Chromatography in the Express Lane
High Resolution, Fast LC

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LC Columns Application Engineer

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Organization and Laboratory Needs

Agilent CrossLab

Organization needs

- Increased capacity
- Shorter time to market
- Increased profitability
- Looking for improvements

Laboratory needs

- Better use of resources
- Increase productivity
- Reduce costs

Laboratory Needs

Translated to liquid chromatography

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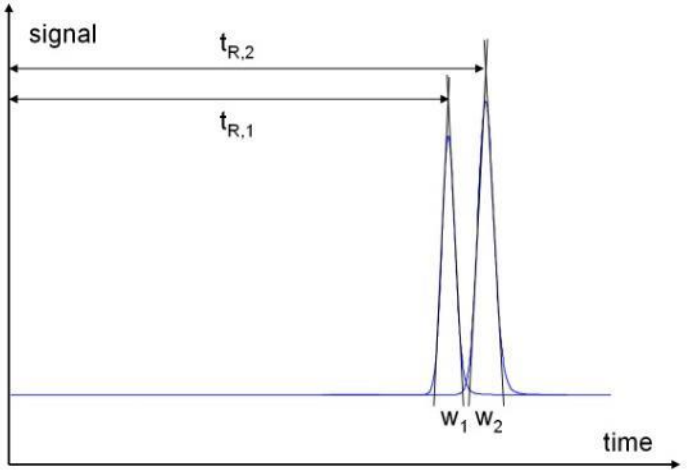
Better use of resources

Increase productivity

Reduce costs

Use all the instruments in the lab

Run fast with high resolution



Laboratory Needs

What's stopping you?

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Better use of resources

Increase productivity

Reduce costs

Use all the instruments in the lab

Run fast with high resolution

Yes, but…

- I don't have all UHPLC instruments
- I can't / don't want to change my methods much

Topics to be Explore

- What is High Resolution Fast LC and what does it look like?
  - Maintain Resolution with Faster Run Time
  - Increased Resolution with Faster Run Time
  - Increasing Speed by 2X – 10X Original Run Time
- What Technology Makes This Possible?
- Application to Isocratic LC Separations
- Application to Gradient LC Separations
- Instrument Consideration

Column Technology Advances - Stationary Phase

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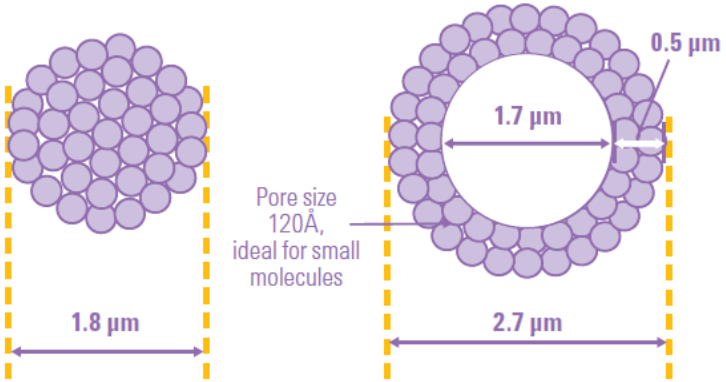
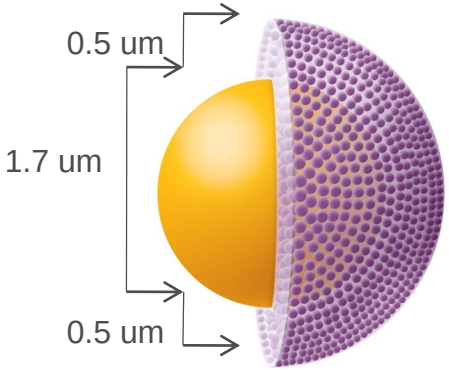
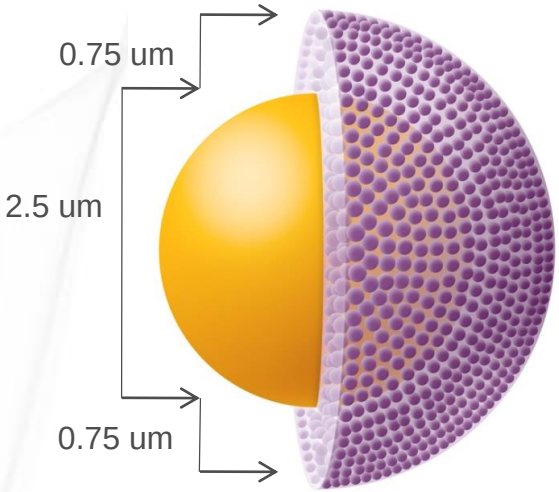
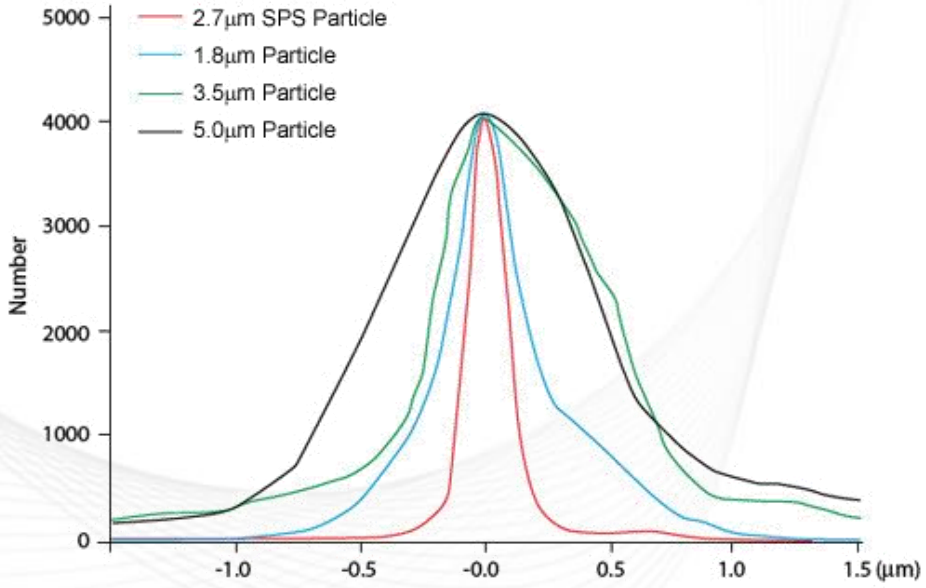
Sub-2um and Superficially Porous Particles









Poroshell 120 Compared with Sub 2-micron Very Similar Performance

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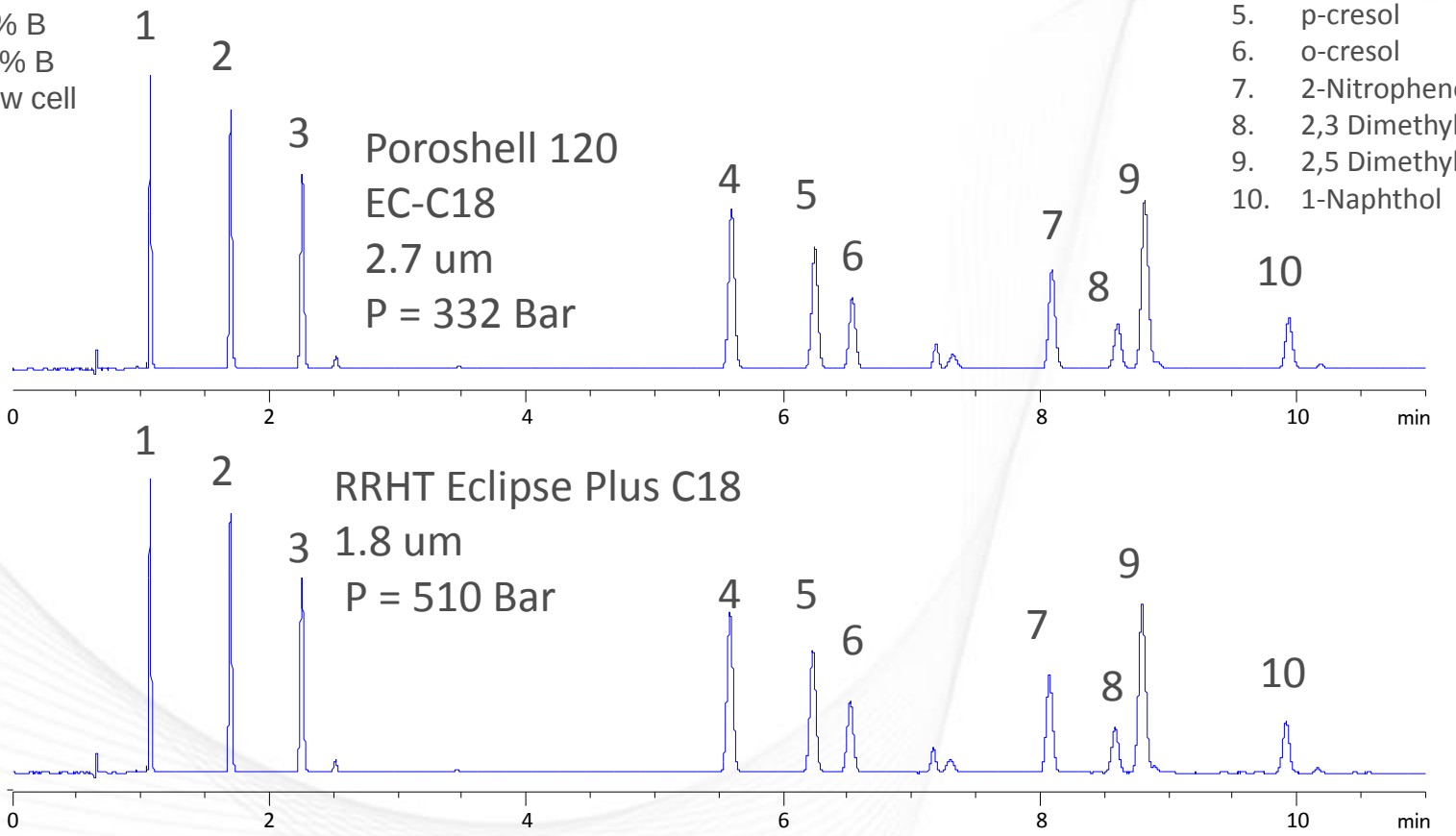
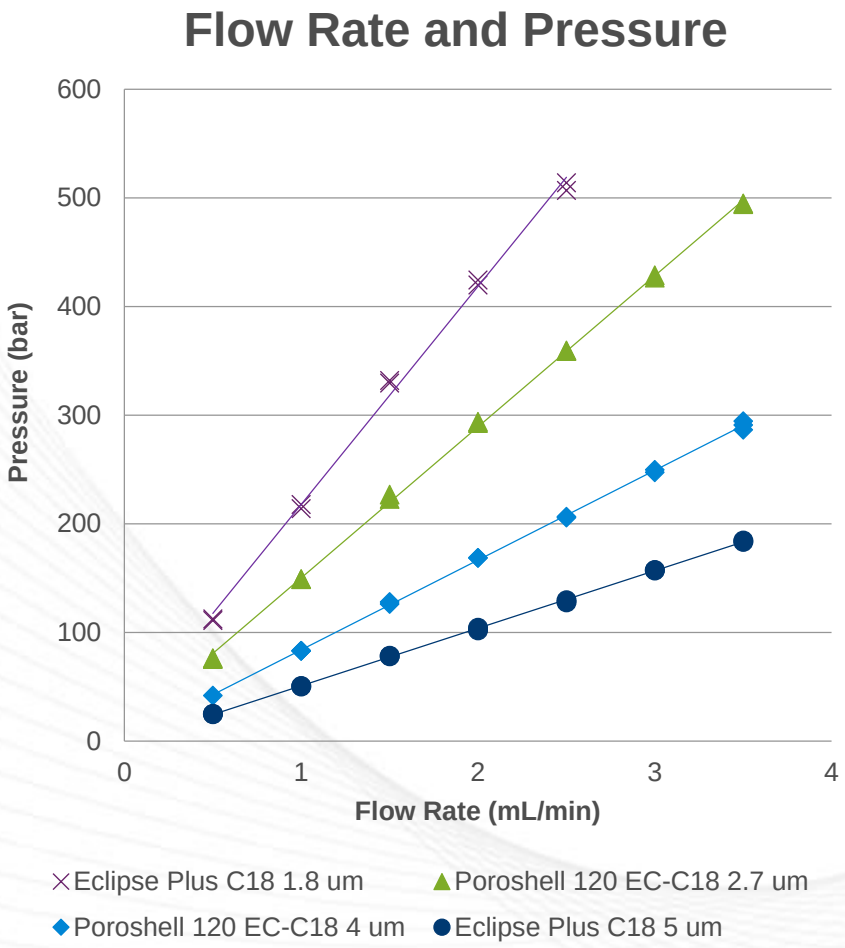
Columns: 4.6 x 100 mm
A: Water 0.1% Formic Acid
B: Acetonitrile 0.1% formic Acid
Gradient 2 ml/min
Initial 8 % B
10 min 30% B
275 nm 2mm flow cell
Injection: 10 uL
Agilent 1200 SL
40 °C

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



Use All the Instruments in the Lab
Pressure Considerations



Column Dimensions: 4.6 x 100 mm

Mobile phase A: 0.1 % formic acid in water

Mobile phase B: 0.1 % formic acid in acetonitrile

Temperature: 35°C

Pressure reading at 5% B

Application note 5991-5510EN

INCREASED RESOLUTION!
When Do You Need It? How Do You Get It?

- Complex Samples—Large No. of Peaks in Short Time Frame
- Closely Related Compounds
- Changes in Bonded Phase Have Not improved Resolution
- Changes in Mobile Phase Have Not Improved Resolution
- Temperature Has Not Helped Change Selectivity

Introduction

Resolution R

Resolution is a key goal in chromatographic separations

- R is proportional to the square root of N.
- R is influenced by retention factor k.
- R is influenced by separation factor α.
- Resolution numbers **greater than 1.50** indicate a **baseline between the peaks**.
- Numbers **less than 1.50** indicate there is some **peak co-elution**

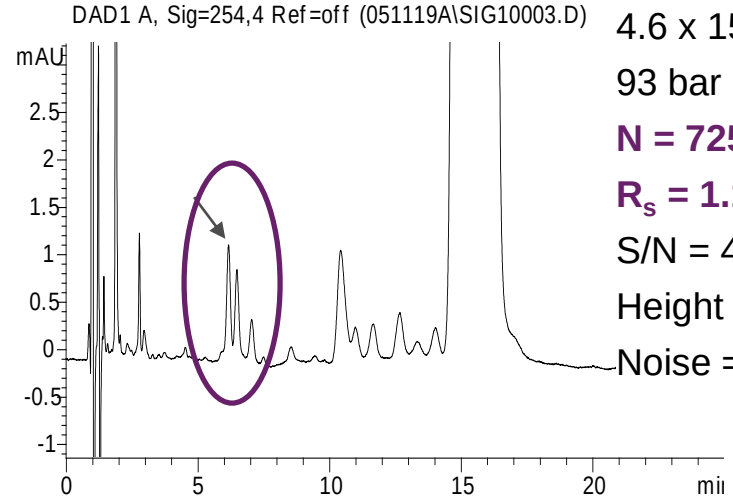
$$R = \frac{1}{4} \cdot \sqrt{N} \cdot \frac{k_2}{1 + k_2} \cdot \frac{\alpha - 1}{\alpha}$$

Selectivity

Efficiency

Retention

Reduce Particle Size and Maintain Column Length Increased "N" in Isocratic Separation



4.6 x 150, **5um**

93 bar

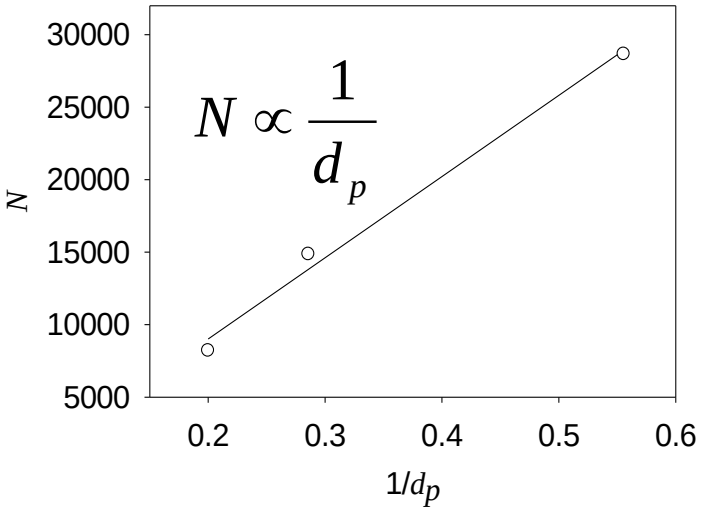
N = 7259

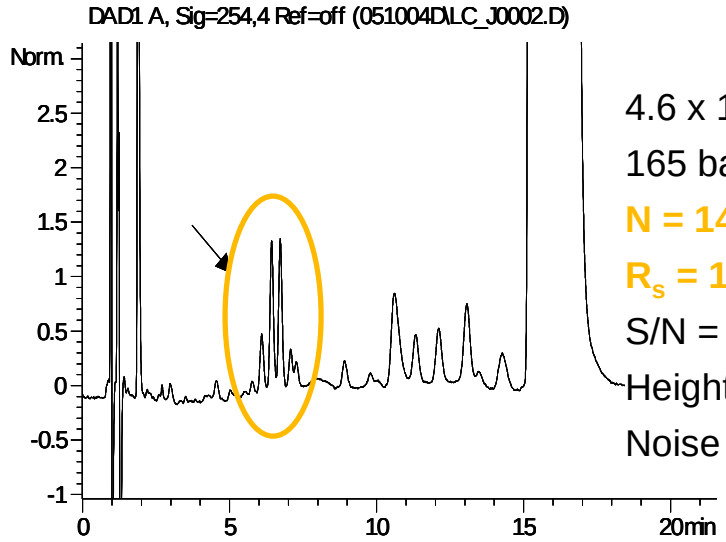
R_s = 1.15

S/N = 42

Height = 1.25

Noise = 24uAU





4.6 x 150, **3.5um**

165 bar

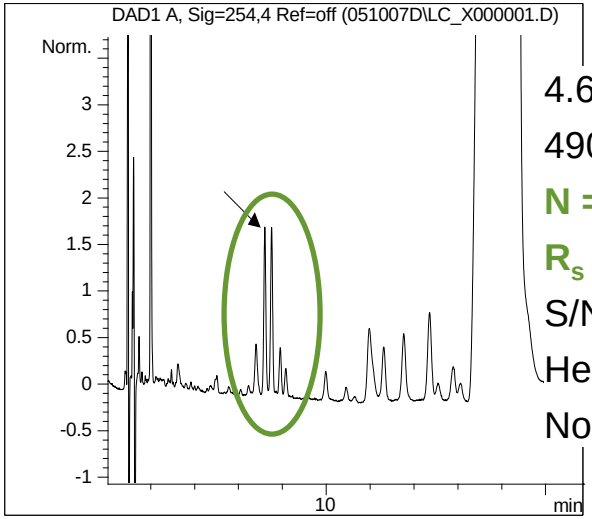
N = 14862

R_s = 1.37

S/N = 50

Height = 1.34

Noise = 20uAU



4.6 x 150, **1.8um**

490 bar

N = 28669

R_s = 1.80 (+57%)

S/N = 44

Height = 1.80

Noise = 30uAU

Where to begin?
Assess Your Current Method

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Assess your current method

4.6 x 150 mm, 5 μm column

1.5 mL/min

RT last = 14 minutes

Questions to ask?

What is the mobile phase composition?

What is the current backpressure?

Injection Volume?

Data Rate/Peak Width?

What is your limiting resolution with current method?

What size column can deliver the resolution you need?

Can your current instrument be used to apply a shorter column with smaller particle size?

Which changes in method parameters are necessary and can you get the same or similar performance and results?

"UHPLC" Increased Performance

What does this mean?

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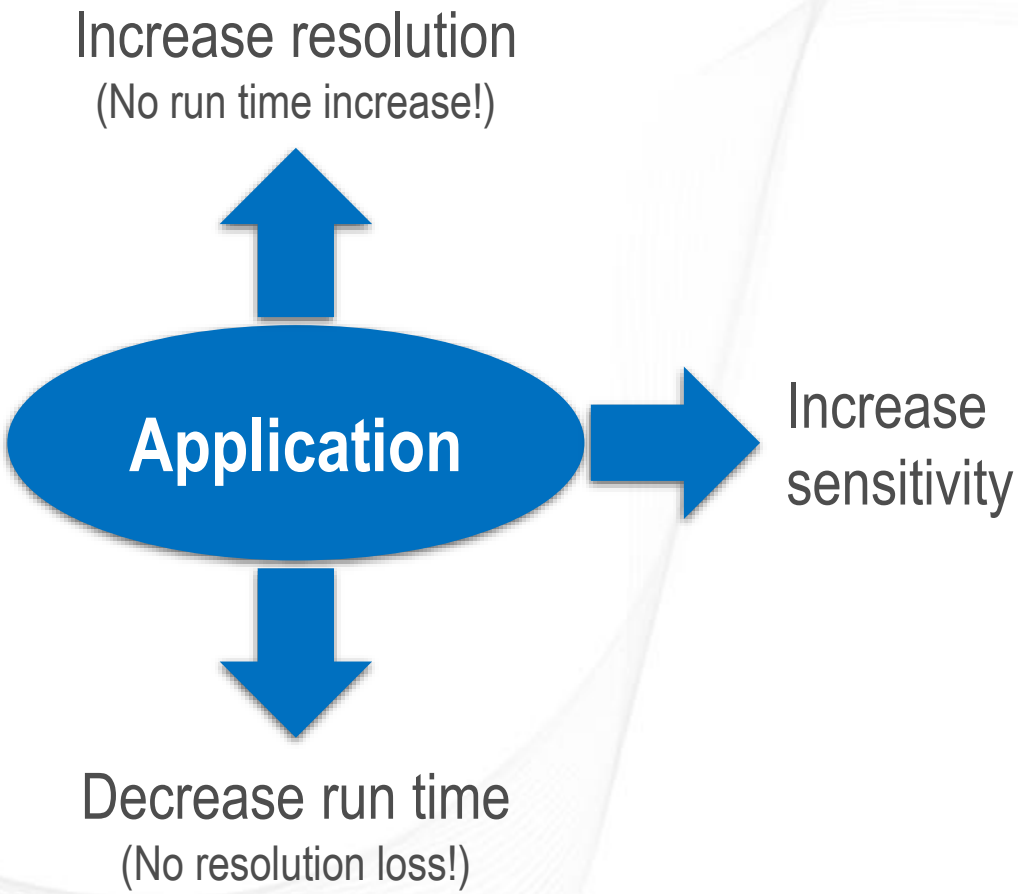

Increase Resolution – No Run Time Increase!

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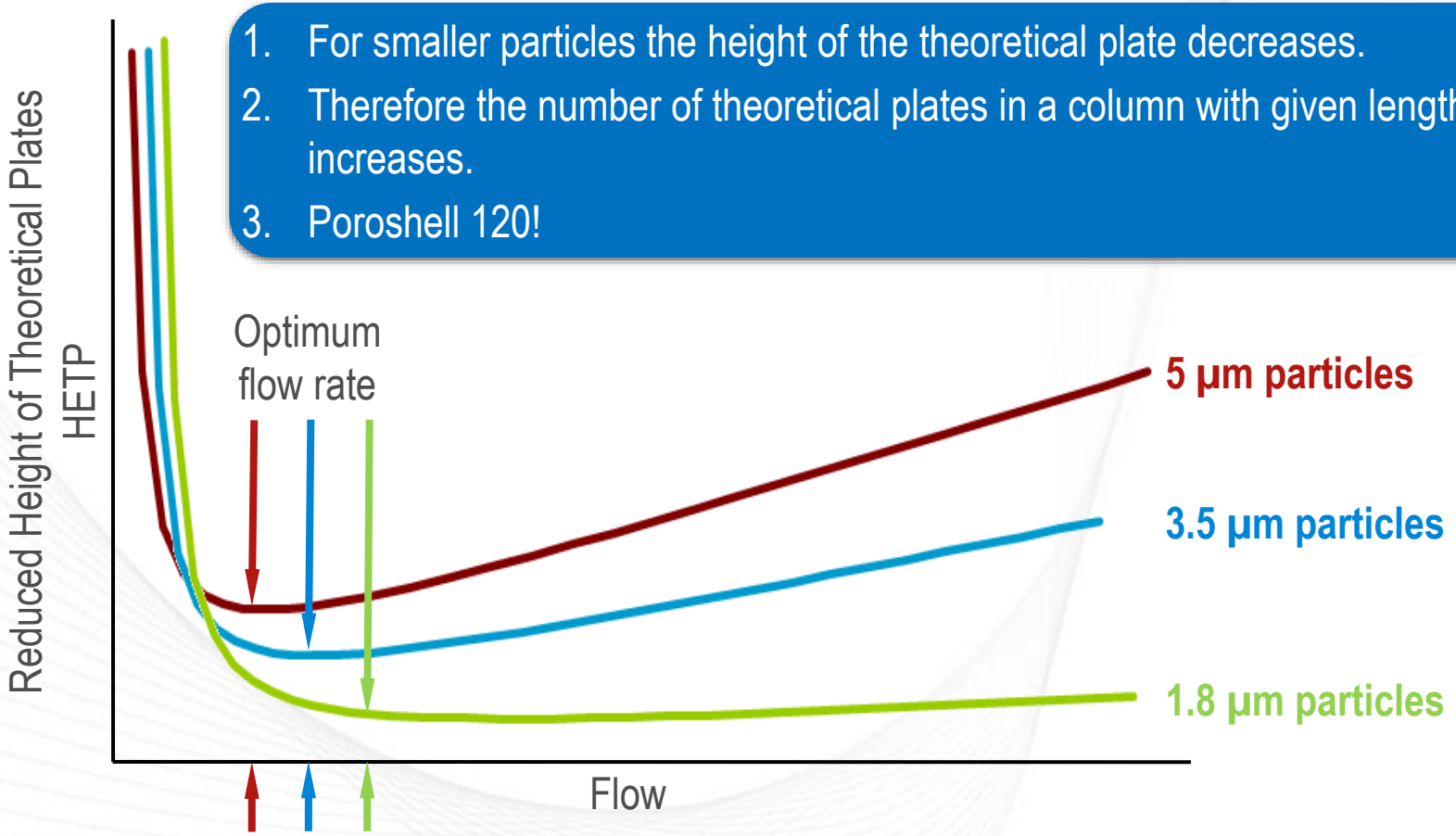
Column with higher number of theoretical plates required

1. For smaller particles the height of the theoretical plate decreases.
2. Therefore the number of theoretical plates in a column with given length increases.
3. Poroshell 120!



Increase Resolution

No Run Time Increase!

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Column with higher number of theoretical plates required

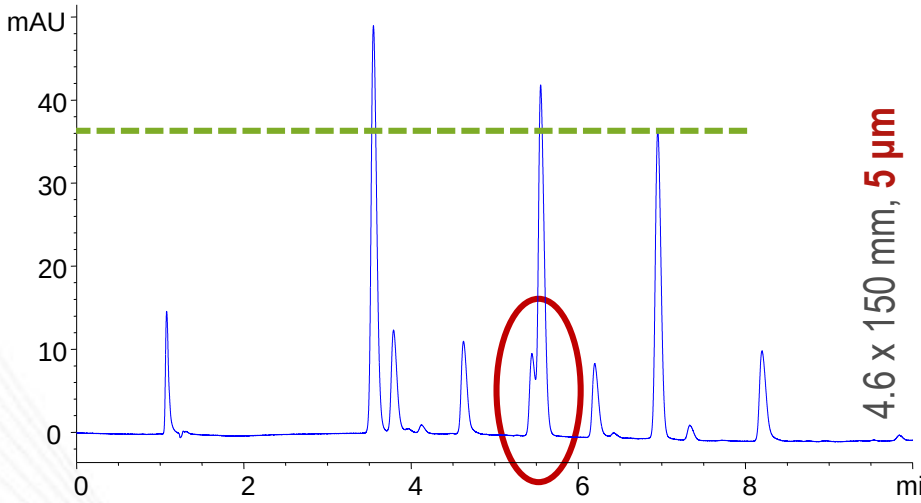
Column length (mm)	Number of plates N 5 µm particles	Number of plates N 3.5 µm particles	Number of plates N 1.8 µm particles
250	21750		
150	**13050** →	18650 →	36250
100	8700	12400	24150
50	4350	6200	12100

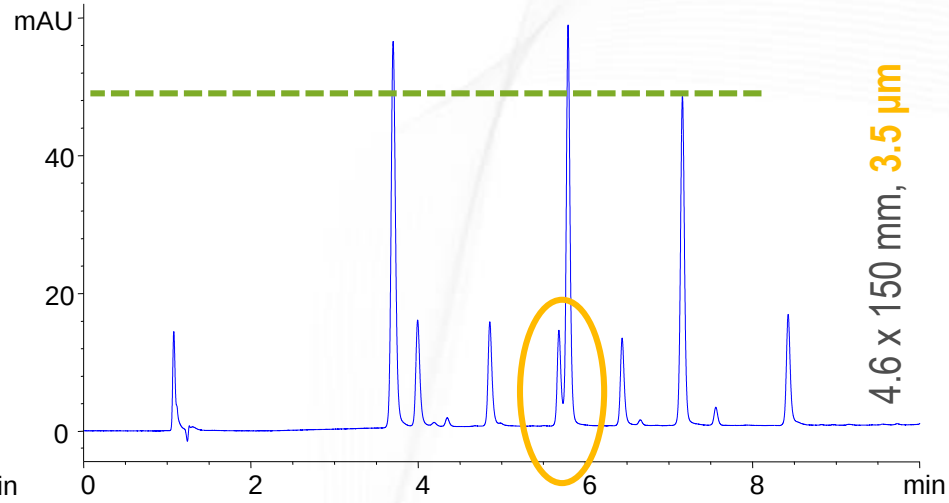
Increase in column length will also increase run time (adjust gradient time)

Decrease in particle size will increase pressure

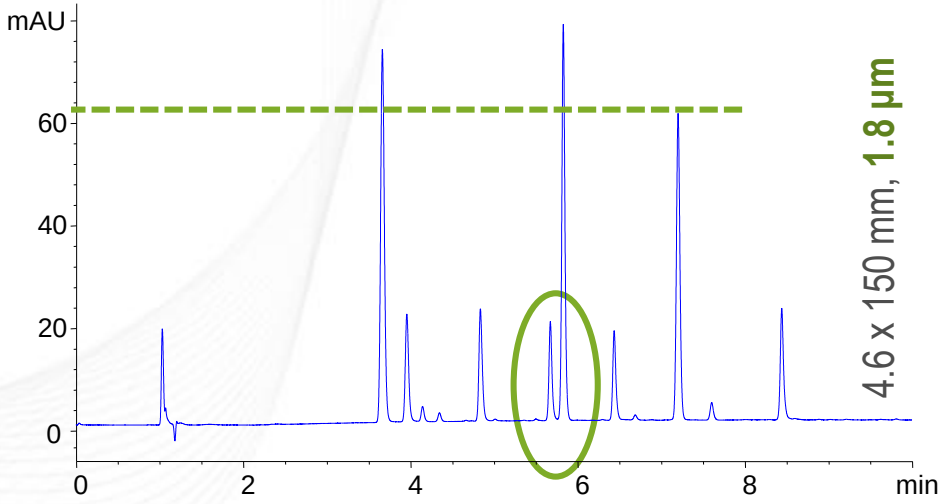
Increase Resolution

No Run Time Increase!





- **Increased** resolution due to reduced peak width
- **Increased** peak height due to reduced peak width gives increased sensitivity



Example 1: Salmeterol Xinafoate

EP Related substances

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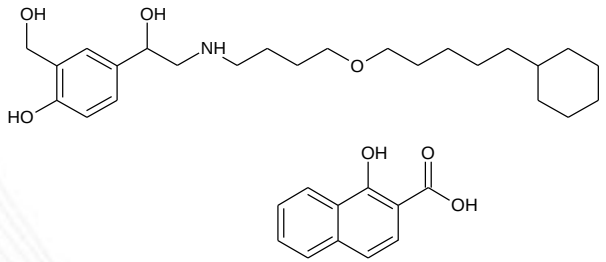
Structure



Suitability requirements

RRT: Check the RRT
Peak to valley ratio: Minimum 10 between imp E and Salmeterol
Chromatogram: Obtained is similar to the chromatogram supplied with system suit sample.

Method

Mode: LC
Detector: UV 278 nm
Column: 4.6-mm x 15-cm; 5-µm packing L1
Flow rate: 2 mL/min
Inj. size: 10 µL

Solution A:
7.71 g/L solution of ammonium acetate

Solution B:
28.84 g/L solution of sodium dodecyl sulphate , adjust the pH to 2.7 with glacial acetic acid

Mobile phase A:
Solution A : Solution B: Acetonitrile; 24:24:52

Mobile phase B:
Acetonitrile

Gradient

Time (min)	Sol. A (%)	Sol. B (%)
0	70	30
1	70	30
20	5	95
25	5	95
27	70	30
35	70	30

Example 1: Salmeterol Xinafoate

EP Related substances

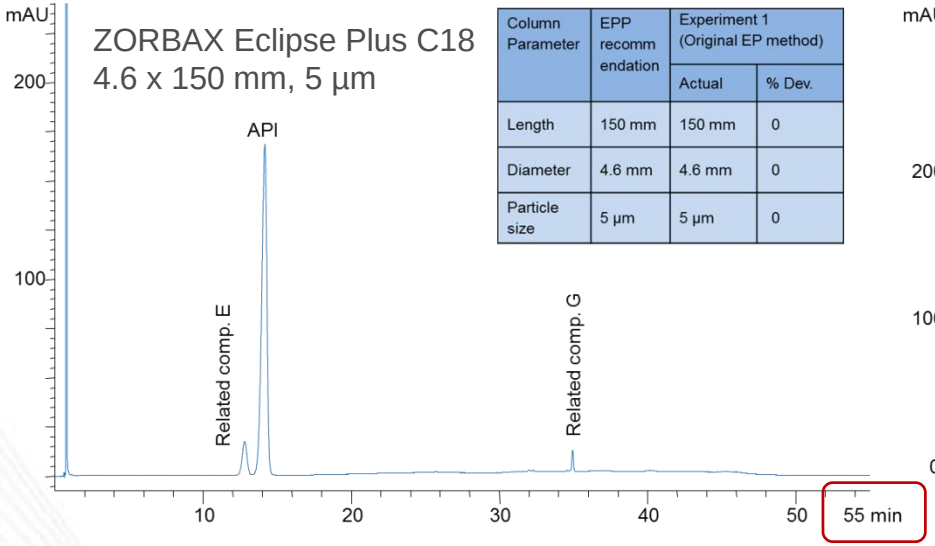
- EP 2.2.46E allows only changes of ± 25 %.
- Column inner diameter according to EP method is 4.6 mm.
- Going down to a 3 mm column would already be outside the allowed limit (- 34 %).
- Therefore no change in column diameter allowed.

Example 1: Salmeterol Xinafoate

EP Related substances

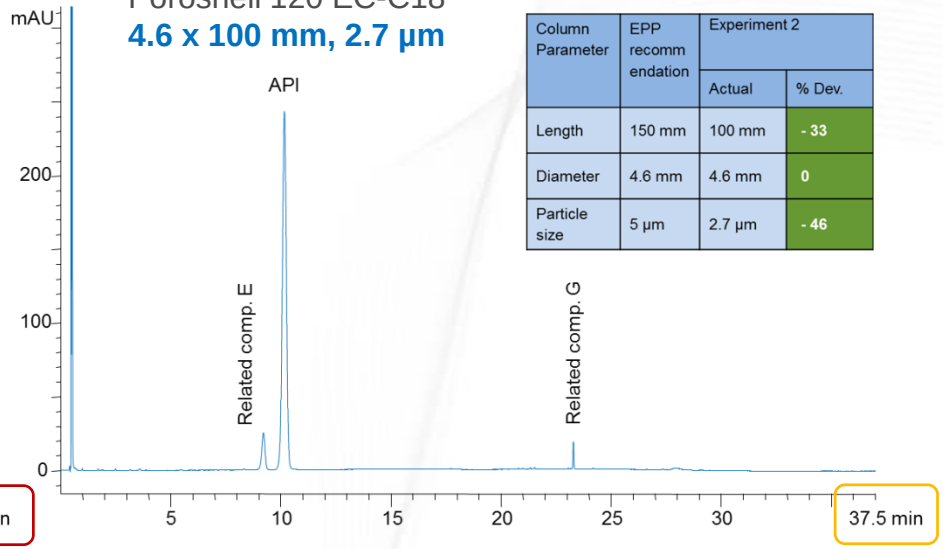
ZORBAX Eclipse Plus C18
4.6 x 150 mm, 5 µm

Column Parameter	EPP recommendation	Experiment 1 (Original EP method)	
		Actual	% Dev.
Length	150 mm	150 mm	0
Diameter	4.6 mm	4.6 mm	0
Particle size	5 µm	5 µm	0



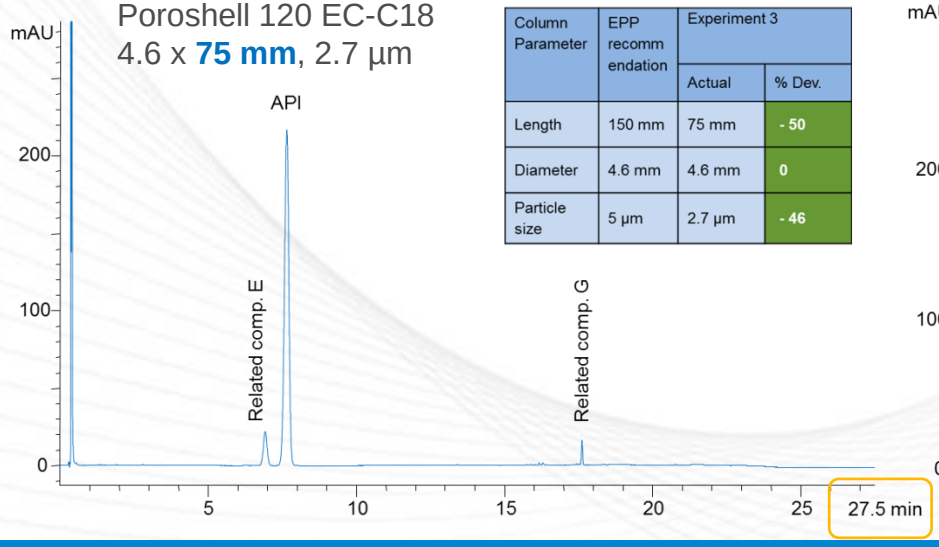
Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 µm

Column Parameter	EPP recommendation	Experiment 2	
		Actual	% Dev.
Length	150 mm	100 mm	- 33
Diameter	4.6 mm	4.6 mm	0
Particle size	5 µm	2.7 µm	- 46



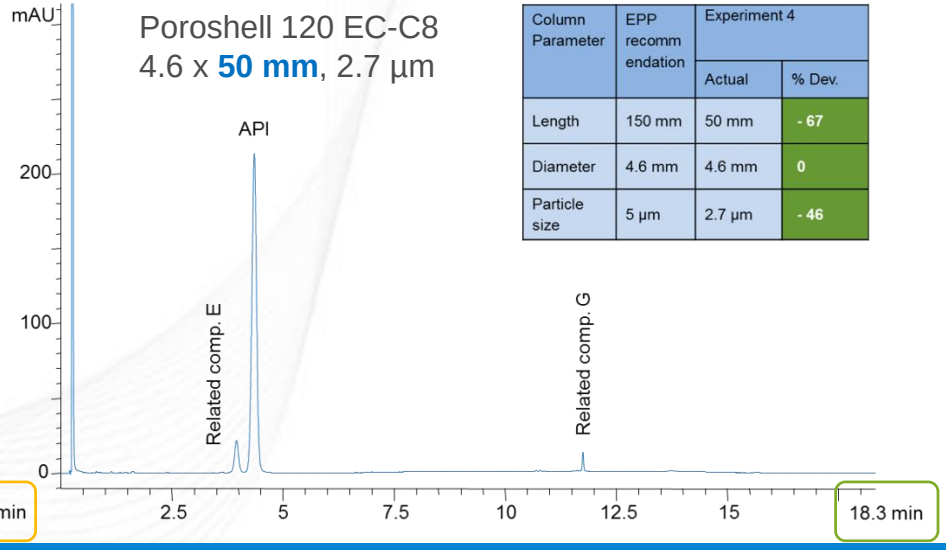
Poroshell 120 EC-C18
4.6 x **75 mm**, 2.7 µm

Column Parameter	EPP recommendation	Experiment 3	
		Actual	% Dev.
Length	150 mm	75 mm	- 50
Diameter	4.6 mm	4.6 mm	0
Particle size	5 µm	2.7 µm	- 46



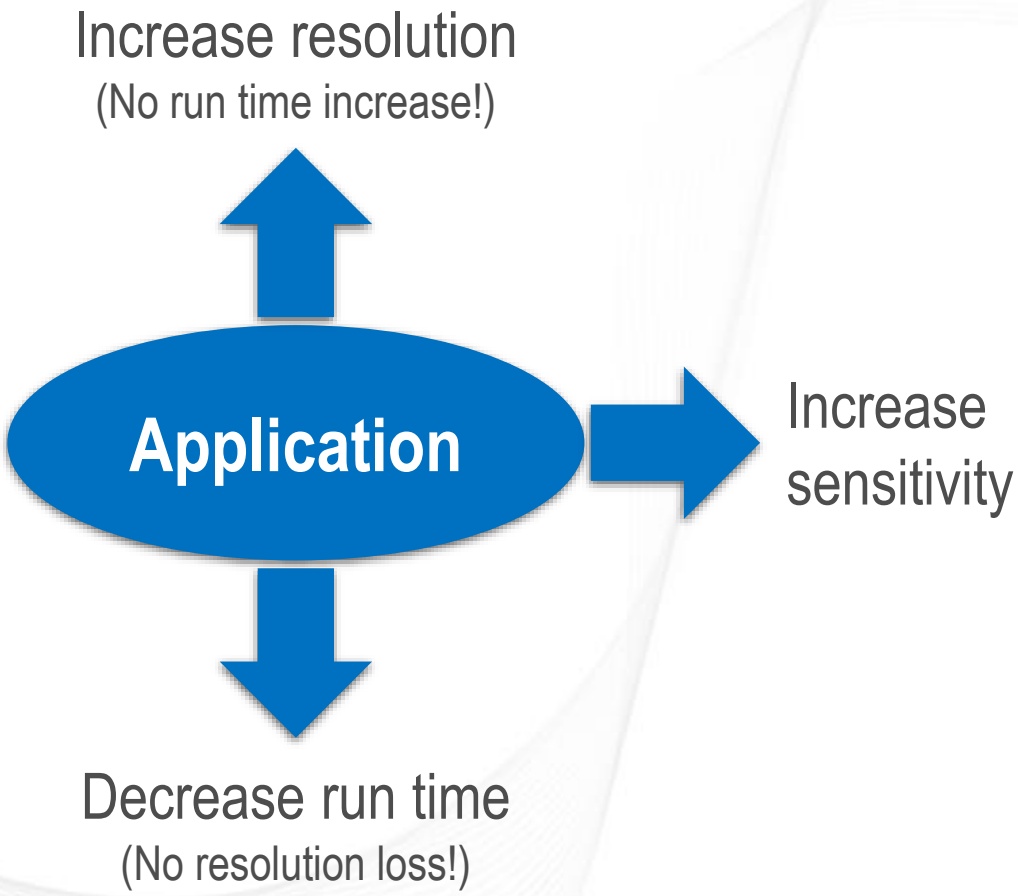
Poroshell 120 EC-C8
4.6 x **50 mm**, 2.7 µm

Column Parameter	EPP recommendation	Experiment 4	
		Actual	% Dev.
Length	150 mm	50 mm	- 67
Diameter	4.6 mm	4.6 mm	0
Particle size	5 µm	2.7 µm	- 46



"UHPLC" Increased Performance

What does this mean?

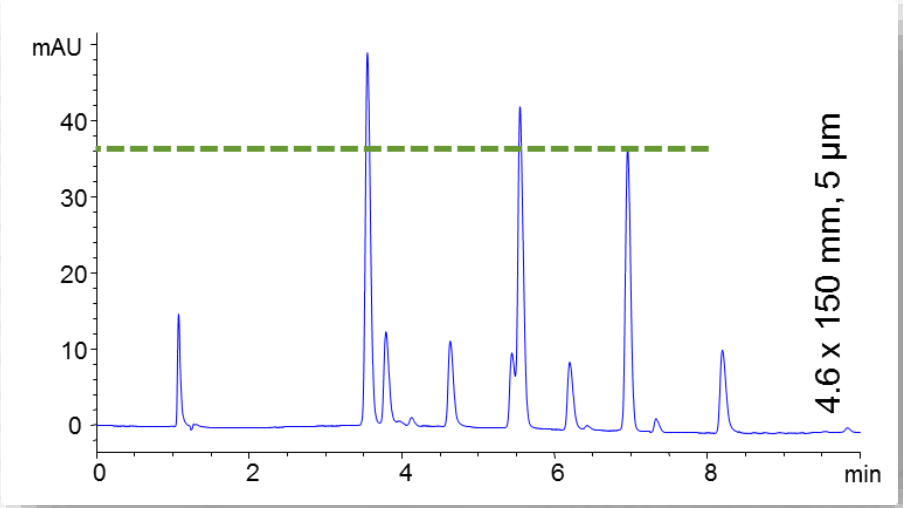


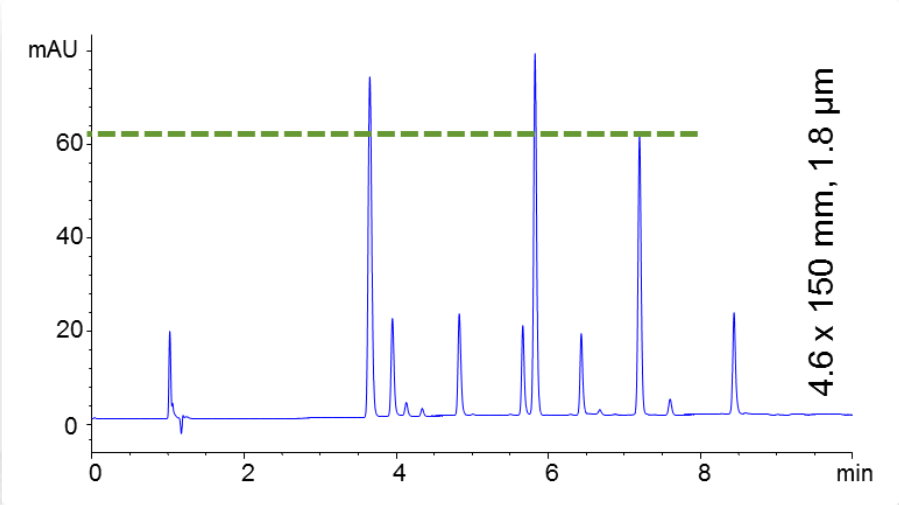
Increase Sensitivity

Smaller particles give increased peak height

$$A_{max} \sim \frac{c_i V_i}{d_c^2} \sqrt{\frac{N}{2\pi}}$$

A:	Absorption
c_i:	Sample concentration
V_i:	Injection volume
V_R:	Retention volume
N:	Number of theoretical plates





"UHPLC" Increased Performance

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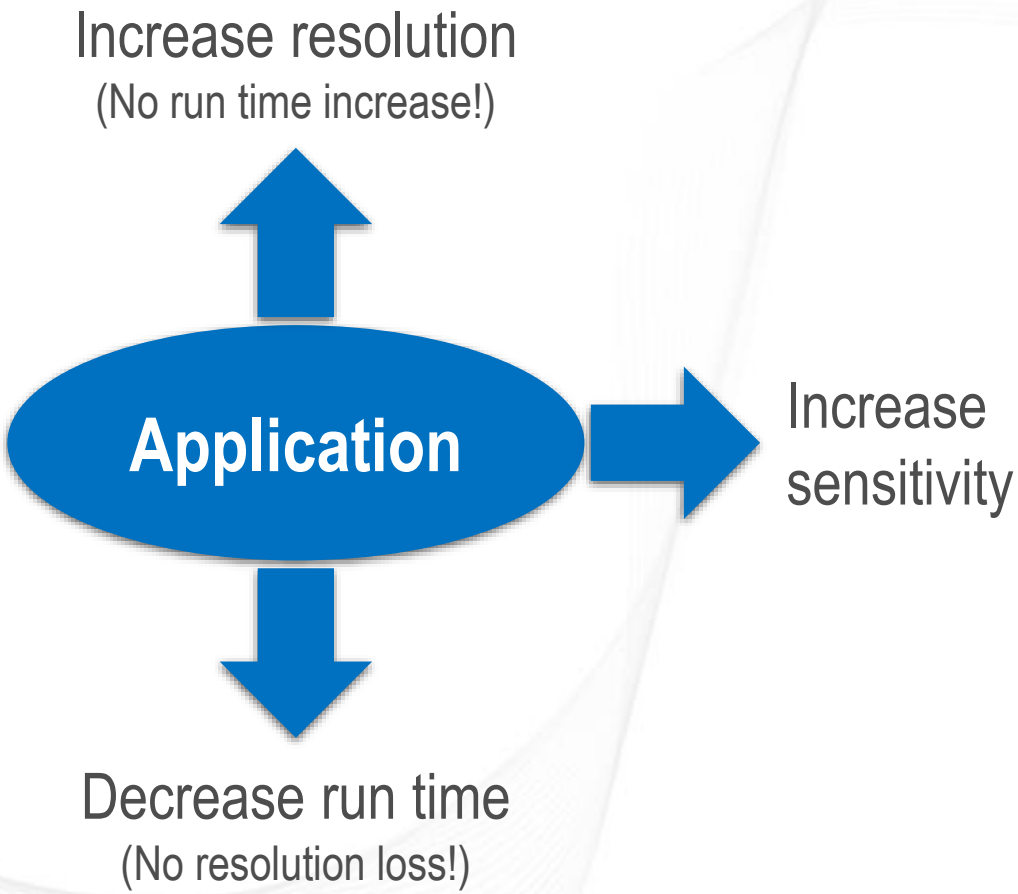
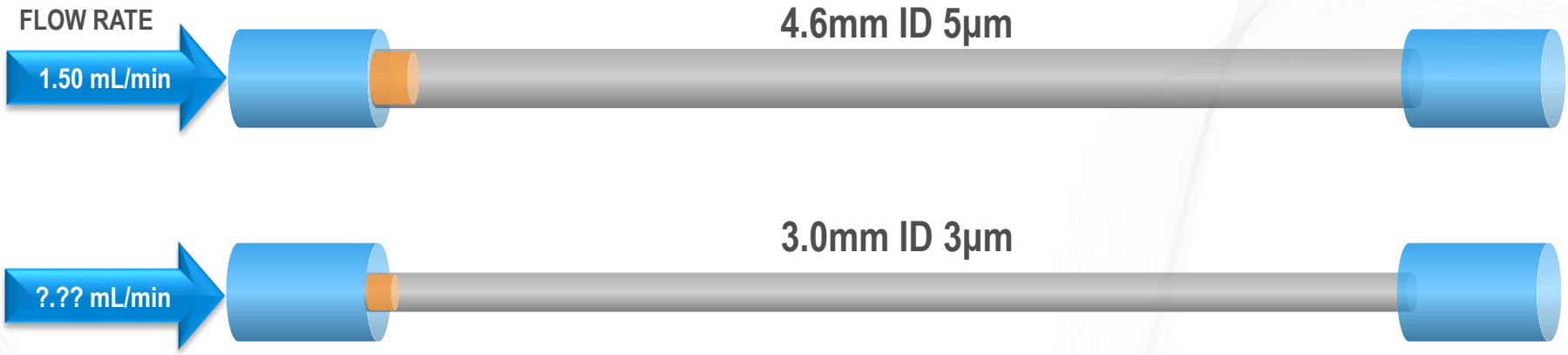


Scaling Flow Rate to the Column Dimension

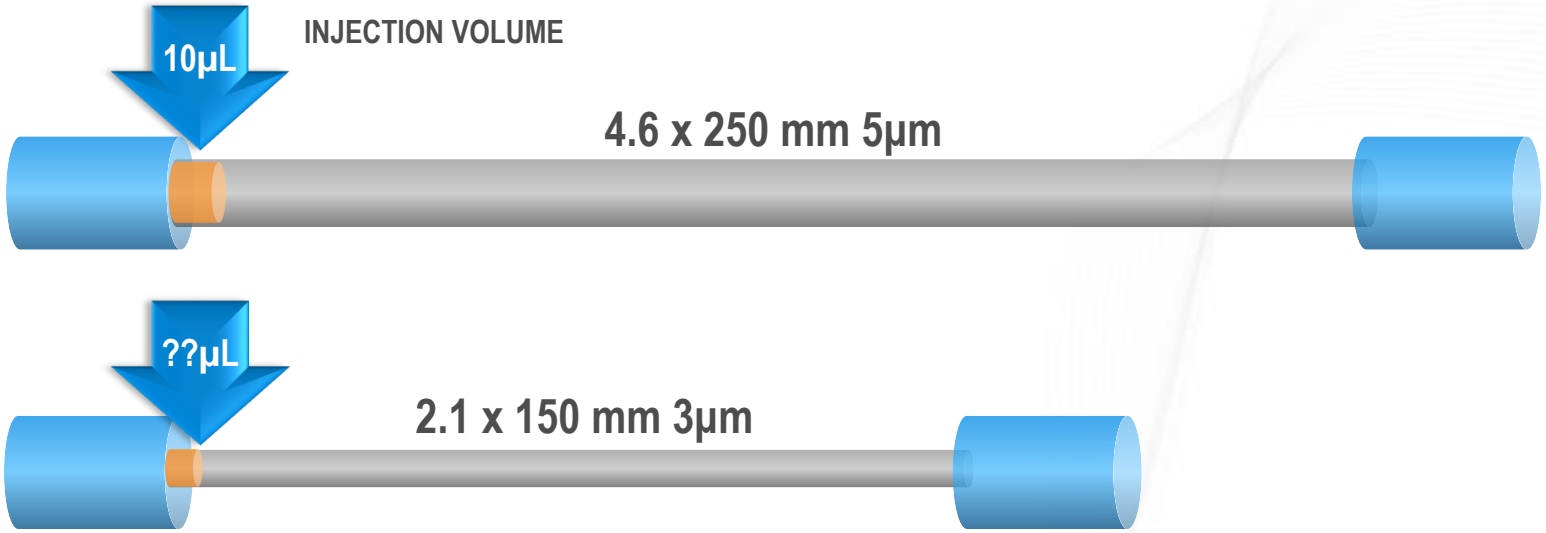


Target flow rate (*F*) adapting linear velocity

$$F_2 = F_1 \cdot \frac{d_{c2}^2}{d_{c1}^2}$$

= *1.07* mL/min

Scaling Injection Volume to the Column Dimension



Scale injection volume (V_{Inj}) to cross section and reduced zone dilution in shorter column length *L (band broadening)*

$$V_{Inj_{new}} = V_{Inj_{old}} \cdot \left(\frac{d_{c,new}}{d_{c,old}} \right)^2 \cdot \sqrt{\frac{L_{new} \cdot d_{p,new}}{L_{old} \cdot d_{p,old}}}$$

= 1.3 µL

Analysis Time Depends on Column, Flow Rate, Sample and Resolution Needs

Column - Short efficient columns minimize analysis and equilibration time

Flow Rate - Higher flow rates reduce analysis and equilibration time

Sample - How many components? What volume do you need to inject?

Resolution - UV detection or LC/MS, screening or quantification

Instrument - Minimize extra-column volume and for gradient run minimize dwell volume

Decrease Run Time

No Resolution Loss!

Shorter column with same number of theoretical plates required

Column length (mm)	Number of plates N 5 µm particles	Number of plates N 3.5 µm particles	Number of plates N 1.8 µm particles
250	21750		
150	**13050**	18650	36250
100	8700	12400	24150
50	4350	6200	12100

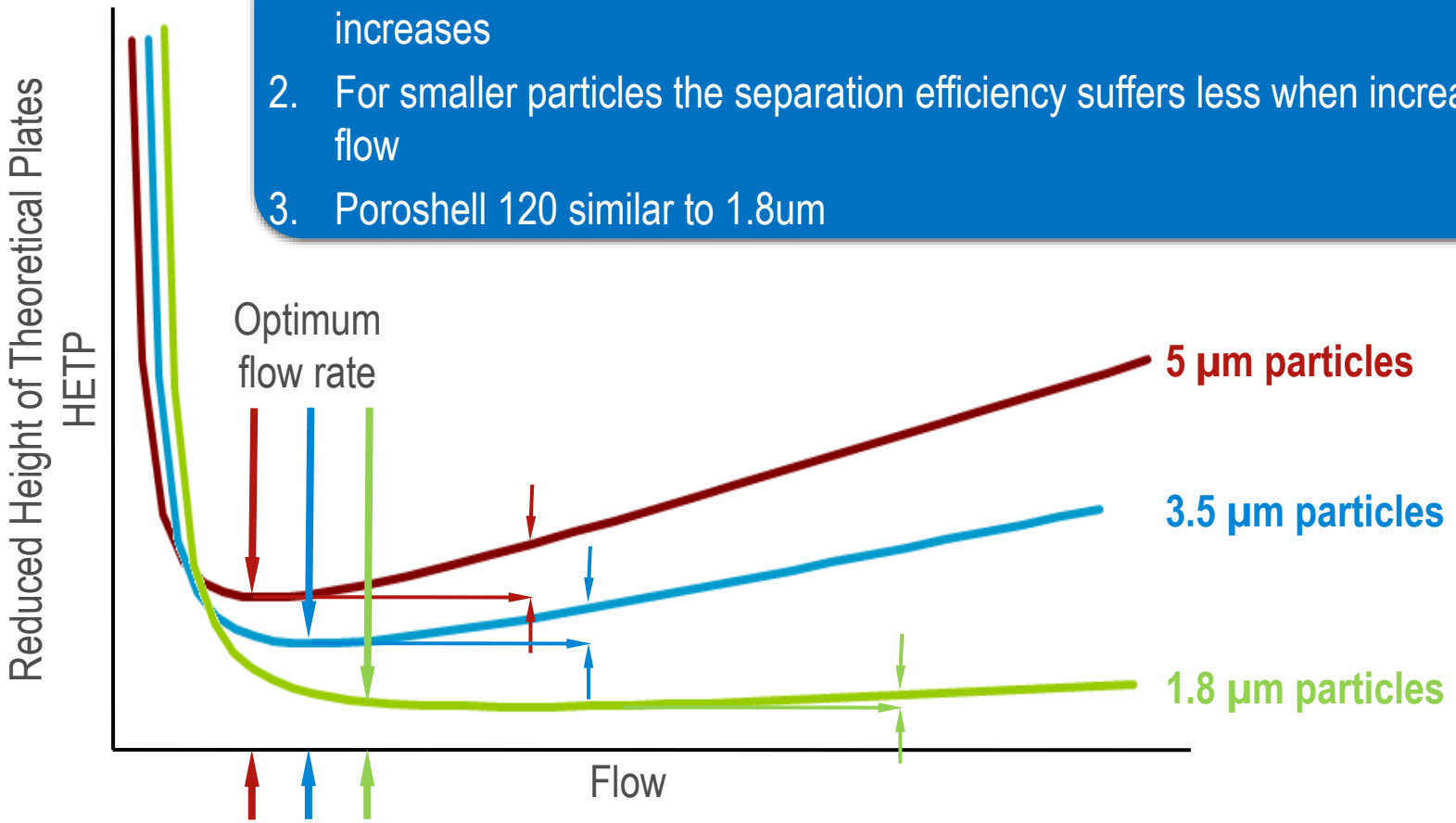
Decrease in column length will decrease run time (adjust gradient time)

Decrease in particle size will maintain resolution

Decrease Run Time – No Resolution Loss!

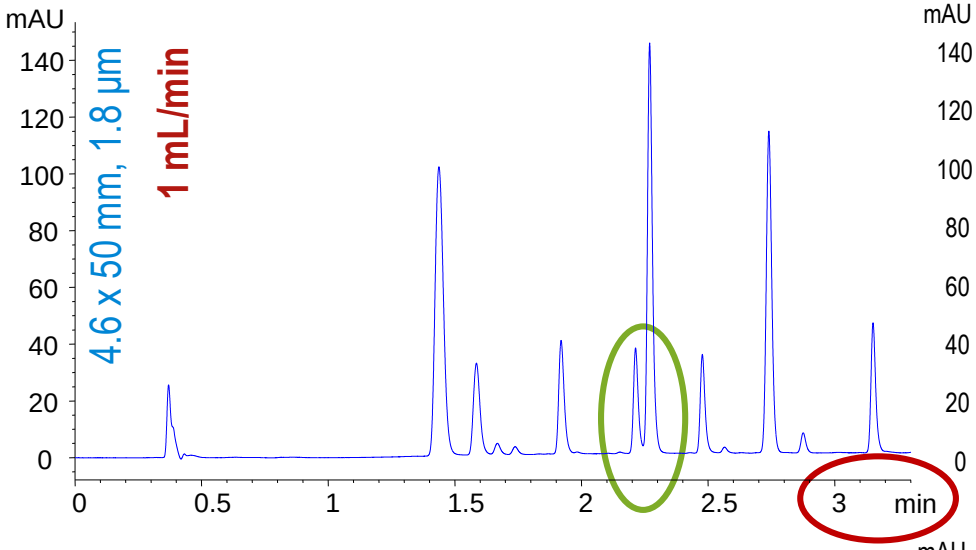
Ideal flow rate for sub-2-micron particles

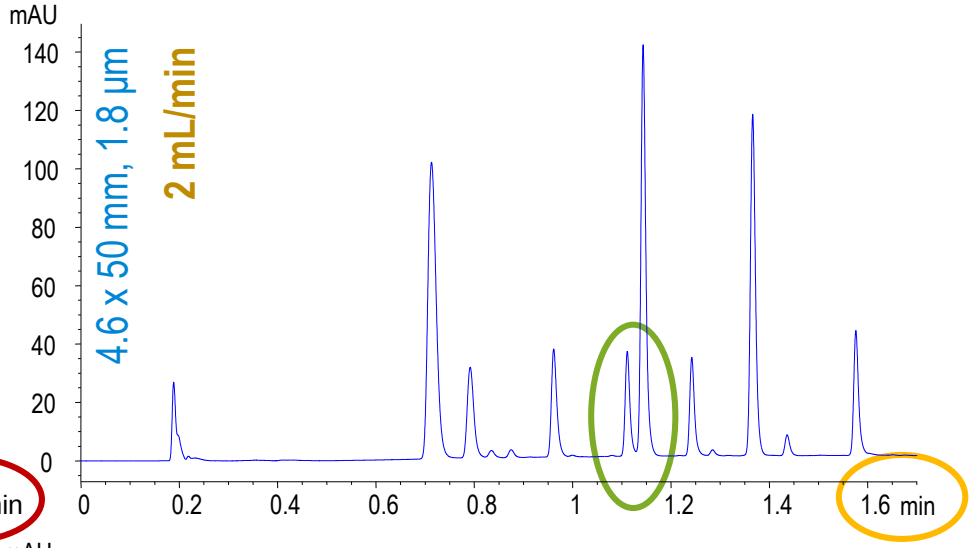
1. For smaller particles the optimum flow rate (lowest height of a theoretical plate) increases
2. For smaller particles the separation efficiency suffers less when increasing the flow
3. Poroshell 120 similar to 1.8um



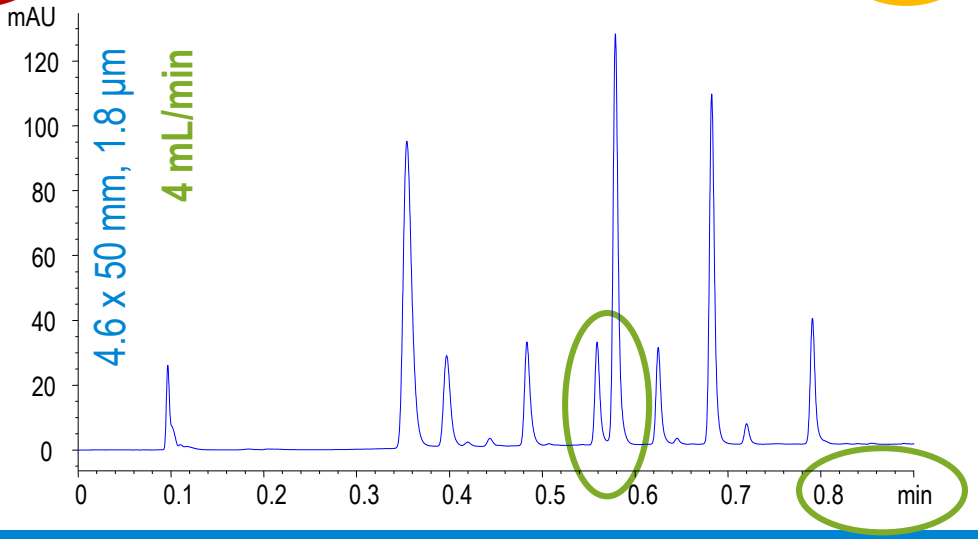
Decrease Run Time

No Resolution Loss!





- Decreased run time due to increased flow rate
- No loss in resolution, number of theoretical plates not influenced by flow rate



Resolution Relationship for Gradient Elution

$$R \approx \frac{\sqrt{N}}{4} \alpha k^*$$

k* - represents the fact that k changes constantly during a gradient

Gradient Retention

$$k^* = \frac{t_g F}{S\,(\Delta\%B)\,V_m}$$

$\Delta\%B$	=	difference between initial and final % B values
S	=	constant (≈ 4 for 100 - 500 Da)
F	=	flow rate (ml/min.)
t_g	=	gradient time (min.)
V_m	=	column void volume (ml)

This Relationship Says that to Keep Relative Peak Position in the Chromatogram Unchanged

Any Decrease in

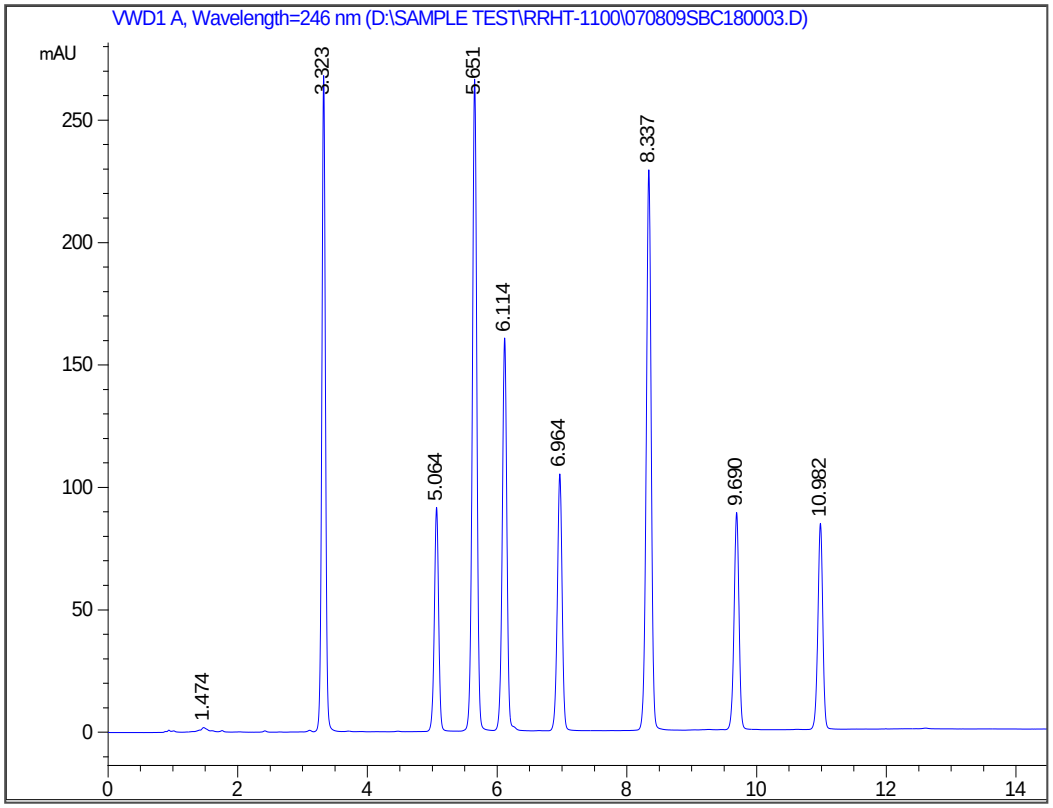
- Column length
- Column volume (i.d.)
- $\Delta\Phi$ (same column)

Can be Offset by a Proportional

- Decrease in $\mathbf{t_G}$ or F
- Increase in $\Delta\Phi$
- Decrease in t_G or **F**
- Increase in $\Delta\Phi$
- Decrease in $\mathbf{t_G}$ or F

$$k^* = \frac{t_G \bullet F}{S \bullet \Delta\Phi \bullet Vm}$$

Conventional Column - 4.6 x 150mm, 5µm, SB-C18



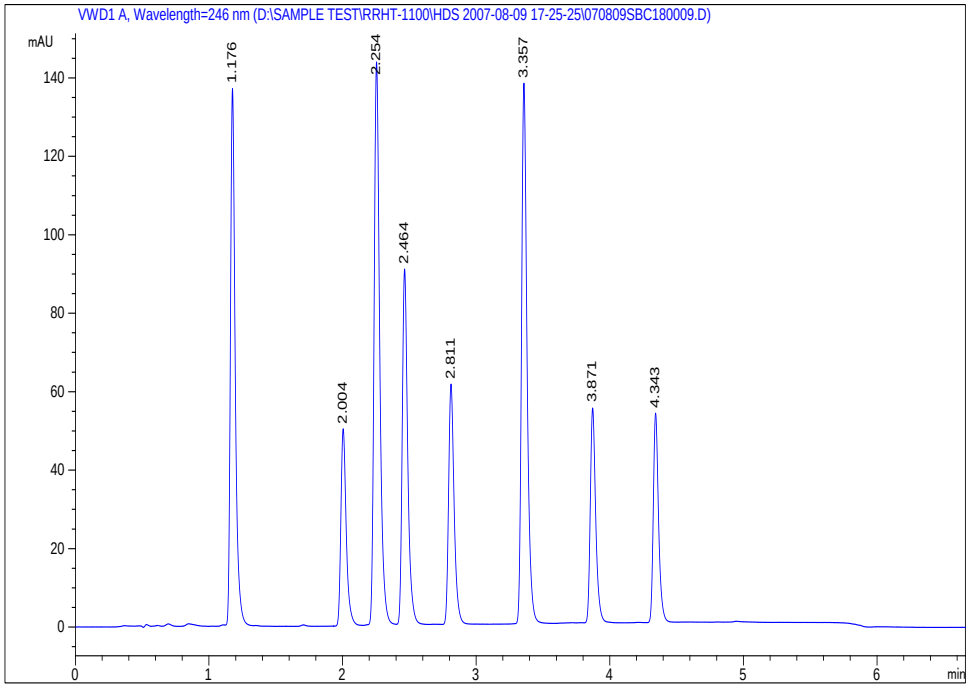
Flow Rate	1.0 ml/min
Injection Volume	15uL
Temperature	30° C
Wavelength	246nm
Sample rate	2.5 Hz

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

Shorten Column and Gradient Time by Same Factor

1/3 Column Length- 1/3 Gradient Time

RRHT Column – 4.6 x 50mm, 1.8µm, SB-C18



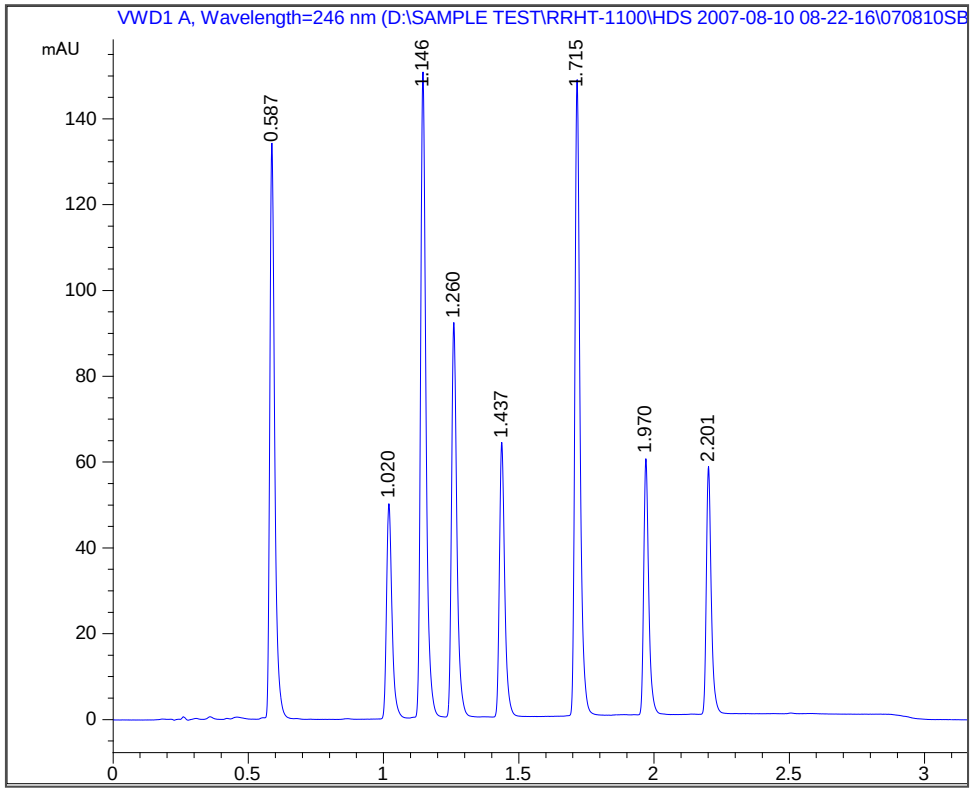
Flow Rate	1.0 ml/min
Injection Volume	5uL
Temperature	30° C
Wavelength	246nm
Sample rate	*13.74 Hz*

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

Increase Column Flow-Reduce Gradient Time

Double Flow (2mL/min) – ½ Gradient Time

RRHT 4.6 x 50mm, 1.8µm, SB-C18



Flow Rate	2.0 ml/min
Injection Volume	5uL
Temperature	30° C
Wavelength	246nm
Sample rate	13.74 Hz

Time (min)	% Acetonitrile
0	50
1.67	90
2.25	90
2.27	50
3.34	50

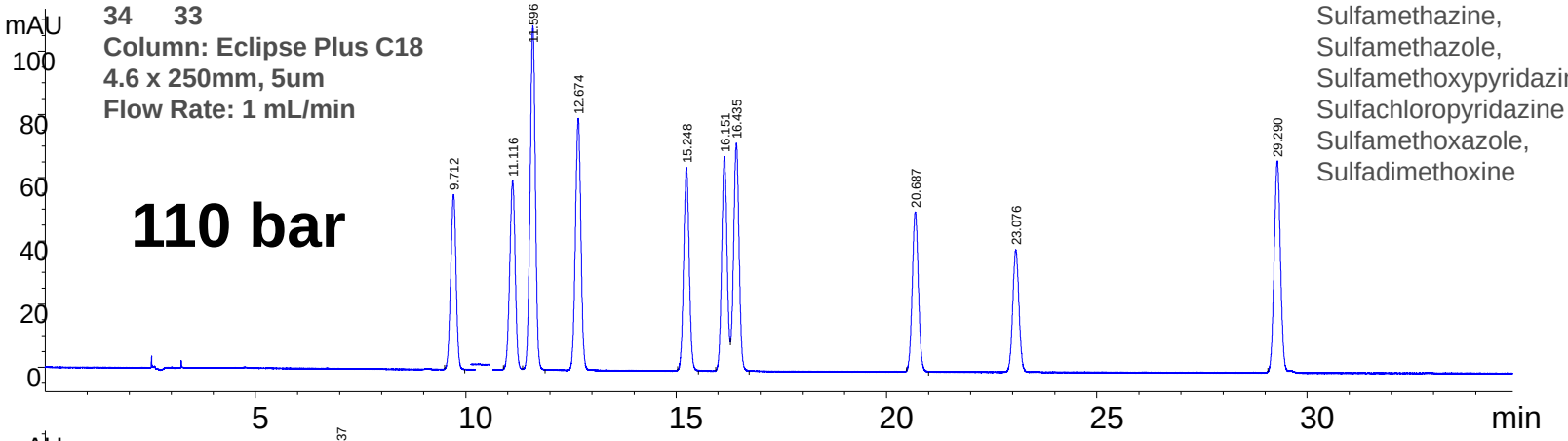
Comparison of 4.6 x 250 mm 5 um to Poroshell 120 EC-C18 4.6 x 100 mm, 2.7um

Time %B
0 8
33 33
34 33
Column: Eclipse Plus C18
4.6 x 250mm, 5um
Flow Rate: 1 mL/min

Mobile Phase:
A: 0.1% formic acid in Water
B: 0.1% formic acid in ACN

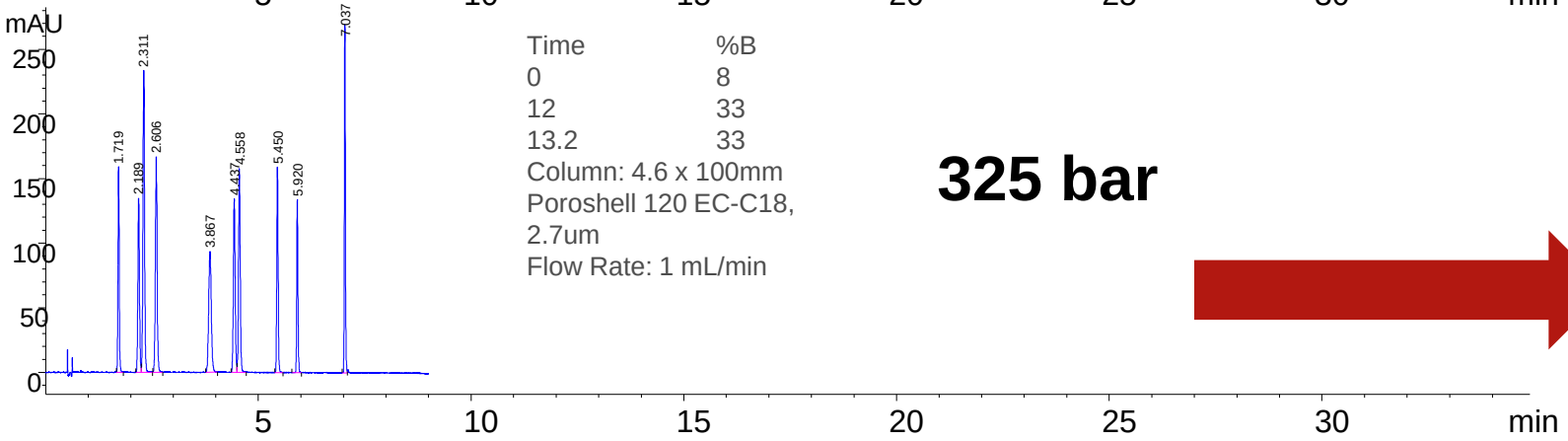
Sulfadiazine,
Sulfathiazole
Sulfapyridine
Sulfamerazine,
Sulfamethazine,
Sulfamethazole,
Sulfamethoxypyridazine,
Sulfachloropyridazine
Sulfamethoxazole,
Sulfadimethoxine

110 bar

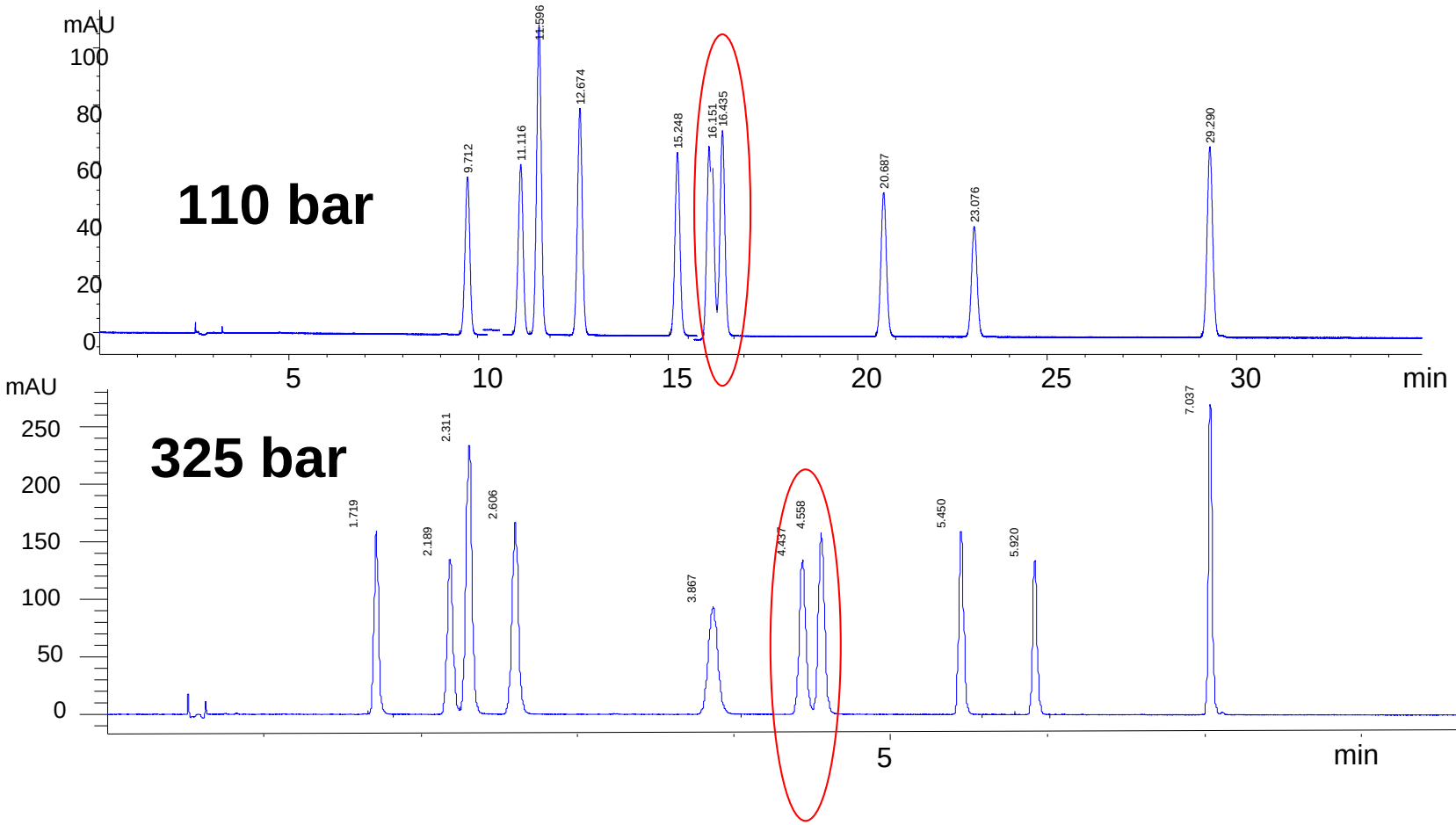


Time %B
0 8
12 33
13.2 33
Column: 4.6 x 100mm
Poroshell 120 EC-C18,
2.7um
Flow Rate: 1 mL/min

325 bar



Expand High Speed Chromatograms for True Comparison to Slower Separation



Agilent 1290 Infinity II LC

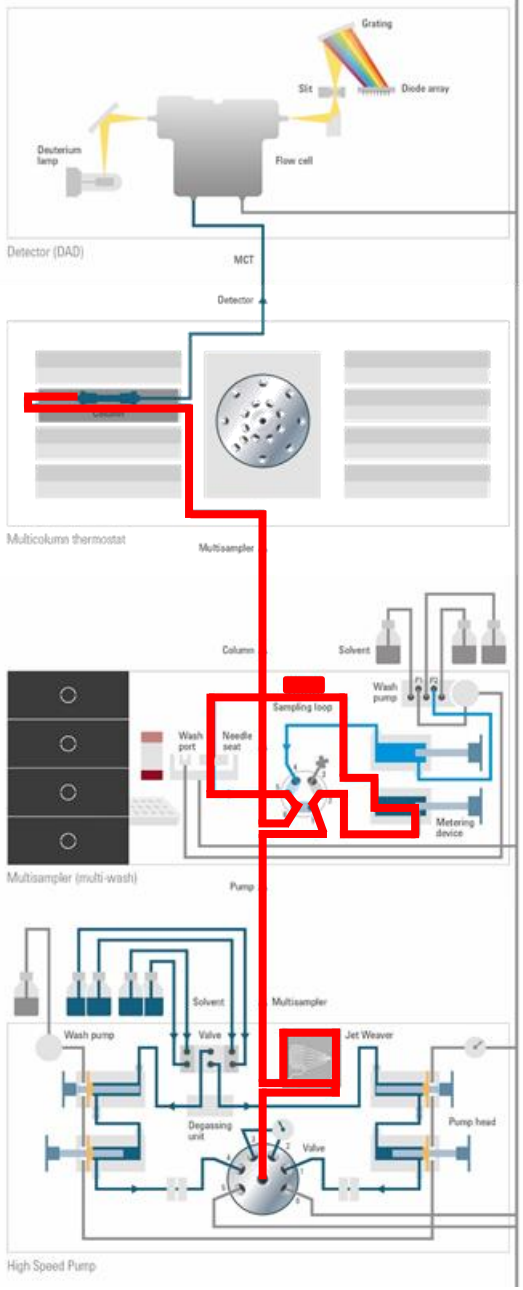
System Design

Gradient Delay Volume

Affects our results:

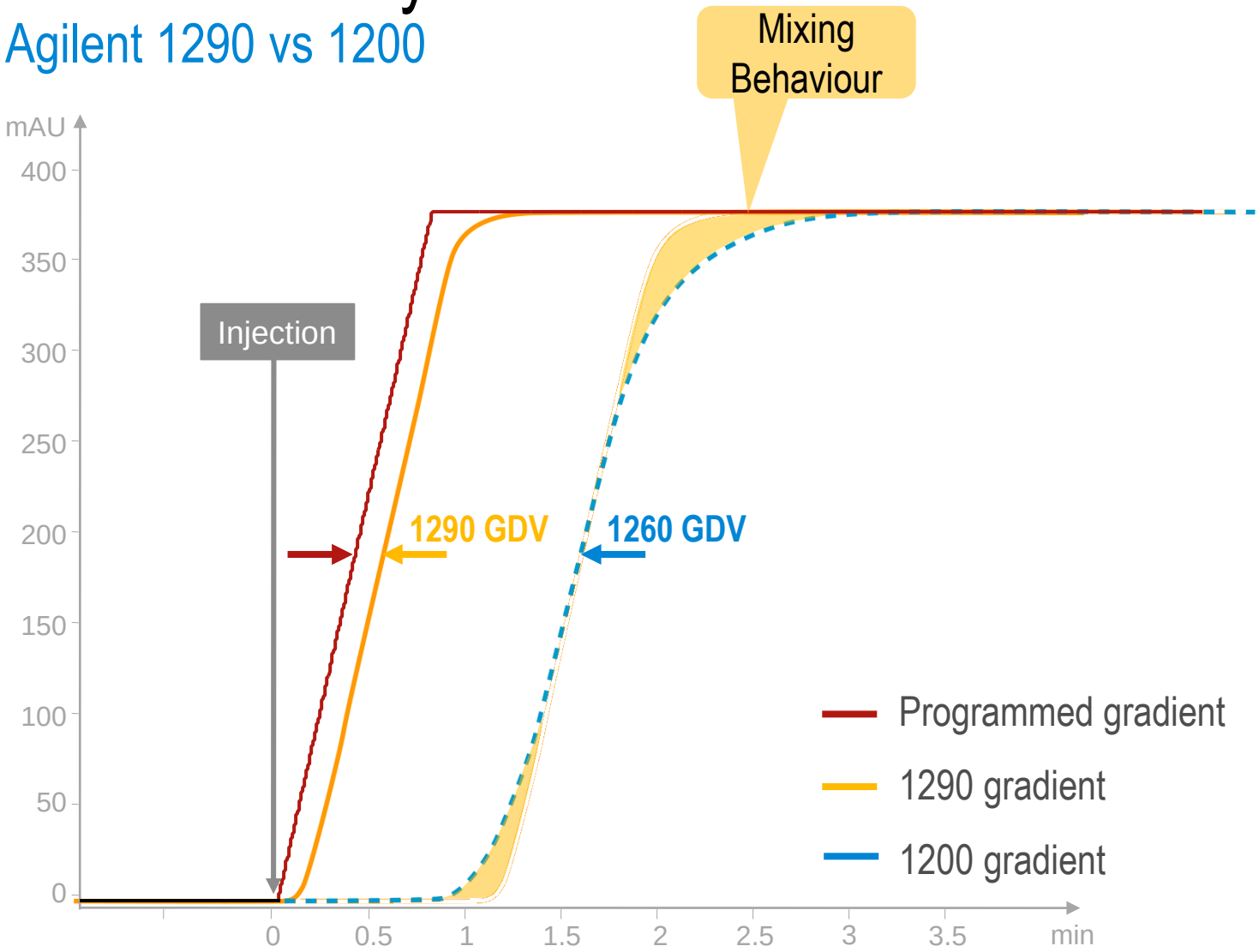
- an isocratic hold step at the beginning of every gradient
- sharpness of the gradient
- required equilibration time and therefore total cycle time

Early eluting peaks are more affected than later eluting peaks



Gradient Delay Volume

Agilent 1290 vs 1200



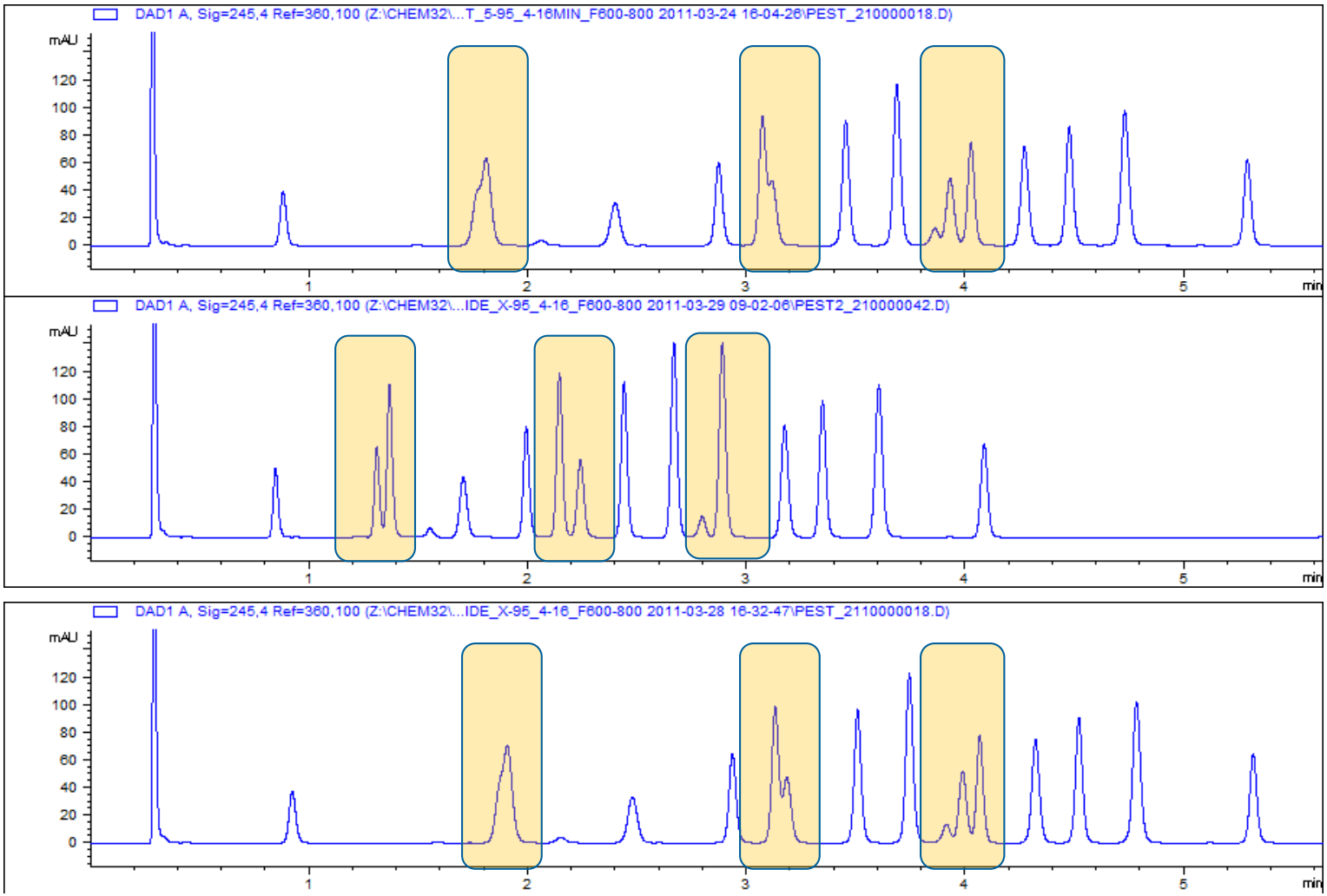
Method Transfer Example, Pesticides

2.1x100 mm Zorbax Eclipse Plus, 1.8 µm column, Flow: 0,8 mL/min

1100 Quaternary 400 bar

1290 Infinity II 1300 bar

1290 Infinity II 1300 bar with ISET

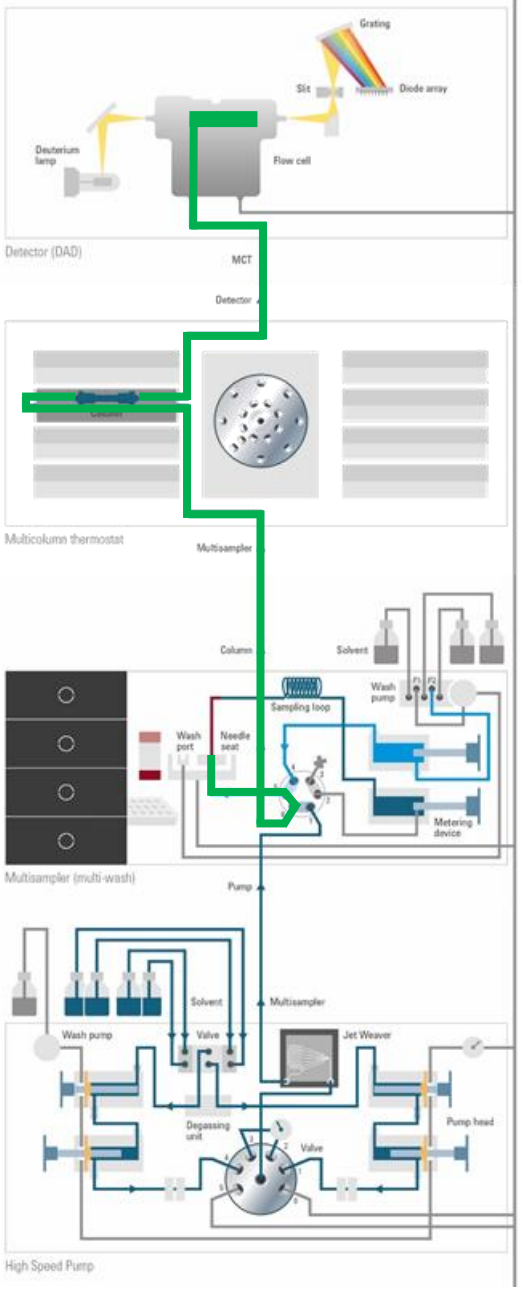


Instrument

System Design

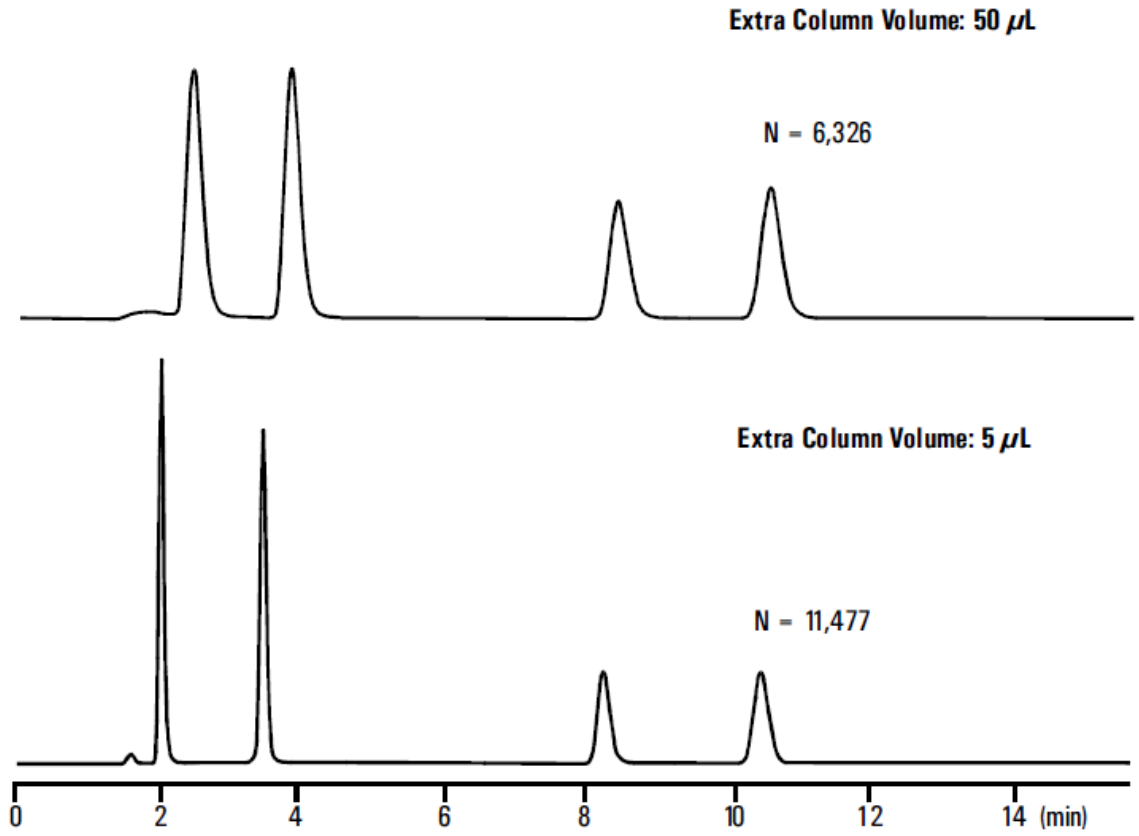
Extra Column Volume

- Extra column band broadening affects resolution and detection sensitivity
- 2.1mm ID columns and smaller are significantly affected by extra column volume effects!



System Design – Extra Column Volume Effects

The Effects of Extra-Column Volume on Narrow-Bore (2.1x150 mm) Column Performance



Volume Characteristics

Column	Internal Column Volume	Calculated Peak Volume (4s)
1.0 x 150 mm	0.09 mL	13 µL
2.1 x 150 mm	0.35 mL	52 µL
3.0 x 150 mm	0.70 mL	112 µL
4.6 x 75 mm	0.80 mL	120 µL
4.6 x 150 mm	1.60 mL	260 µL

Instrument
System Design

Extra Column Volume

- Keep injector to column and column to detector tubing length and ID as small as possible.
- Make sure all fittings are made correctly. ***[A-Line Quick Connect!]***
- Rule of thumb: keep extra column volume below 1/10th of peak volume
- Use flow cell with appropriate cell volume: Rule of thumb is 1/10th of peak volume

Using Small Volume Columns for Isocratic and Gradient Separations

Critical instrument parameters include:

Isocratic and Gradient:

- Injection Conditions
- *Data rate*
- Optimized Lamp/Light Path
- *Flow cell size*

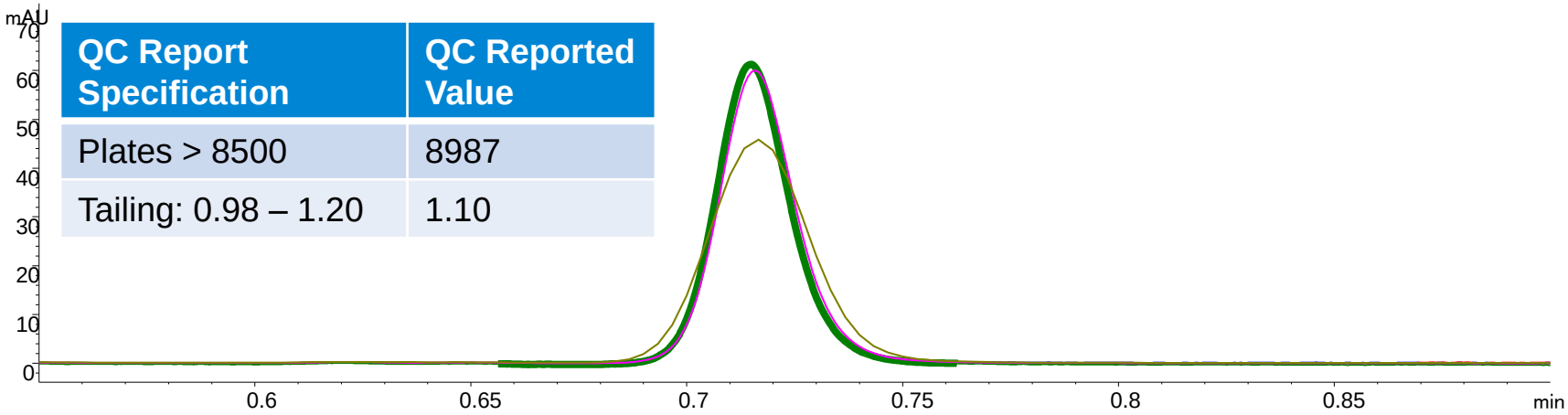
Gradient only:

- Mixing volume (part of gradient delay volume)
- Gradient Dwell Volume

To Ensure the Best Performance of a 2.1 x 50 mm, 2.7 µm Poroshell 120 EC-C18 Column: Optimize the Data Collection Rate

System Modifications	Pressure (bar)	k' (naphthalene)	TF(naphthalene)	N(naphthalene)
Starting Method: DAD = 5 Hz	159	2.3	1.06	3962
Increase DAD to 20 Hz	159	2.3	1.14	7786
Increase DAD to 80 Hz	**159**	**2.3**	**1.15**	**8317**

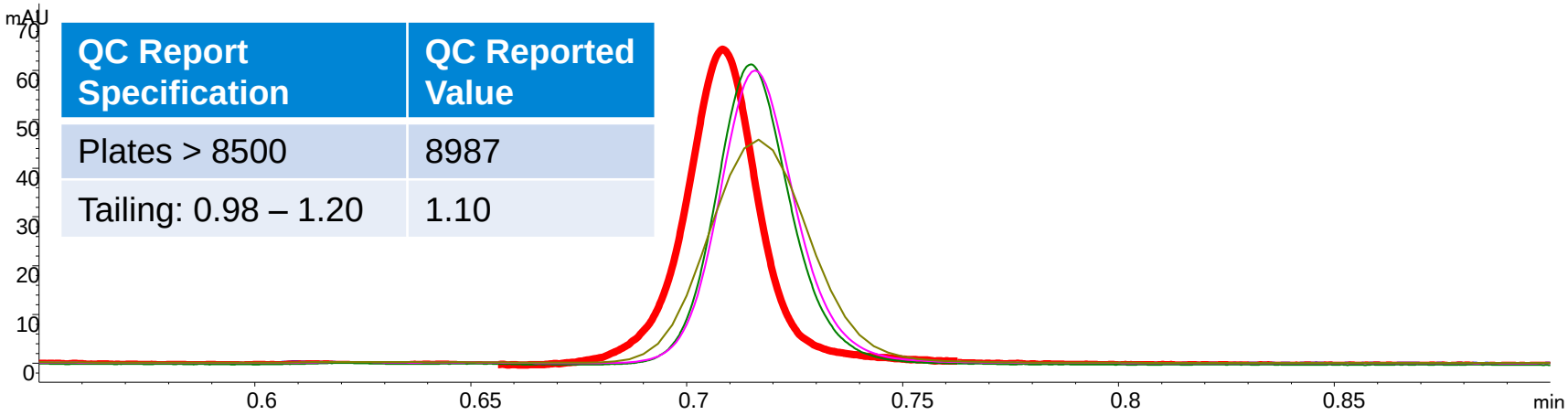
QC Report Specification	QC Reported Value
Plates > 8500	8987
Tailing: 0.98 – 1.20	1.10



A: water, B: acetonitrile, 0.50 mL/min at 65% B, 0.5 µL injection of Newport QC standard), 80 µg/mL naphthalene, 25 °C, DAD: 254, 8 nm at 80, 20 or 5 Hz

To Ensure the Best Performance of a 2.1 x 50 mm, 2.7 µm Poroshell 120 EC-C18 Column: Reduce LC System Extra-Column Volume

System Modifications	Pressure (bar)	k' (naphthalene)	TF(naphthalene)	N(naphthalene)
Starting Method: DAD = 5 Hz	159	2.3	1.06	3962
Increase DAD to 20 Hz	159	2.3	1.14	7786
Increase DAD to 80 Hz	159	2.3	1.15	8317
Exchange flow path for 0.08 mm id cap + 0.6 µL flow cell	**188**	**2.4**	**1.00**	**9387**



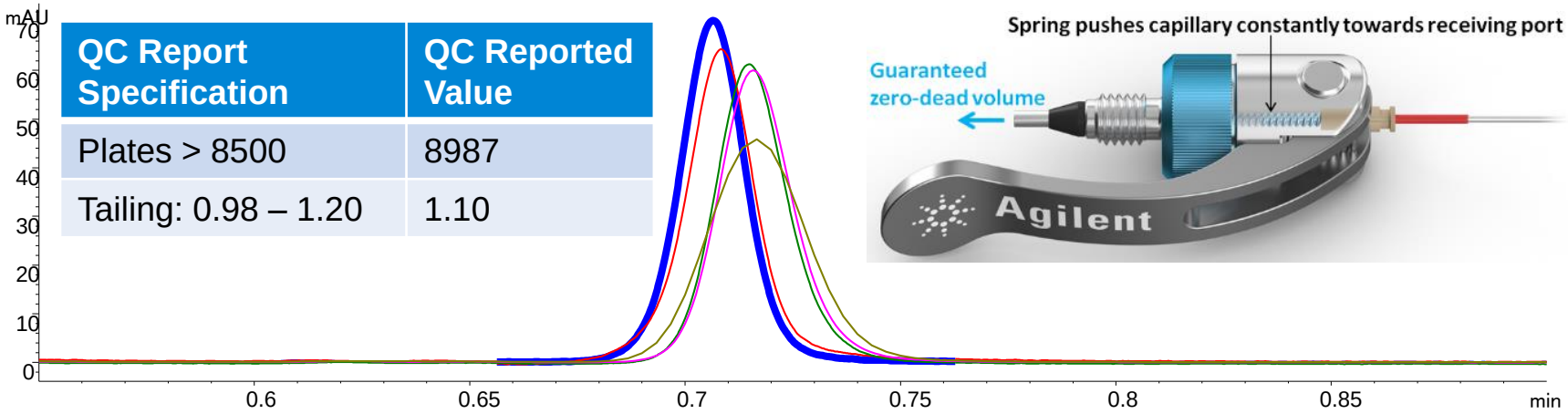
QC Report Specification	QC Reported Value
Plates > 8500	8987
Tailing: 0.98 – 1.20	1.10

A: water, B: acetonitrile, 0.50 mL/min at 65% B, 0.5 µL injection of Newport QC standard), 80 µg/mL naphthalene, 25 °C, DAD: 254, 8 nm at 80, 20 or 5 Hz

To Ensure the Best Performance of a 2.1 x 50 mm, 2.7 µm Poroshell 120 EC-C18 Column: Improve LC Capillary Connections

System Modifications	Pressure (bar)	k' (naphthalene)	TF(naphthalene)	N(naphthalene)
Starting Method: DAD = 5 Hz	159	2.3	1.06	3962
Increase DAD to 20 Hz	159	2.3	1.14	7786
Increase DAD to 80 Hz	159	2.3	1.15	8317
Exchange flow path for 0.08 mm id cap + 0.6 µL flow cell	188	2.4	1.00	9387
Install A-Line Fitting	**182**	**2.4**	**1.00**	**9917**

QC Report Specification	QC Reported Value
Plates > 8500	8987
Tailing: 0.98 – 1.20	1.10



A: water, B: acetonitrile, 0.50 mL/min at 65% B, 0.5 µL injection of Newport QC standard), 80 µg/mL naphthalene, 25 °C, DAD: 254, 8 nm at 80, 20 or 5 Hz

Agilent 1290 Infinity II LC

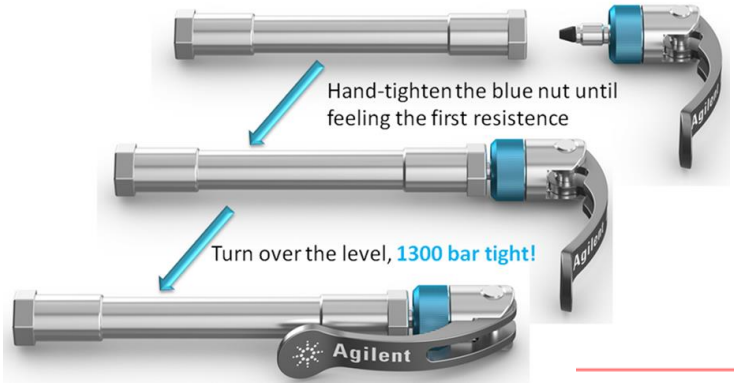
Lowest Dispersion for Highest Resolution

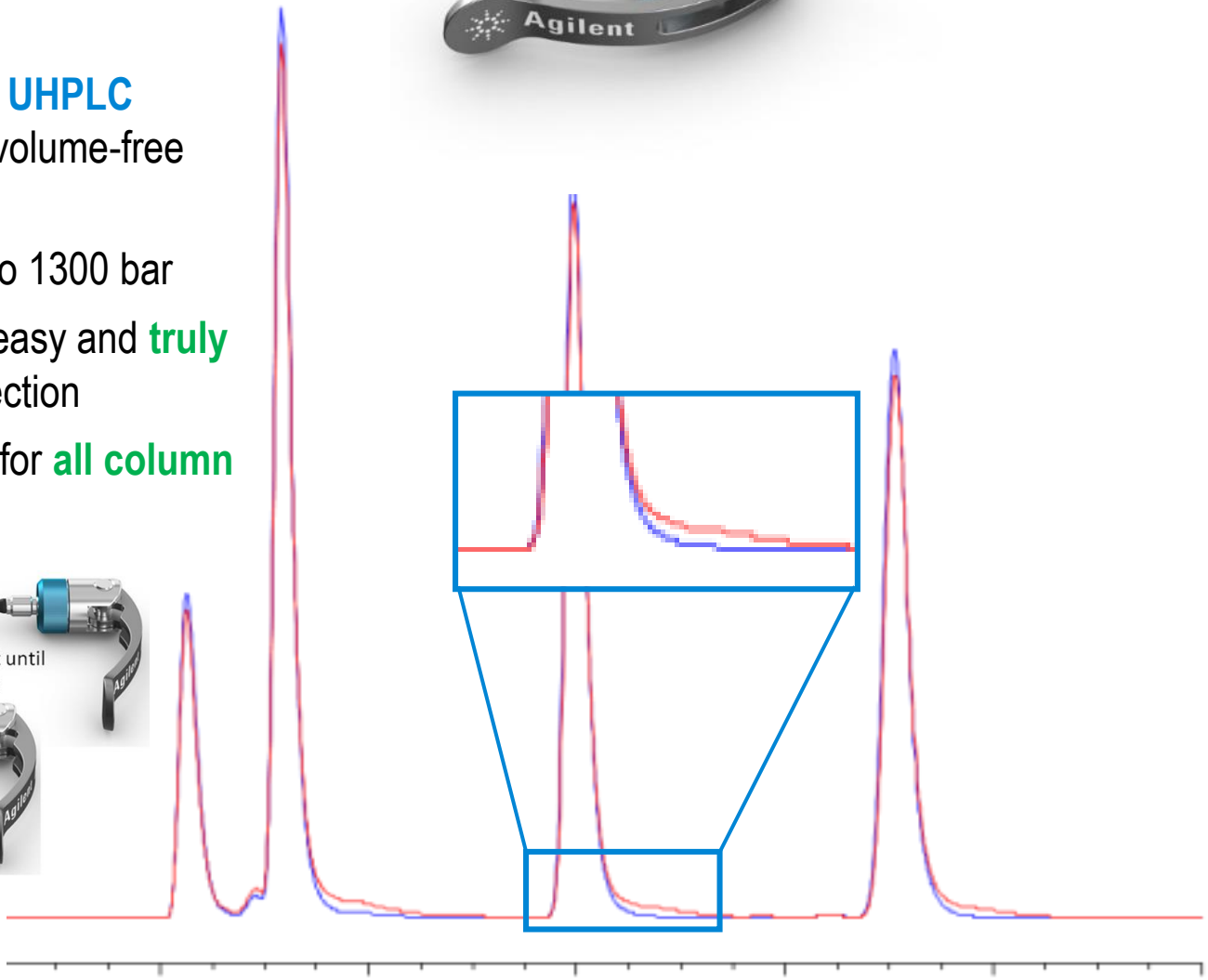
A-Line Quick Turn

A-Line Quick Connect

Agilent **A-Line Quick Connect UHPLC column fittings** for truly dead-volume-free fluidic connections

- **Tool-free** connection up to 1300 bar
- Spring loaded design for easy and **truly zero-dead volume** connection
- Removable and reusable for **all column types**





Optimal Performance
What Instrument Parameters Do I Optimize?

Isocratic and gradient separations

- Maximum efficiency expected from reduced particle size and column length
  - Requires minimizing the extra column volume of the HPLC system

Gradient separations

- Minimize gradient delay volume for fast gradients and fast re-equilibration time
- Minimize overall analysis time (run cycle time) by choosing the appropriate instrument hardware
- Minimize overall cycle time by choosing a higher flow rate and minimizing re-equilibration time and needed gradient range

High Resolution, Fast LC
Agilent 1.8um and Poroshell 120 (2.7um) Columns

- Make High Resolution, Fast LC Possible on All Instruments
- Reduce Analysis Time
- Speed Up Method Development
- Improves Methods on HPLC
- Provides Best Benefits on UHPLC
- Coupled with UHPLC Instruments Allow Higher Flow Rates for Higher Resolution Without Long Run Times

Agilent Technical Support

800-227-9770 (Toll Free US & Canada)

- **For LC columns**
- *Select option 3, then option 3, option 2*

lc-column-support@agilent.com

- ***For GC Columns***

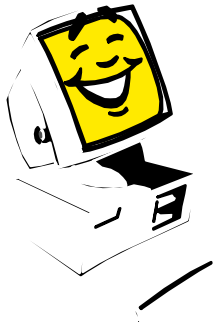
Select option 3, then option 3, option 1

gc-column-support@agilent.com

- ***For Sample Prep***

Select option 3, then option 3, option 3

- spp-support@agilent.com



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Appendix

Determining the Dwell Volume of Your System

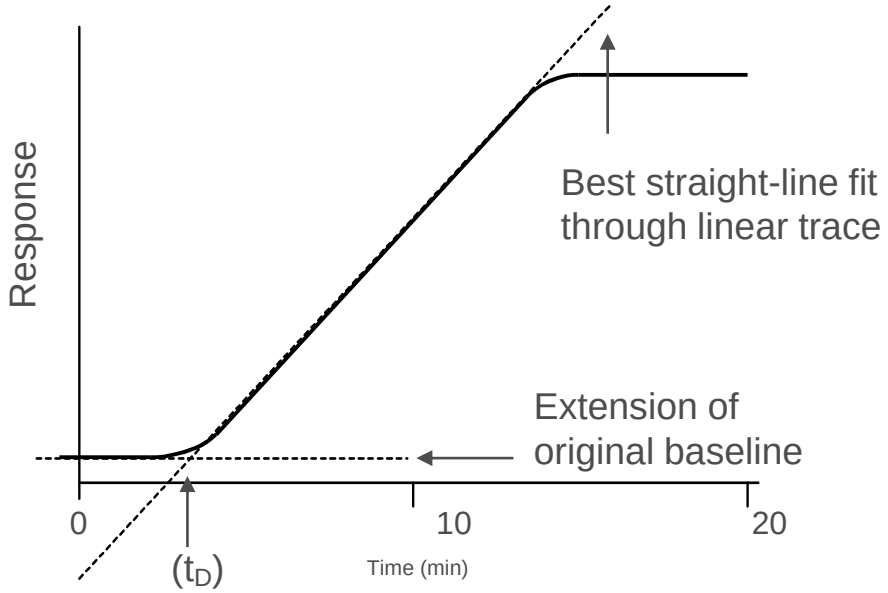
- Replace column with short piece of HPLC stainless steel tubing
- Prepare mobile phase components

  A. water - UV-transparent

  B. water with 0.2% acetone - UV-absorbing
- Monitor at 265 nm
- Adjust attenuation such that both 100% A and 100% B are on scale
- Run gradient profile 0 - 100% B/10 min at 1.0 mL/min
- Record

001814S1.PPT

Measuring Dwell Volume



Intersection indentifies
dwell time (t_D)

$V_D = t_D \times F$

V_D = Dwell Volume

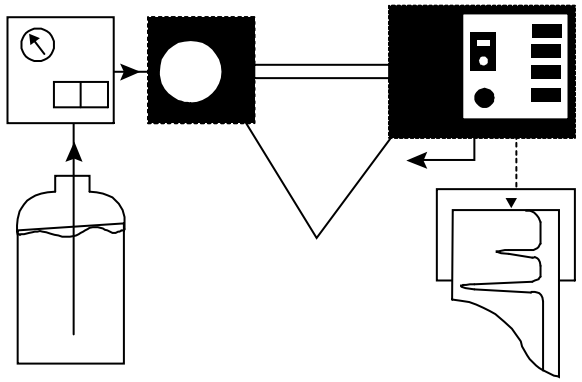
001815S1.PPT

Another Consideration: Extra-Column Volume

Extra-column volume is less of a concern when operating high-throughput narrow-bore columns at flow rates $\geq$ 1.0 mL/min

However the following are still recommended . . .

- Use the lowest volume detector provided by your instrument manufacturer
- Minimize tubing between injector and detector
- Inject samples in mobile phase or weaker solvent



Determination of Extra Column Volume of an HPLC System

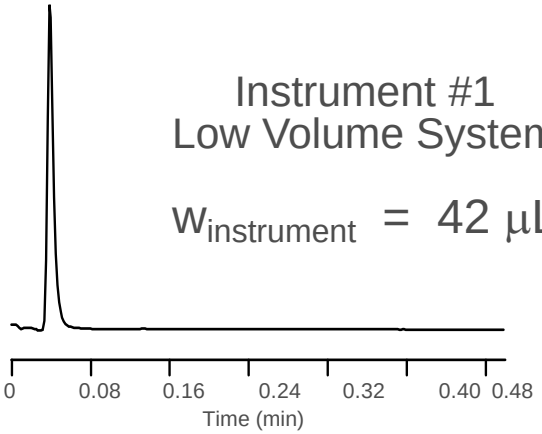
- **Remove HPLC column from instrument**
- **Join injector and detector tubing with zero-dead-volume union**
- **Inject (0.5 - 2 μL) of toluene in 100% acetonitrile**
- **Determine width of peak at base ($w_{instrument}$)**
- **Peak bandwidth follows:**

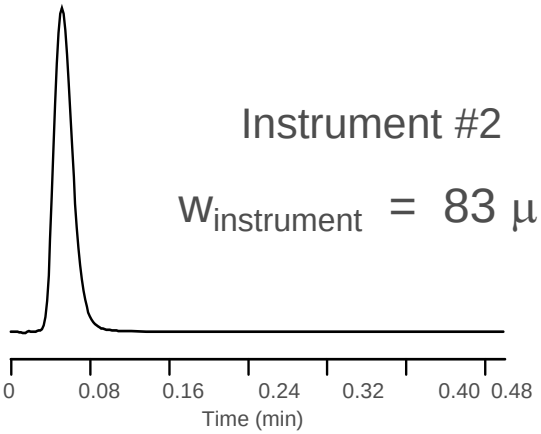
$$W^2_{tot} = W^2_{col} + W^2_{instrument}$$

001812S1.PPT

Determination of the Extra Column Volume of an HPLC System

Toluene in Acetonitrile





$w^2_{tot} = w^2_{col} + w^2_{instrument}$

For peak having a k′ = 2

3%

$w^2_{tot} = (180)^2 + (42)^2$

w_{tot} = 185 µL

15%

$w^2_{tot} = (73)^2 + (42)^2$

w_{tot} = 84 µL

10%

4.6 x 150 mm, 5 µm

$w^2_{tot} = (180)^2 + (83)^2$

w_{tot} = 198 µL

51 %

4.6 x 50 mm, 3.5 µm

$w^2_{tot} = (73)^2 + (83)^2$

w_{tot} = 110 µL