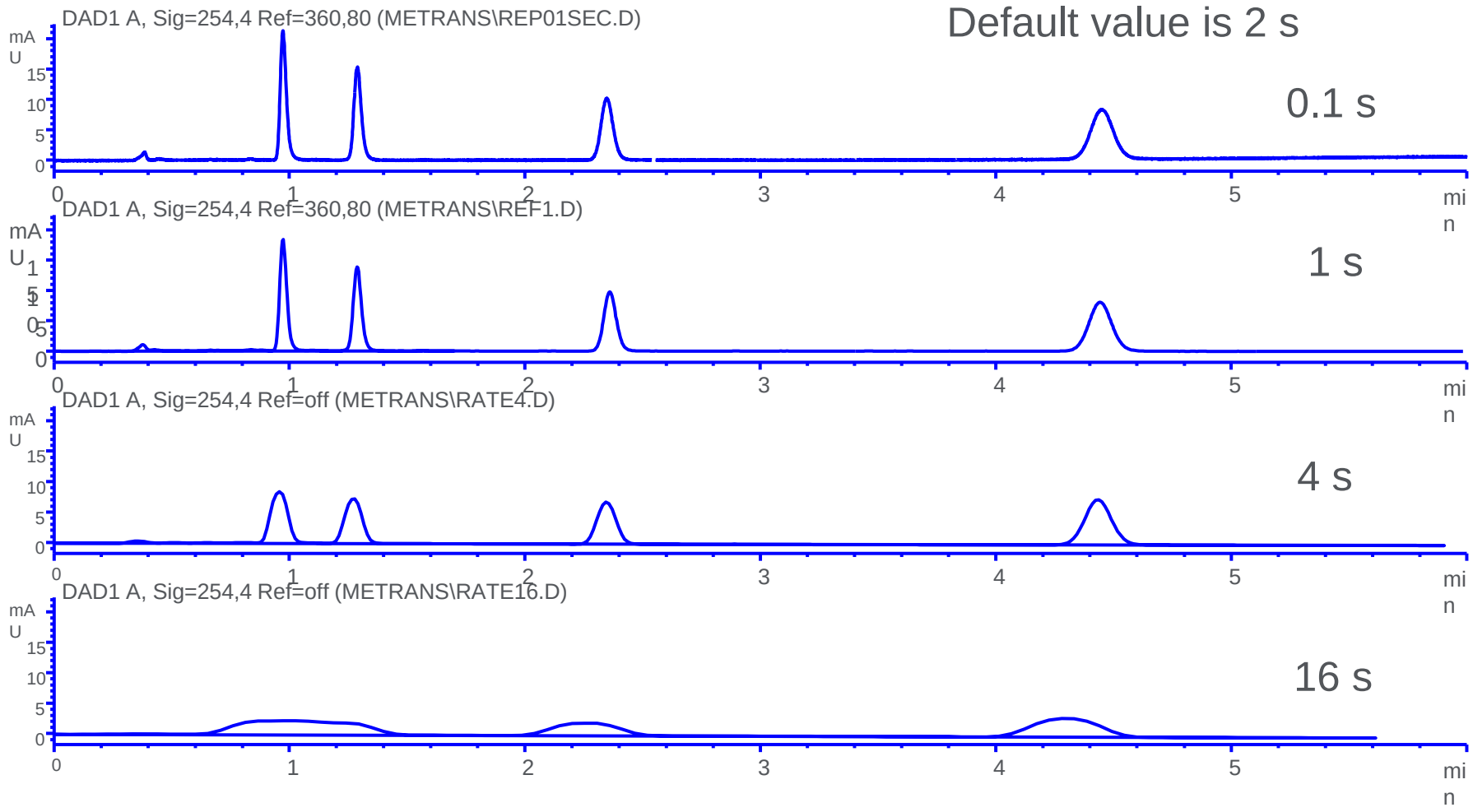
80 Hz versus 10 Hz (20 Hz) data rate

- Peak width: – 55% (– 30%)
- Resolution: + 90% (+ 30%)
- Peak capacity: + 120% (+ 40%)
- App. column eff.: + 260% (+ 70%)

Data Rate	Peak Width	Resolution	Peak Capacity
80 Hz	0.300	2.25	60
40 Hz	0.329	2.05	55
20 Hz	0.416	1.71	45
10 Hz	0.666	1.17	29
5 Hz	1.236	0.67	16

Sample: Phenones Test Mix
Column: ZORBAX SB-C18, 4.6 x 30, 1.8 µm
Gradient:: 50-100% ACN in 0.3 min
Flow rate: 5 mL/min

Influence of Data Rate on Peak Appearance



Some Things Change, Some Things Stay the Same
Reduce Analysis Time, Increase Throughput

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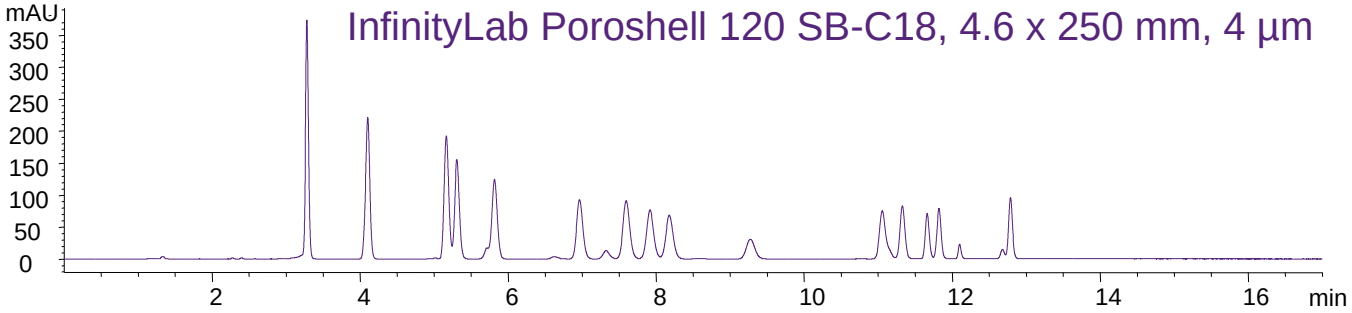
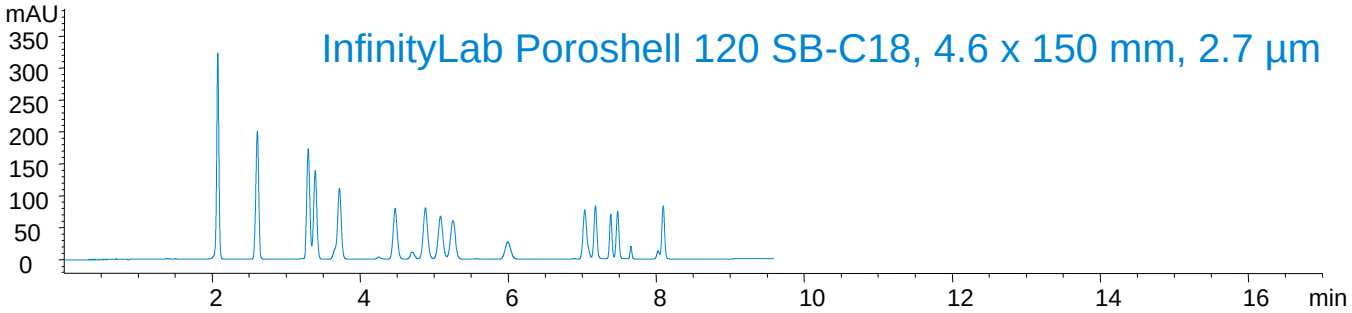
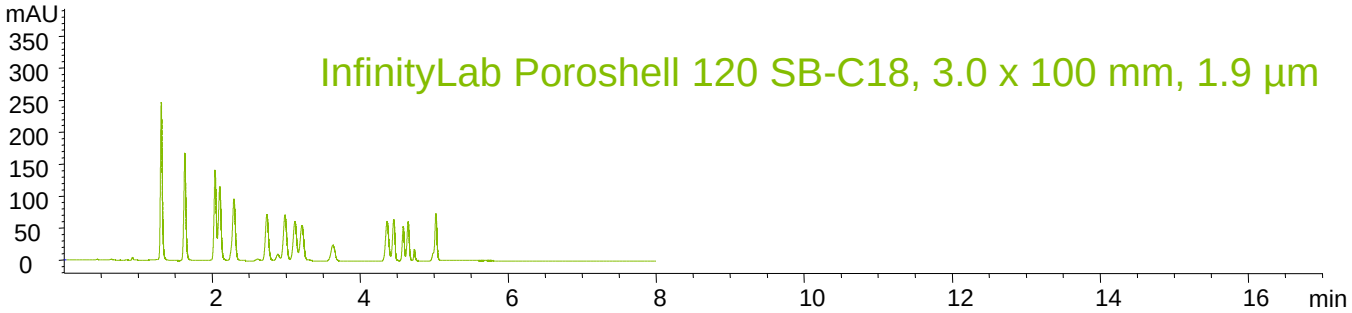
Particle size scalability







Mobile Phase: Water-ACN;
0-8.33min: 57% ACN
8.33-10 min: 57%- 75% ACN
10-16.7 min: 75% ACN
Flow rate: 1.5 mL/min
Injection volume: 5 uL
Column Temp: 50 C
Detector: UV 360 nm

Analytes

1. Formaldehyde-2,4-dinitrophenylhydrazone
2. Acetaldehyde-2,4-dinitrophenylhydrazone
3. Acrolein-2,4-dinitrophenylhydrazone
4. Acetone-2,4-dinitrophenylhydrazone
5. Propionaldehyde-2,4-dinitrophenylhydrazone
6. Crotonaldehyde-2,4-dinitrophenylhydrazone
7. Methacrolein-2,4-dinitrophenylhydrazone
8. Butyraldehyde-2,4-dinitrophenylhydrazone
9. 2-Butanone-2,4-dinitrophenylhydrazone
10. Benzaldehyde-2,4-dinitrophenylhydrazone
11. Cyclohexanone 2,4-dinitrophenylhydrazone
12. Valeraldehyde-2,4-dinitrophenylhydrazone
13. *o*-Tolualdehyde 2,4-dinitrophenylhydrazone
14. *m,p*-Tolualdehyde 2,4-dinitrophenylhydrazone
15. Hexaldehyde-2,4-dinitrophenylhydrazone

Some Things Change, Some Things Stay the Same
Reduce Analysis Time, Increase Throughput

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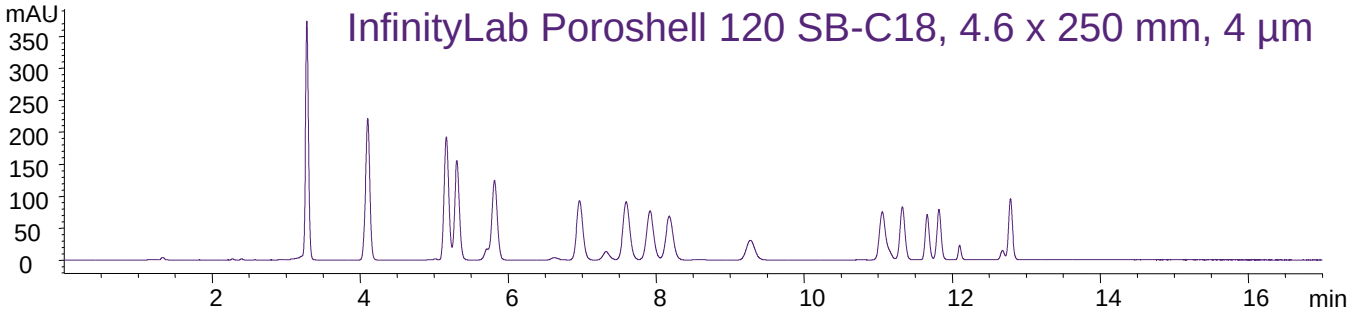
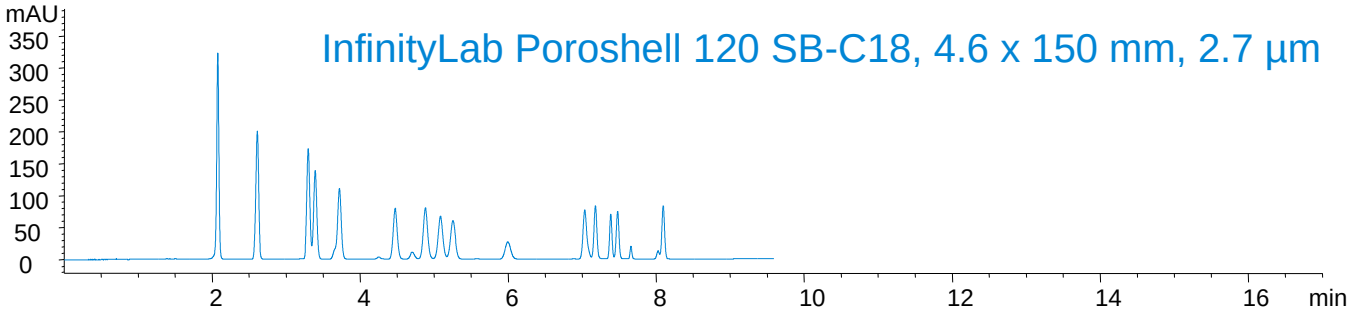
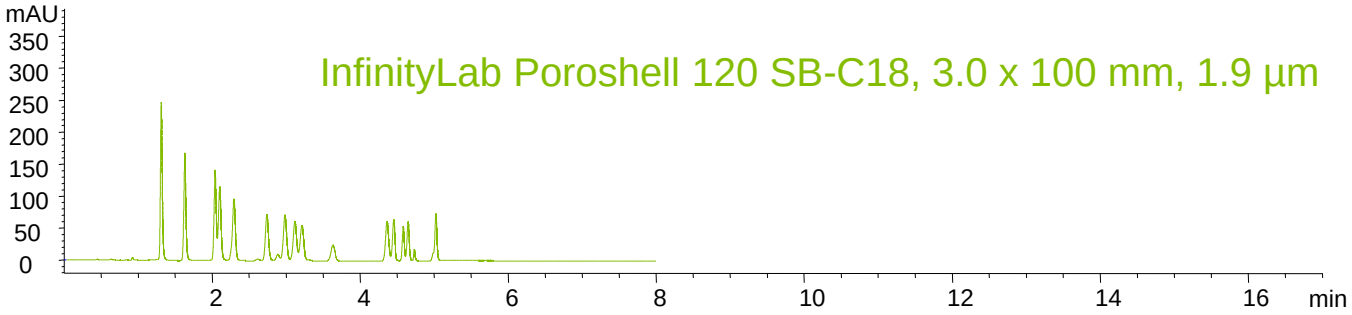
Particle size scalability



HPLC (4 µm)	Value	Difference
Run time	14 min	--
Response/injection volume	80 mAU/µL	--
Solvent consumption	21 mL	--
Samples per 8 h day	**24**	**--**



UHPLC (2.7 µm)	Value	Difference
Run time	8.75 min	– 37.5%
Response/injection volume	113 mAU/µL	+41%
Solvent consumption	13.1 mL	– 37.5%
Samples per 8 h day	**48**	**+24**



LD UHPLC (1.9 µm)	Value	Difference
Run time	5.25 min	– 62.5%
Response/injection volume	295 mAU/µL	+269 %
Solvent consumption	3.36 mL	– 84 %
Samples per 8 h day	**80**	**+56**

Considerations When Transferring Methods to Small(er) Volume Column

Isocratic separations

Sample load (V_{inj}, [analyte])

Extracolumn volume

- Injection volume
- Tubing volume
- Flow cell volume

Injector precision

- Can vary with V_{inj}

Gradient separations

Same as isocratic separations plus…

Delay volume

- Same instrument (different pressures)
- Different instrument (for example, 1100 to 1290)

Gradient time

- Adjust relative to equation for gradient retention
- Keep k* constant

Gradient delay time

- Gradient delay time must be same as for larger column separation
- Ratio of gradient volume/column volume must be same as for larger column

Column equilibration time (post time)

Change Column ID; Match Conditions

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Column id	Column volume	Peak volume, k=1	Typical injection volume.*	Typical injection volume range	Flow rate for equivalent ν**
4.6 mm	1500 µL	148 µL	20 µL	5–50 µL	1.0 mL/min
3.0 mm	640 µL	44 µL	10 µL	3–30 µL	0.42 mL/min
2.1 mm	320 µL	22 µL	2 µL	0.5–15 µL	0.21 mL/min
1.0 mm	70 µL	4 µL	0.5 µL	0.1–3 µL	47 µL/min
0.5 mm	15 µL	1 µL	150 nL	40–500 nL	12 µL/min
0.3 mm	6 µL	0.3 µL	50 nL	15–250 nL	4.2 µL/min
0.1 mm	700 nL	32 nL	10 nL	1–10 nL	472 nL/min
0.075 mm	400 nL	18 nL	2 nL	0.5–5 nL	266 nL/min

Column length = 150 mm

Tips

** Typical injection volume = 10–30% of peak volume of first eluting peak*

*** Maintain equivalent mobile phase linear velocity when scaling column diameter*

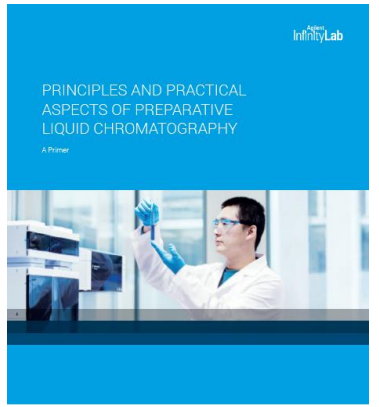
Flow rate column 2 = $(\text{diameter column 2})^2/(\text{diameter column 1})^2$ x flow rate column 1

Conclusions

- Improved results and lower costs may justify time spent updating methods
- Check for ways to save time and solvent with any method
- Apply current technology when appropriate – scale down column dimensions and particle size to meet speed and sample requirements
- Column similarity and scalability
- Evaluate new column technologies and configurations for increased speed and reduced solvent usage
- Plan for compatibility with instrumentation, HPLC, UHPLC, LC/MS

Agilent Resources for Support

- Resource page http://www.agilent.com/chem/agilentresources
  - Quick reference guides, product catalogs
  - Online selection tools, How-to videos
  - Column user guides - https://www.agilent.com/en-us/support/liquid-chromatography/kb005965
  - Bio-Column user guides - https://www.agilent.com/en/support/liquid-chromatography/kb005960
- Tech support http://www.agilent.com/chem/techsupport
- InfinityLab Supplies Catalog (5991-8031EN)
- Agilent University http://www.agilent.com/crosslab/university
- Youtube – Agilent Channel
- Your local FSE and specialists
- Prep HPLC guide (5994-1016EN)
- Subscribe to Agilent Peak Tales podcast at peaktales.libsyn.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

Option 5 for chemical standards

Available in the USA and 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

chem-standards-support@agilent.com