Things You May Not Know

or May Have Forgotten

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LC Columns Application Engineer
June 13, 2019

Agilent
Trusted Answers

Outline

Column dimensions

- Scaling up or down

Take care of your column

- Dirty column

Mobile phase

Instrument considerations

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Column Dimensions

Scaling

- ID
- Length
- Load
- Flow
- Particle Size

Isocratic v. gradient

- Equilibration

Changing Column Diameter
Important Values to Know

Column ID	Column Volume	Peak Volume, k=1	Typical Injection Vol.*	Typical Inj Vol Range	Flow Rate for equivalent ν**
4.6 mm	1500 μL	148 μL	20 μL	5 – 50 μL	1.0mL/min
3.0 mm	640 μL	44 μL	10 μL	3 – 30 μL	0.42mL/min
2.1 mm	320 μL	22 μL	2 μL	0.5 – 15 μL	0.21mL/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, N =13,000 for 5um

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

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

Column Volume

$$V_m = \pi \cdot r^2 \cdot L \cdot {\sim}0.6$$

Column volume is calculated as the volume of a cylinder less the space occupied by the packing material. As an example, Agilent ZORBAX Eclipse Plus C18 packing material occupies 40% of the column, the remaining 60% of the cylinder would be considered as column volume.

Flow Rate, Important for Method Transfer

Adjusting flow for different column diameters

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

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

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Important 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

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

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Scale-up Calculations – Example

Analytical column:
Zorbax SB-C18 3x150 mm, 5 µm

Preparative column:
Zorbax SB-C18 21.2x150 mm, 5 µm

$$\frac{\dot{V}_1}{\dot{V}_2} = \frac{r_1^2}{r_2^2}$$

Flow: 0.6 ml/min → Flow: ~30 ml/min

Amount: 500 µg/Injection → Amount: 25 mg/Injection

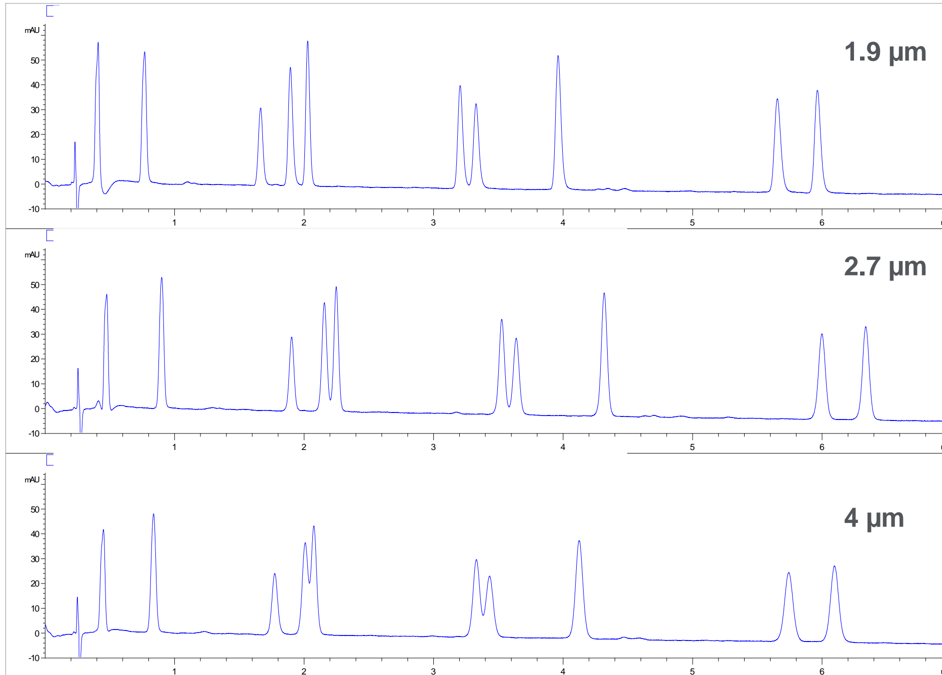
V_1 = flow column 1 = 0.6 ml/min
x_1 = max. amount column 1 = 500 µg
r_1 = radius column 1 = 1.5 mm

$$\frac{x_1}{\pi \cdot r_1^2} = \frac{x_2}{\pi \cdot r_2^2} \cdot \frac{1}{C_L}$$

V_2 = flow column 2 = ?
x_2 = max. amount column 2 = ?
r_2 = radius column 2 = 10.6 mm

C_L = ratio lengths of columns = 1

Decreasing Particle Size Increases Efficiency



Columns:
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 1.9 µm
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 2.7 µm
Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 4 µm
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

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

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Smaller Particle Size Columns Improve Resolution
But Pressure Increases

Up to 60% higher resolution than in conventional HPLC

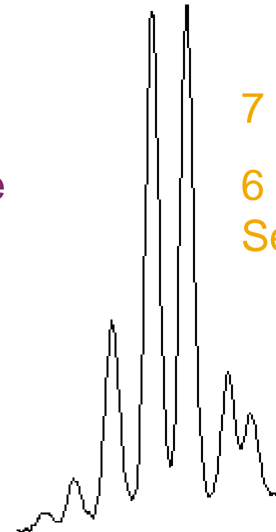
Isocratic Impurity Method

Zoom of critical time range @ 7min

4 Impurities

2 Not Baseline Separated!

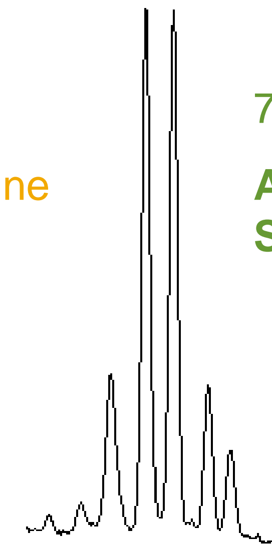
4.6 x 150, 5um
93 bar
N = 7259
Rs = 1.15
S/N = 42



7 Impurities

6 Not Baseline Separated!

4.6 x 150, 3.5um
165 bar
N = 14862
Rs = 1.37
S/N = 50



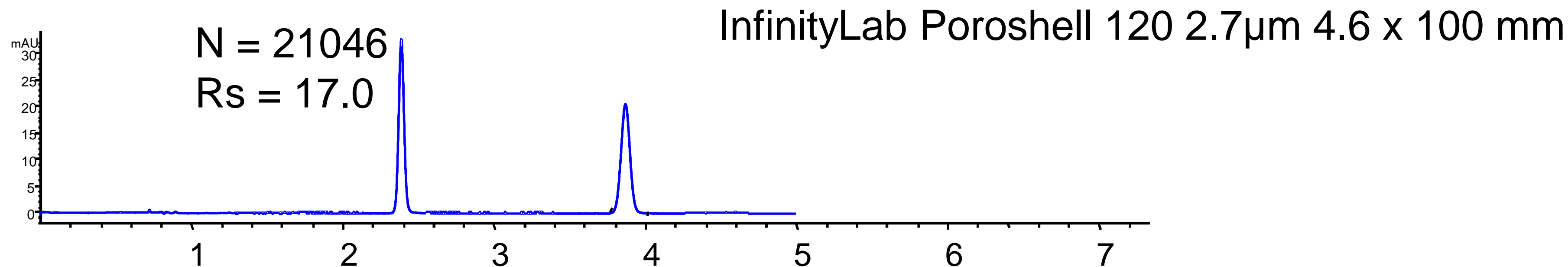
7 Impurities

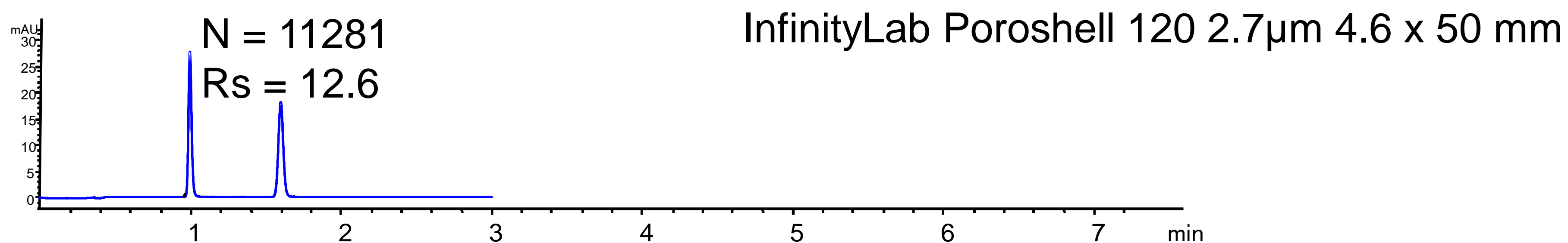
All 7 Baseline Separated!

4.6 x 150, 1.8um
490 bar
N = 28669
Rs = 1.80 (+57%)
S/N = 44

Column Length
Isocratic

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





Mobile phase: 50:49:1 MeCN:H2O Acetic Acid

Flow pate: 1.2 mL/min, **All Pressures < 300 bar**

Peak 1. Naproxen 2. Butyrophenone

Maintain Gradient Retention (k*)
Keep relative peak position in chromatogram unchanged and shorten analysis

Any Decrease in		**Can be Offset by a Proportional**
Column length		**Decrease in t_G or F**
Column volume (id)		**Decrease in t_G or F**

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

ΔF = change in volume fraction of B solvent
S = constant
F = flow rate (mL/min)
t_g = gradient time (min)
V_m = column void volume (mL)

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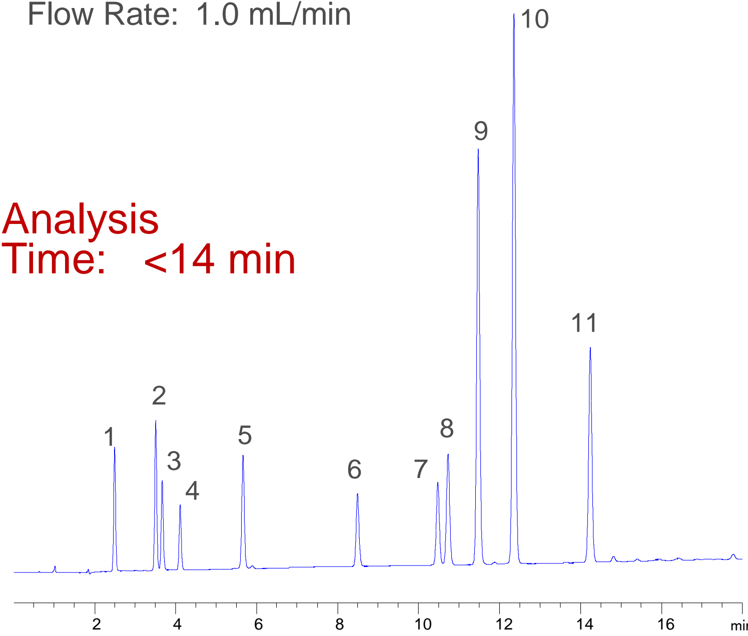
Reduce Analysis Time
Gradient

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)

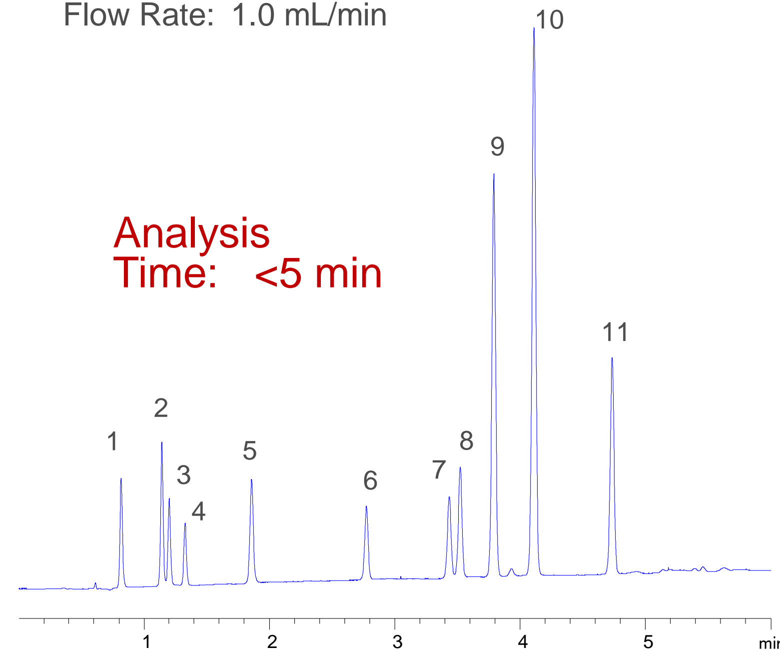
Column: ZORBAX Eclipse Plus-C18
4.6 x 150 mm, 5 µm

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



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

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



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Take Care of Your Column

Symptoms

- Pressure
- Undesirable peak shape changes

Contamination

- Particulates
- Microbial growth

Cleaning

- Column guide

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Pressure or Peak Shape Issues
Determine Cause and Correct

Column contamination, plugged frit, etc.

- Back flush column (check column manufacturer user guide)
  - ✓ Clear plugged frit
  - ✓ Replace frits (not usually recommended and not possible with many columns)
- Wash column
  - ✓ Eliminate column contamination and plugged packing
  - ✓ High molecular weight/adsorbed compounds
  - ✓ Precipitate from sample or buffer

Replace Column

Prevention

- Filter MP & sample
- Column protection

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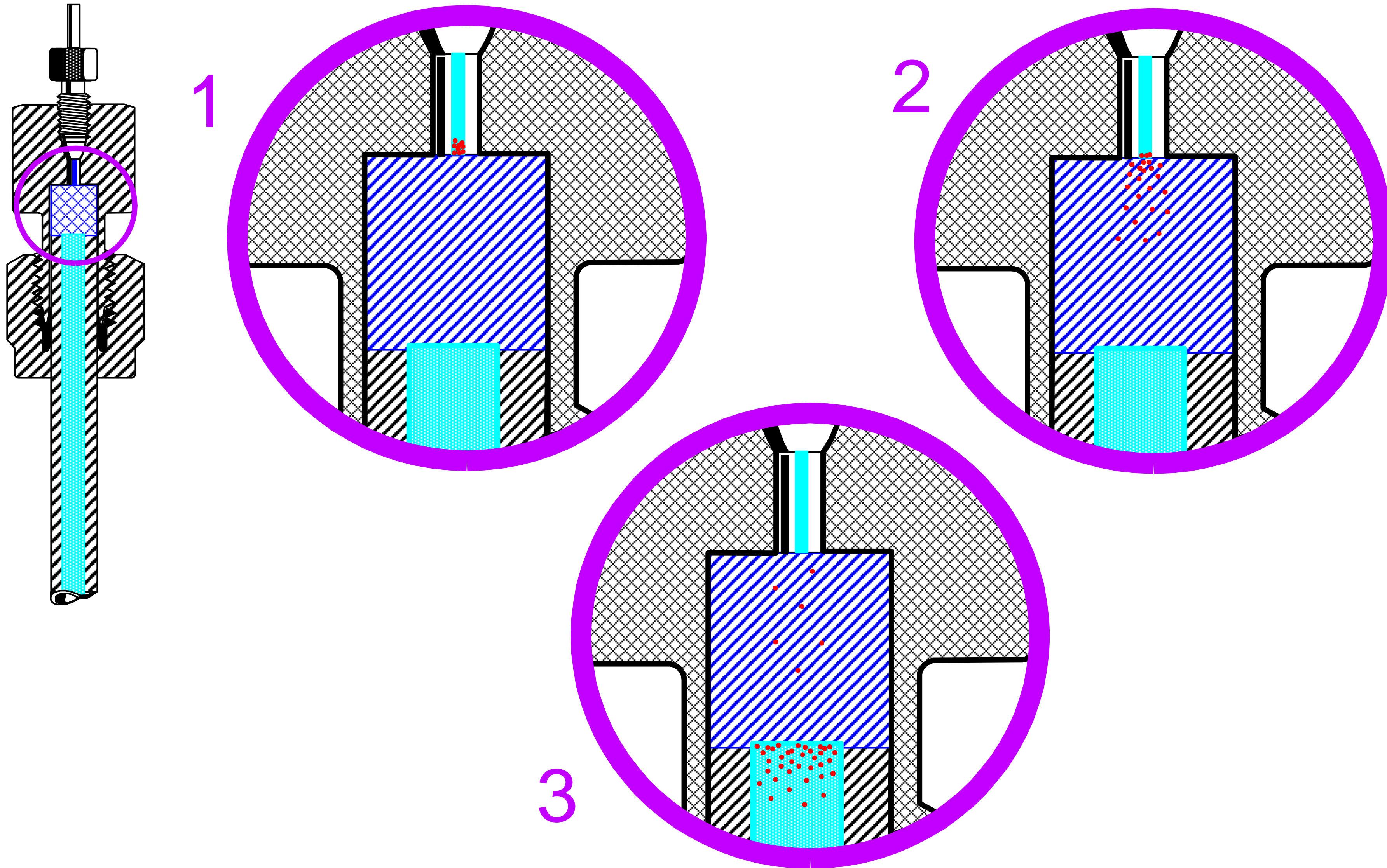
Contamination at the Column Head



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Column Cleaning Example
Reversed Phase

RP solvent choices, in order of increasing strength

- Use at least 10 x V_m of each solvent for analytical column

Remove detector from flow path

1. Mobile phase without buffer salts (water/organic)
2. 100% Organic (Methanol or ACN)
3. Is pressure back in normal range?
4. If not, discard column or consider more drastic conditions:
   75% Acetonitrile:25% Isopropanol, THEN
5. 100% Isopropanol
6. 100% Methylene Chloride*
7. 100% Hexane*

*If using Hexane or Methylene Chloride, the column must be flushed with Isopropanol before and after

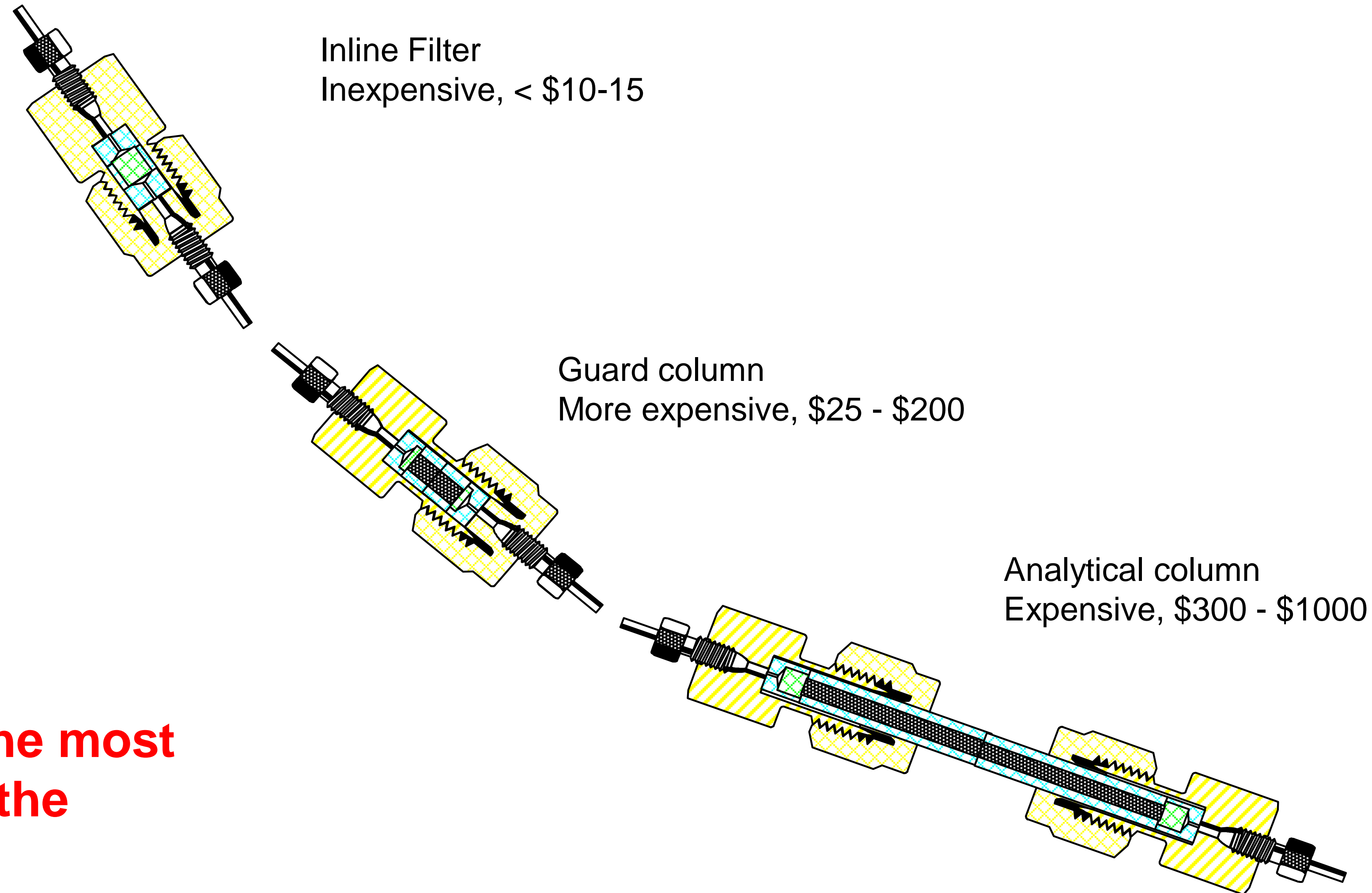
Note – Prior to cleaning, always consult manufacturer's column user guide for solvent recommendations & compatibility

Caring for your columns …

1. Column installation
2. Column use
3. Column storage
4. Column clean-up
5. Common problems
   - Pressure
   - Retention time
   - Resolution
   - Peak shape

REFER TO THE USER GUIDE / CARE GUIDE !

Prevention
Ways to Protect Your Column



Your column is the most effective filter in the whole LC!!!

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Mobile Phase

Characteristics

- Solvent strength
- UV cutoff

Preparation

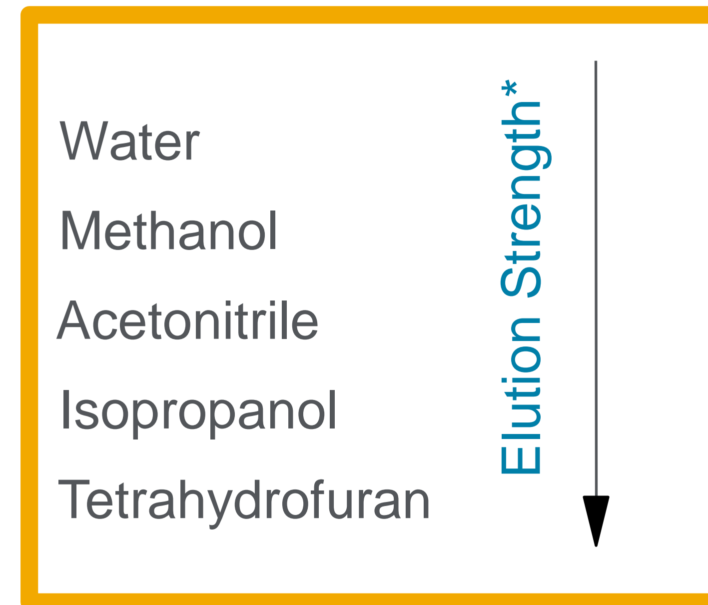
- How to
- Equilibration

Precautions

- Volatility
  - LCMS
  - Δ concentration
- Miscibility
- Microbial growth

Common LC Solvents

Solvent	UV Cutoff (nm)*	Polarity
Acetonitrile	190	
Water	190	78.1
Cyclohexane	195	2.0
Hexane	200	1.9
Methanol	210	32.6
Acetone	331	20.7
Chloroform	240	4.8
Ethanol	210	24.3
Tetrahydrofuran	280	
Toluene	280	

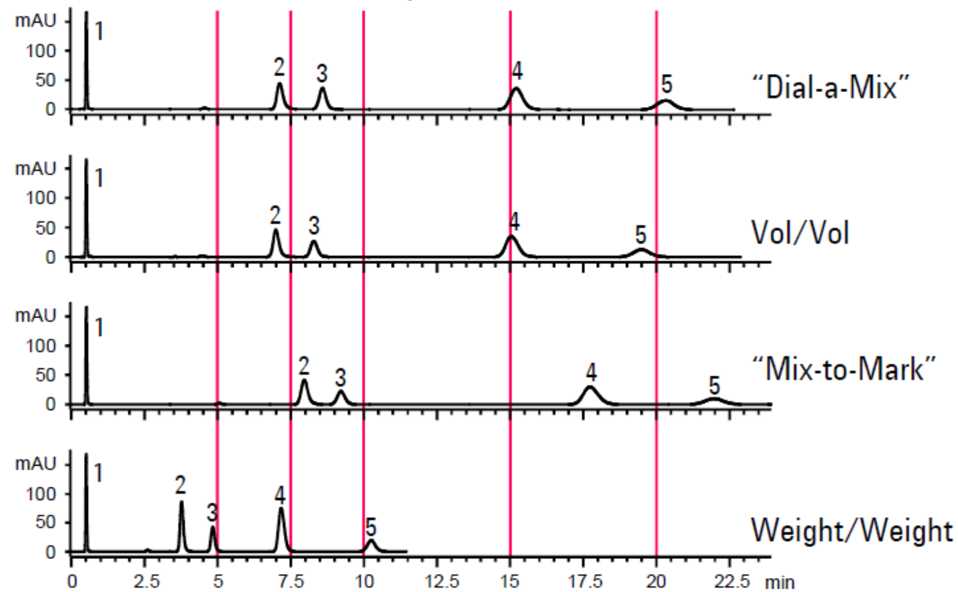


*In HILIC water is the stronger solvent

Buffer Options

Non-volatile		pK_a	Buffer Range
Phosphate	$H_3PO_4 \rightleftharpoons H_2PO_4^-$	$pK_1 = 2.1$	1.1 – 3.1
	$H_2PO_4^- \rightleftharpoons HPO_4^{-2}$	$pK_2 = 7.2$	6.2 – 8.2
	$HPO_4^{-2} \rightleftharpoons PO_4^{-3}$	$pK_3 = 12.3$	11.3 – 13.3
Citrate	CH_2COOH / HOCCOOH / CH_2COOH	$pK_1 = 3.1$	2.1 – 4.1
		$pK_2 = 4.7$	3.7 – 5.7
		$pK_3 = 5.4$	4.4 – 6.4
Borate	H_3BO_3	$pK_1 = 9.2$	8.2 – 10.2
Volatile			
Trifluroacetate	F_3CCOOH	$pK_1 = 0.5$	xx – 1.5
Formate	HCOOH	$pK_1 = 3.8$	2.8 – 4.8
Acetate	CH_3COOH	$pK_1 = 4.8$	3.8 – 5.8
Ammonium	NH_4^+	$pK_1 = 9.2$	8.2 – 10.2

Mobile Phase Preparation
Effect on Chromatography



HPLC System:	Agilent 1100 with quaternary pump
Column:	ZORBAX Eclipse XDB-C8 Rapid-Resolution (3.5µm), 4.6 x 50 mm Agilent Part No. 935967-906
Mobile Phases:	Dial-a-Mix= A: water B: MeOH, pump 50% B Vol/Vol=250 mL water + 250 mL MeOH, pump 100% Mix-to-Mark = 250 mL MeOH, fill to 500 mL with water, pump 100% Premixed (w/w) = 200 g MeOH + 200 g water, pump 100%
Detection:	UV 254 nm
Flow:	1 mL/ min.
Temperature:	ambient

1. Uracil
2. Butylparaben
3. Napthalene
4. Dipropylphthalate
5. Acenaphthene

Method used to prepare MP can significantly affect the elution pattern
- Be consistent
- w/w is more accurate than v/v

Effect of Mobile Phase Preparation on Chromatography
Pub. No. 5988-6476EN

Mobile Phase Preparation

Small changes in mobile phase strength can have a large affect on retention

Volume % of solvents can depend on preparation

Specified volume ACN added to a 1 L volumetric and made to volume with H_2O

≠

Specified volume H_2O added to a 1 L volumetric and made to volume with ACN

≠

500 ml H_2O added to 500 ml ACN

- ✓ Degree of contraction is affected by the relative quantities of each
- ✓ Temperature
- ✓ HPLC grade or better
- ✓ Be consistent/document process
  - Buffer prep procedure in Appendix

Initial Column Equilibration*

In appropriate vessel, test highest % organic/buffer ratio to verify that buffer will not precipitate. With stirring, add organic to buffer first, not vice versa.

In appropriate vessel, test solubility

- Highest % organic/buffer ratio
- With stirring, add organic to buffer, not vice versa
- MeOH generally more miscible than ACN

100% ACN   10% ACN + 90% 10 mM Phosphate

Equilibrate column with, in order:

- 100% organic modifier (if brand new)
- Mobile phase minus buffer
- Buffered mobile phase containing highest % organic modifier (gradient high end)
- Buffered mobile phase containing lowest % organic modifier (gradient low end)

Inject standard or sample several times until RTs stabilize

- For gradient methods, precede former with 1 or 2 blank gradients

*General recommendation. Check manufacturer guidelines.

Shutdown State and Instrument Flushing*

Shutdown State

- Next day use – same buffers
  - Pump mobile phase very slowly (for example, 0.01 – 0.1mL/min)
- When flushing column or for longer term column storage
  - Flush with 20/80 organic/water, then 80/20 organic/water or 100% organic

Instrument flushing

- Replace column with capillary tubing. Leave disconnected from detector
- Flush pumps with water, then connect capillary tubing to detector
- Inject water 2-3 times at maximum injection volume setting
- Flush all pumps with 100% organic for long term storage

*General recommendation. Check manufacturer guidelines.

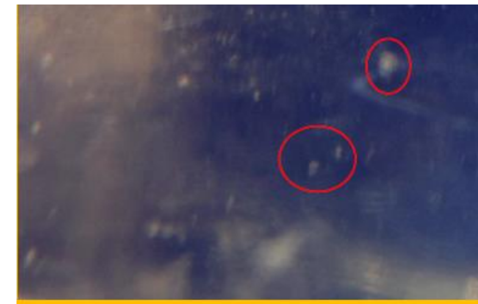
Microbial Growth

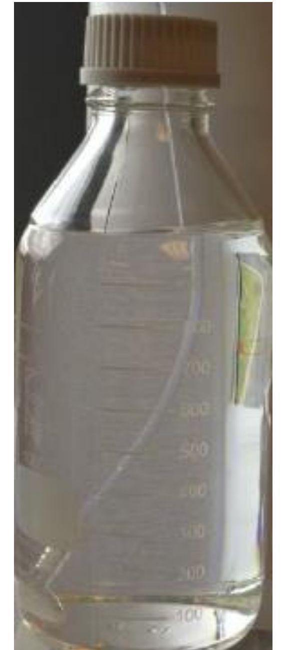
Potential problems

- Increased system pressure or pressure fluctuations
- Increased column pressure, premature column failure
- Can mimic application problems
- Gradient inaccuracies
- Ghost peaks
- Difficult to remove if gets in degasser and rest of system

Prevent and/or Reduce Microbial Growth

- Use freshly prepared mobile phase
- Filter
- Do not leave mobile phase in instrument for days without flow
- Always discard “old” mobile phase
  - Do not add fresh mobile phase to old
- Use an amber solvent bottle for aqueous mobile phase
- If possible, can add
  - 5% organic added to water can be used to reduce bacterial growth
  - Few mg/l sodium azide



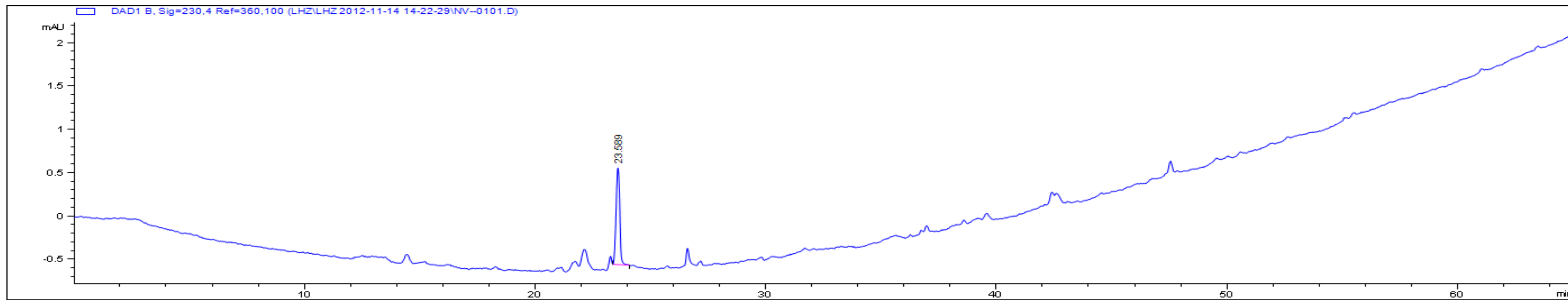


To avoid contaminating your system and column, prevent microbial growth

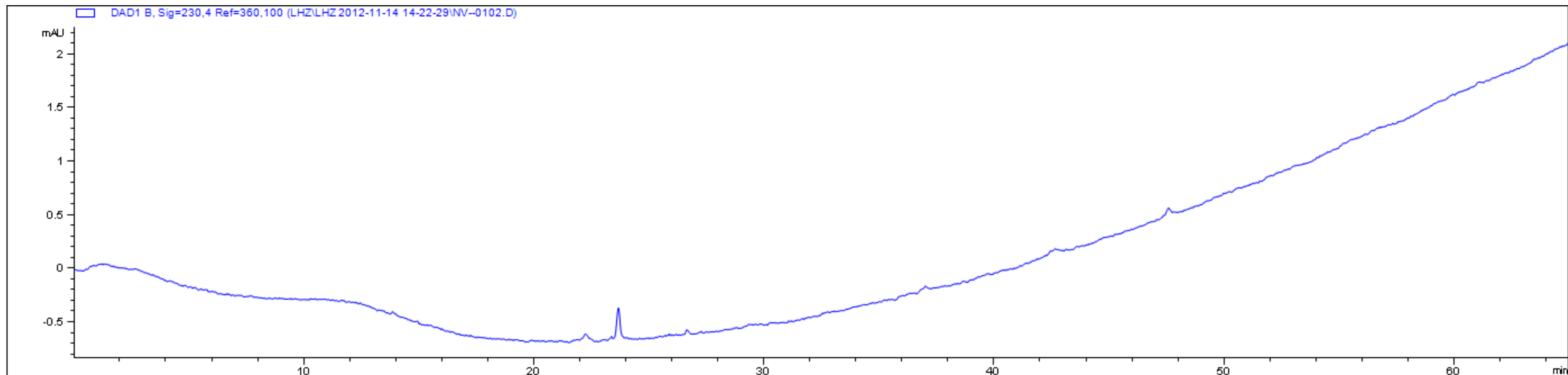
- Check your instrument manual for guidelines

Ghost Peaks

The LC system was equilibrated at starting conditions for 30min, then a gradient run was made. Impurities were trapped and eluted out with the gradient.



When an injection is made with minimal equilibration, a much cleaner baseline was observed.



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Instrument Considerations

Dwell volume

ECV (extra column volume)

Detector

- Settings
- Acquisition rate
- Flow cell

Fittings

Tubing

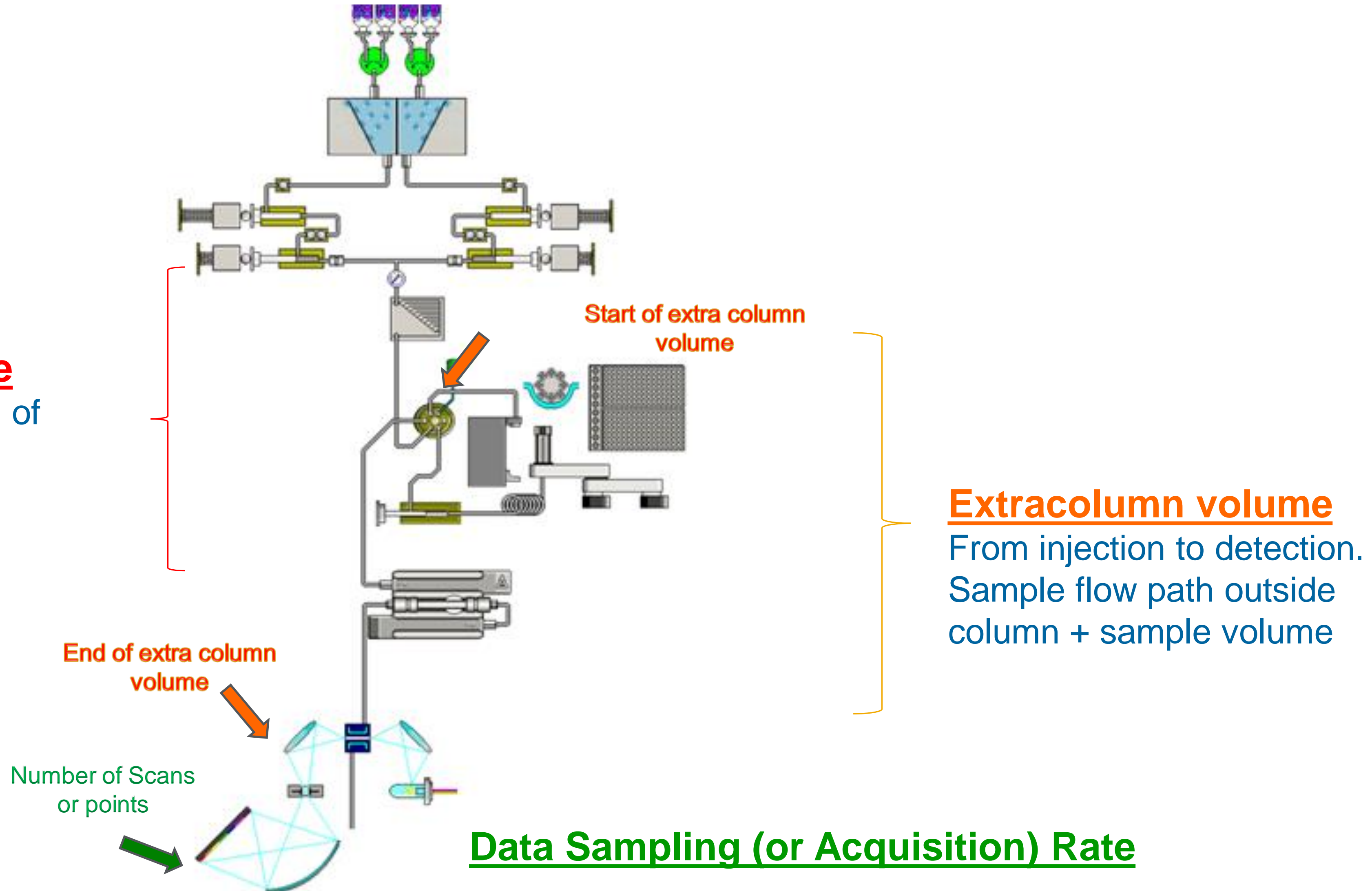
- ID
  - 0.12 mm for smaller volume columns
- Length
  - Shortest

Isocratic v gradient

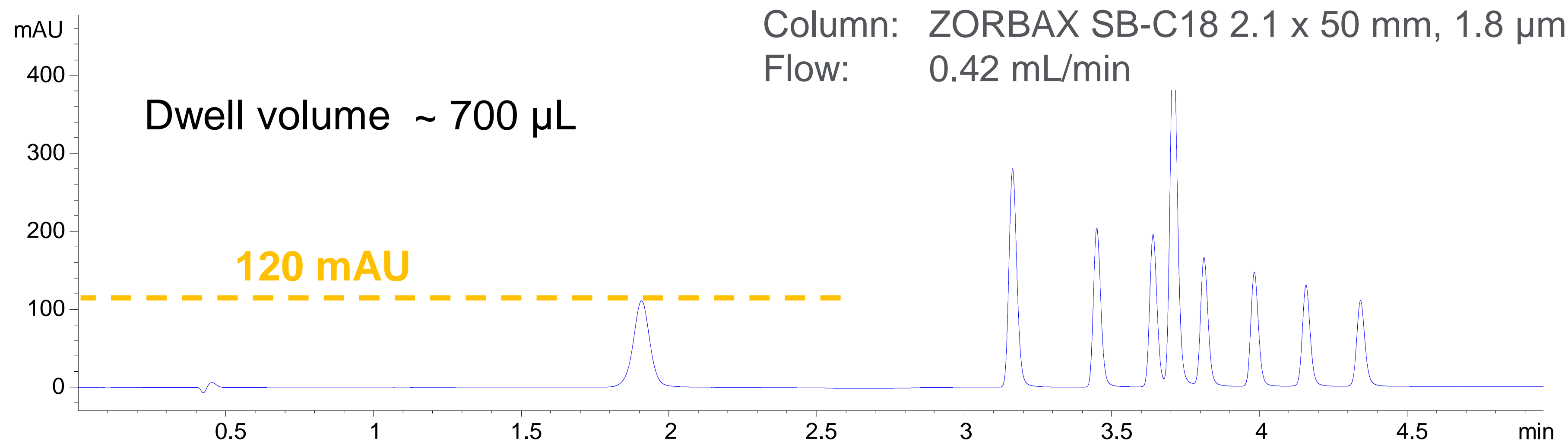
- Dwell volume
- Equilibration

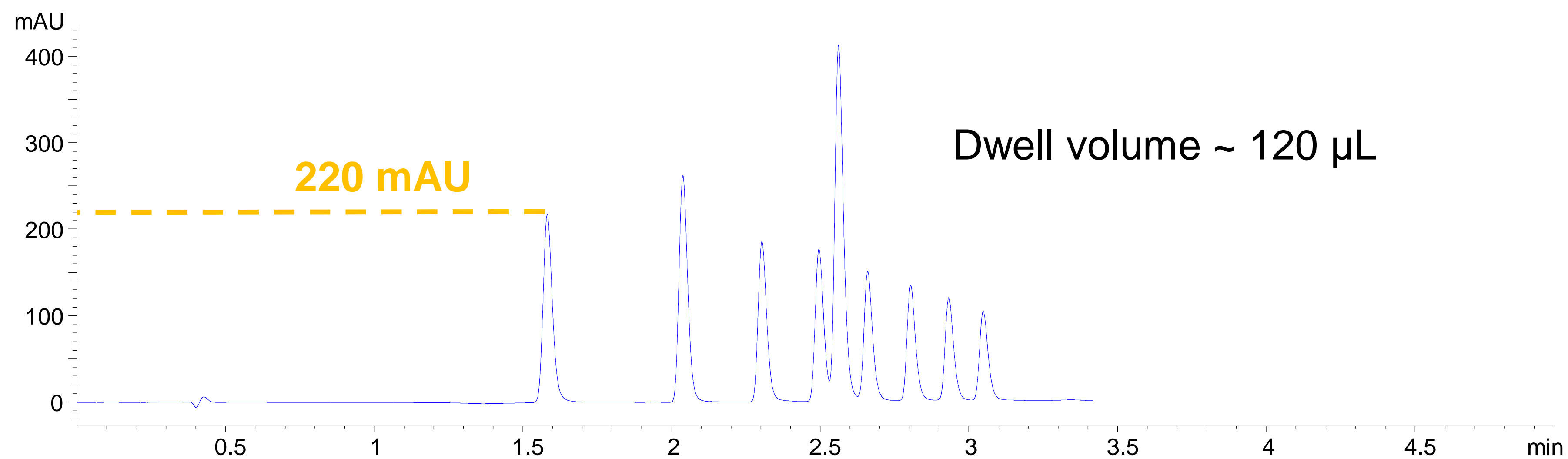
Instrument Considerations

Dwell/delay volume
Volume from formation of gradient to the column



System
Dwell Volume*





*See appendix for instructions

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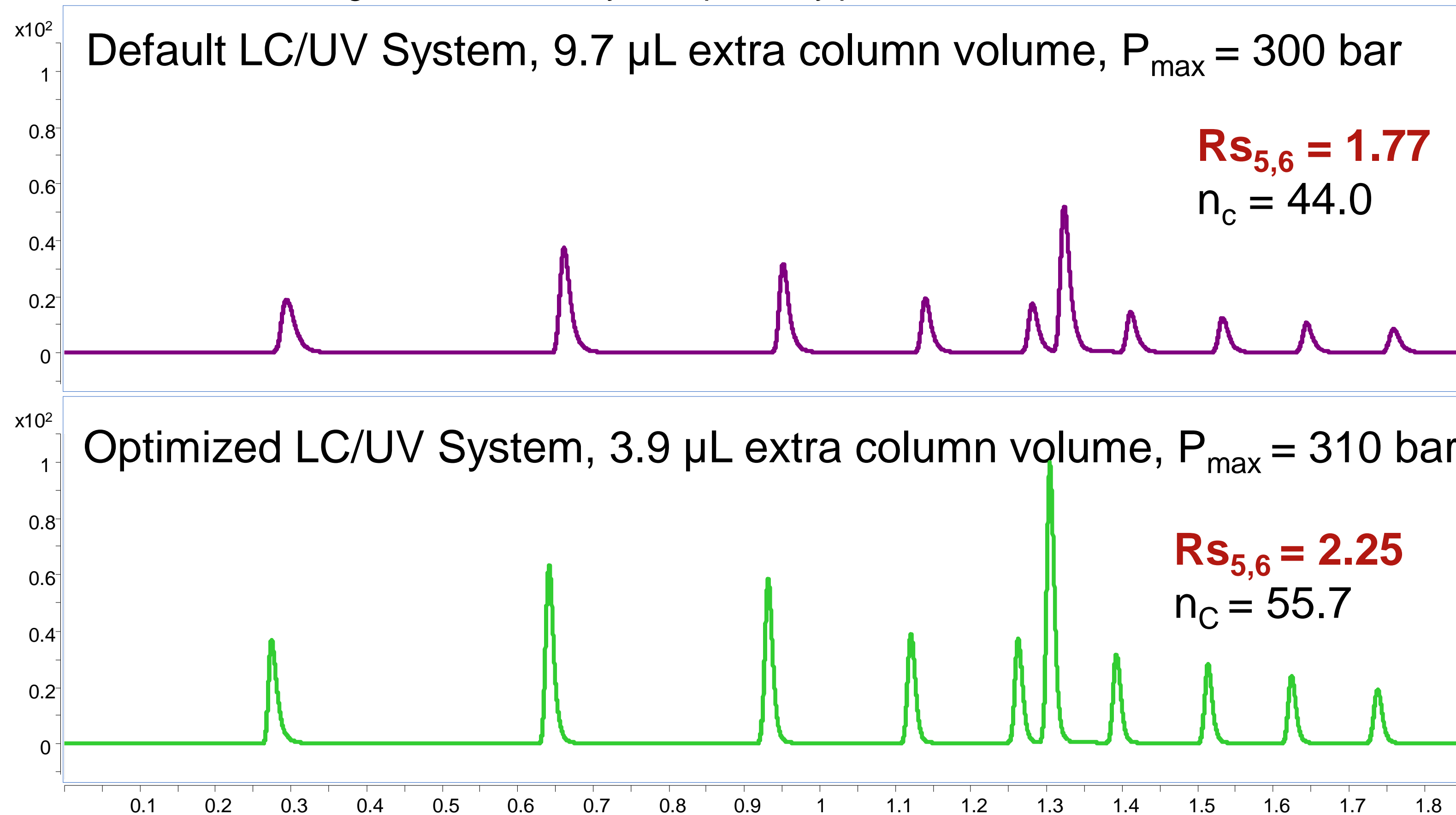
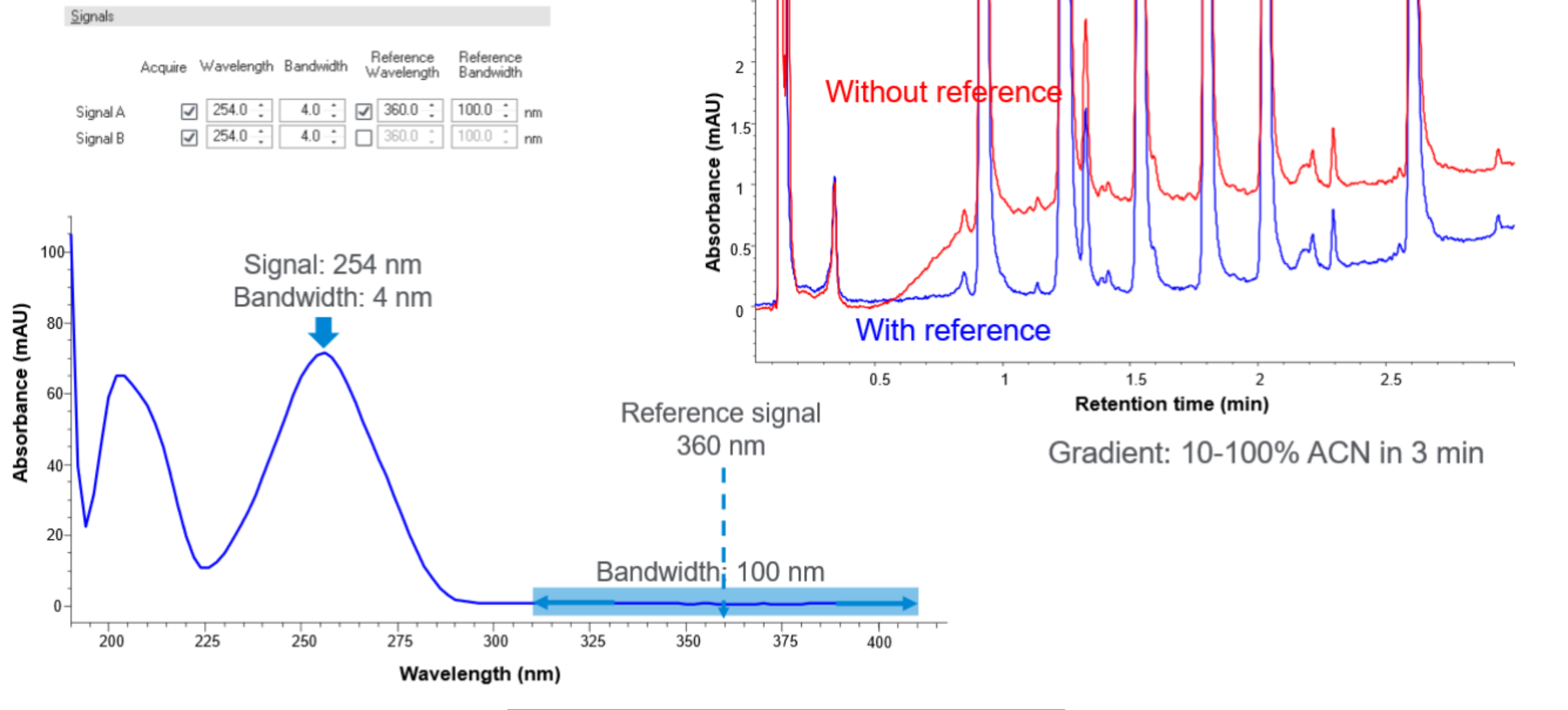
Optimized LC Volume Improves Gradient Resolution

Column: RRHD Eclipse Plus C18, 2.1 x 50mm, 1.8um Gradient: 25-95% CH_3CN in 1.2 min, Flow Rate: 0.4 mL/min LC: Agilent 1290 Infinity Sample: Alkylphenones



>20% improvement in gradient Rs and peak capacity with optimized LC

DAD Settings
Choose the right signal and reference



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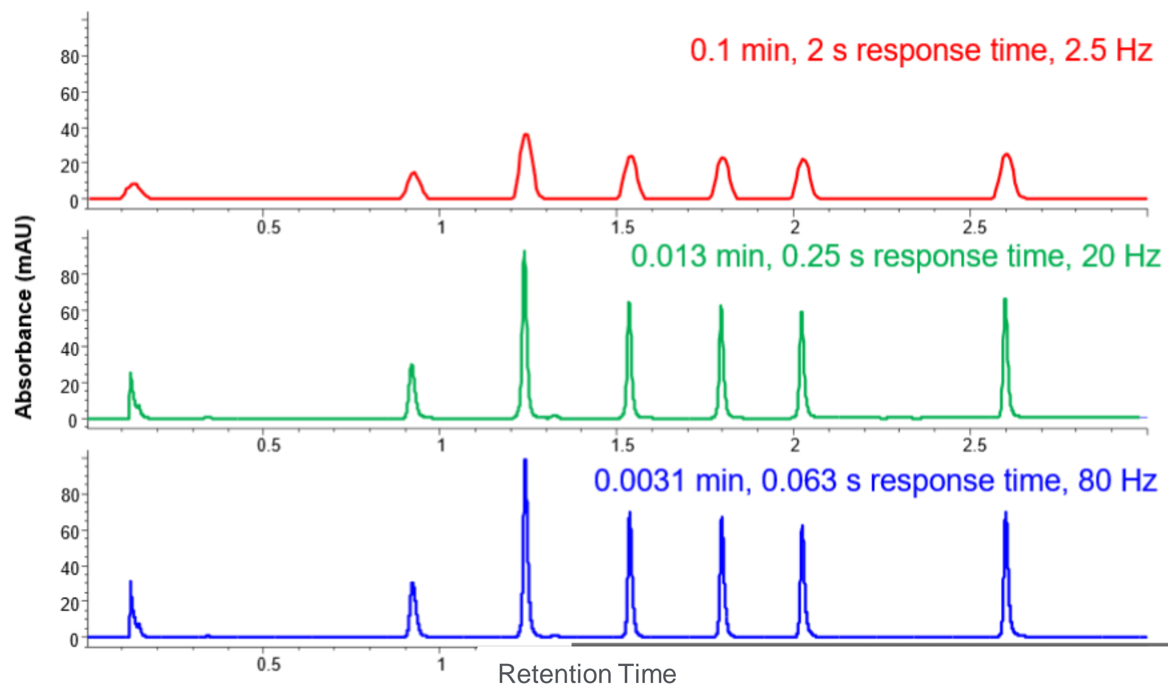
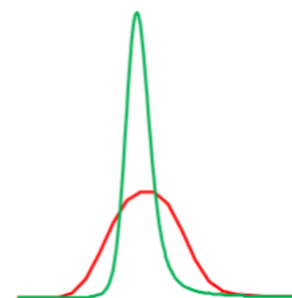
Detector

DAD Setting — Choose the right sampling rate



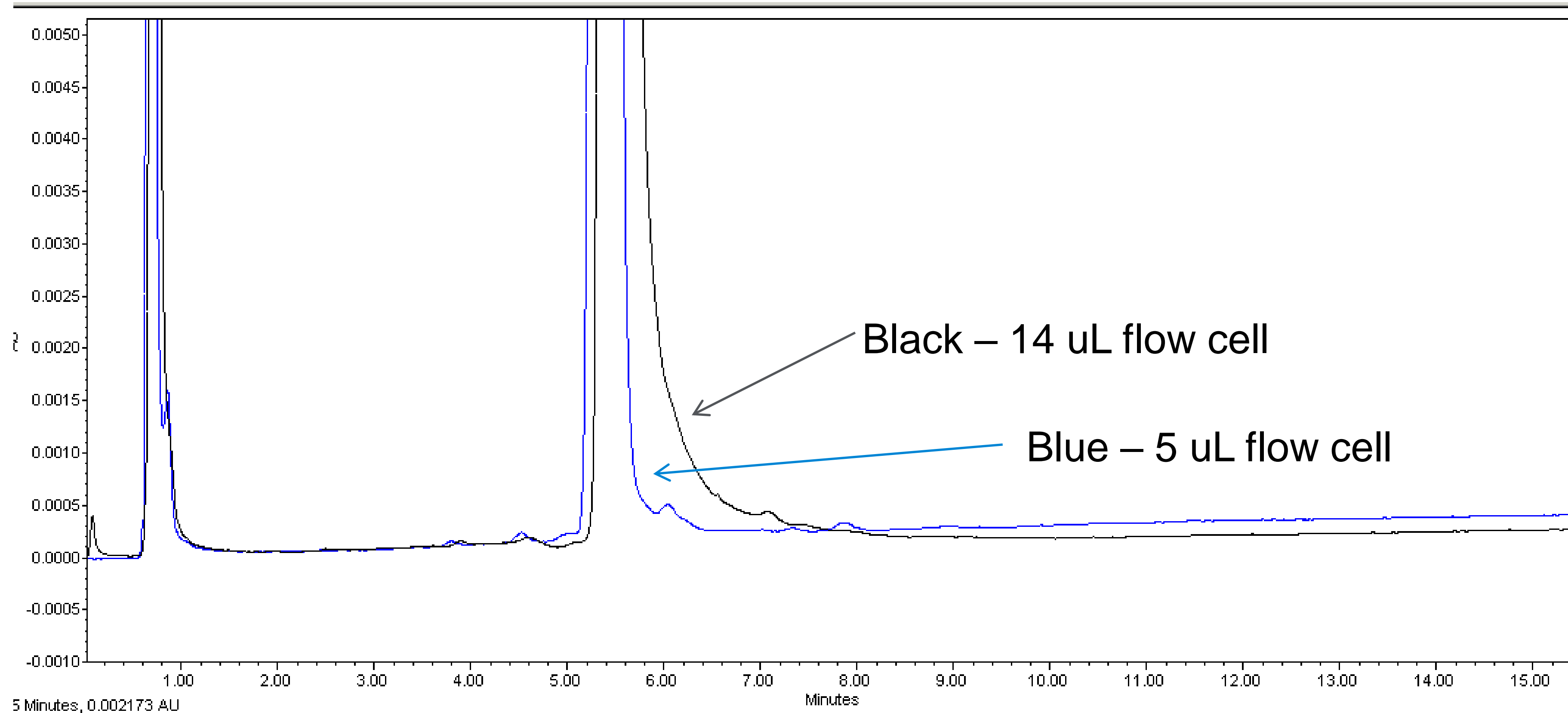


Changes in **Peak Width** and **Resolution**

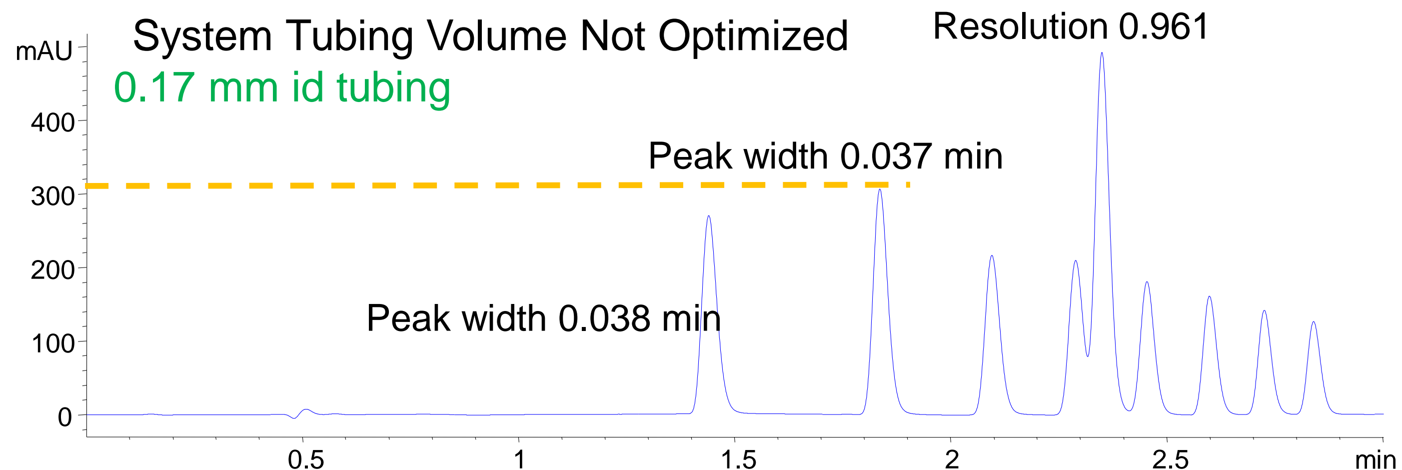
Column: ZORBOX Eclipse Plus C18, 2.1x50 mm, 1.8 µm
Column temperature: 35 °C; Flow rate: 1 mL/min
Gradient: 10-100% ACN in 3 min
Signal: 254 nm, Bandwidth: 4 nm
Reference: 360 nm, Bandwidth: 100 nm

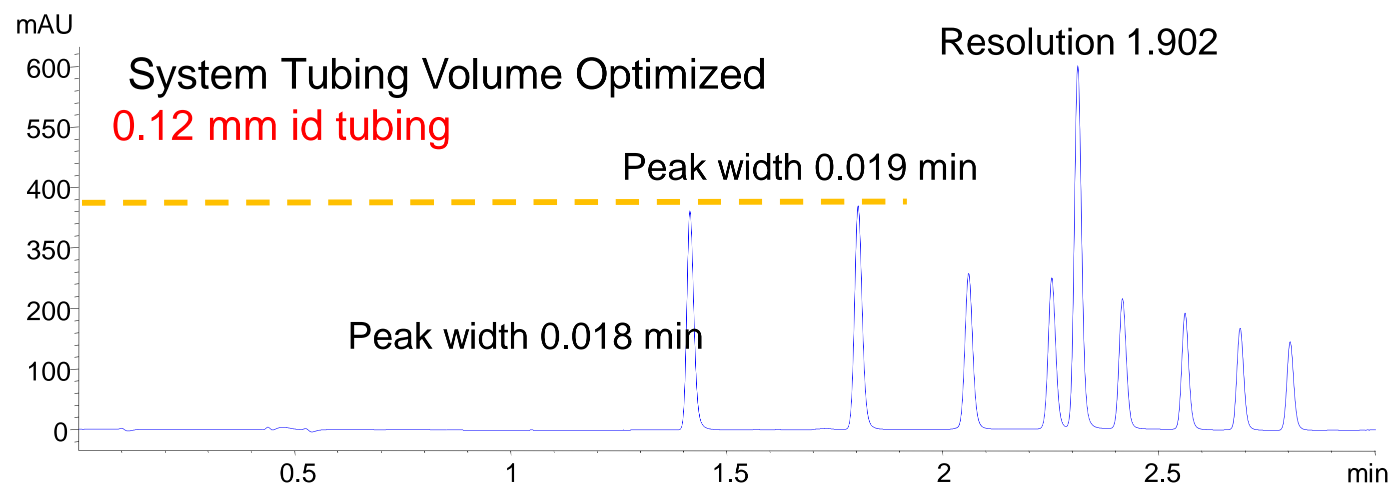
Smaller Column i.d. Requires a Lower Detector Cell Volume

3 x 100mm Column

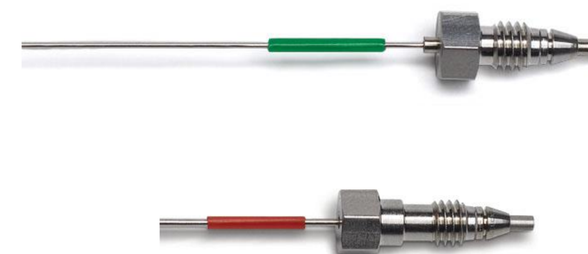


Optimize Tubing Volume for Small Volume Columns





Length	10mm	50mm	100mm	150mm
Tubing ID	Volume	Volume	Volume	Volume
0.17mm (green)	0.227 uL	1.1uL	2.27 uL	3.3 uL
0.12mm (red)	0.113 uL	0.55uL	1.13 uL	1.65 uL



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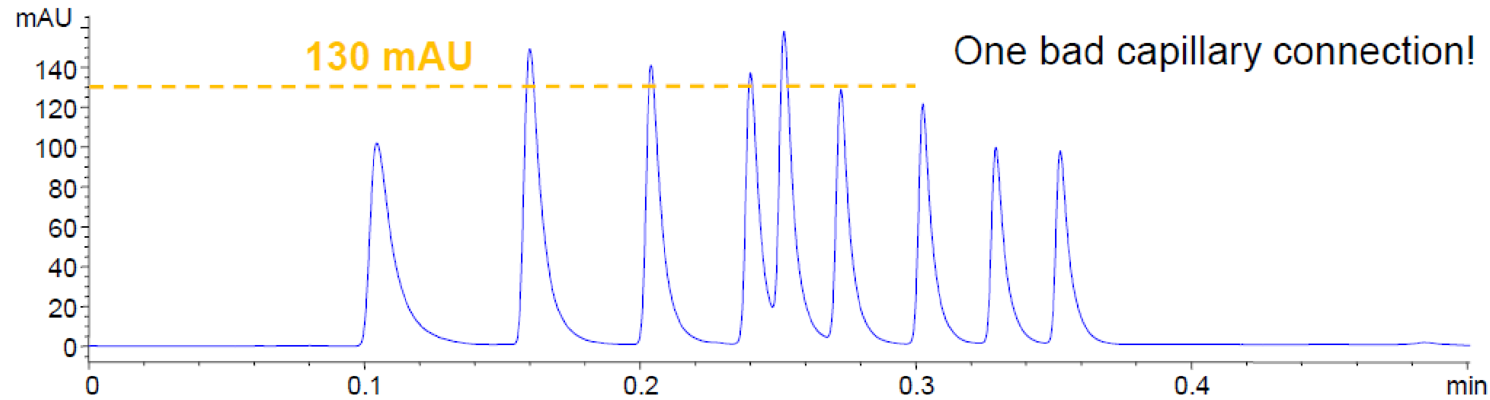
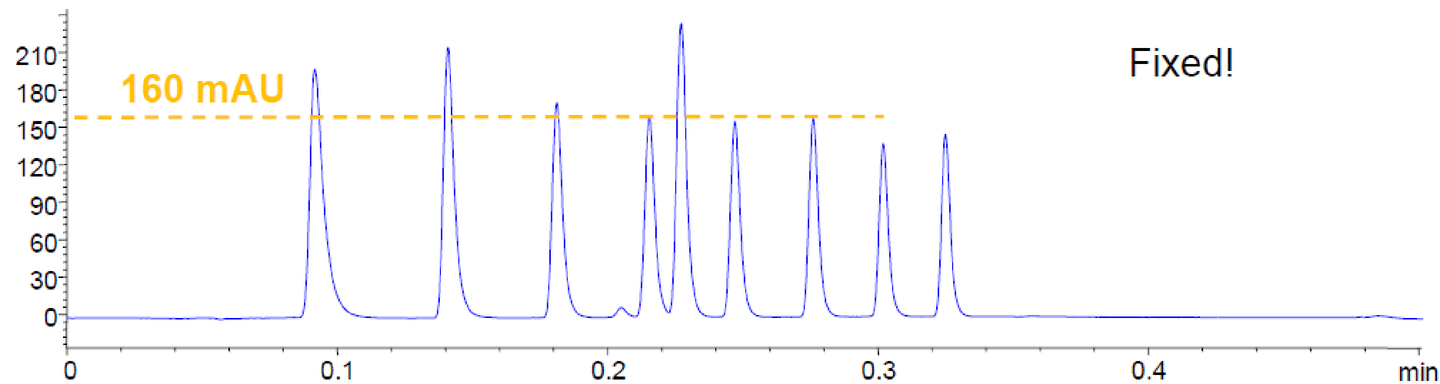
Importance of Correct Connections

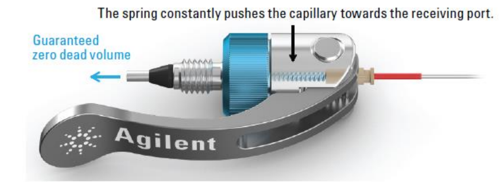




Quick Turn

Agilent Technical Note: Agilent InfinityLab Fittings
Pub No 5991-5525EN

Correct connection every time



Quick Connect

Summary

Column dimensions

- Proportional Δ
- Optimize instrument

Take care of your column

- Consult column guide
- Column protection

Mobile phase

- Consistency
- Characteristics

Instrument

- Dwell volume
- ECV
- Detector

Upcoming Seminars

- ✓ *“From Instrument to Column”: Tracking Down the Problem*
  Mark Powell, July 18, 2019 @11:00 AM EDT
- ✓ *From Proteins to Polymers – GPC/SEC: Understanding Column Selection and Method Considerations for Your Sample*;
  Jean Lane, August 15, 2019 @11:00 AM EDT

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Contact Agilent Chemistries and Supplies Technical Support

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

Option 1 for GC or GC/MS Columns and Supplies

Option 2 for LC or LC/MS Columns and Supplies

Option 3 for Sample Preparation, Filtration, and QuEChERS

Option 4 for Spectroscopy Supplies

gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

Resources for Support

- LC Handbook, Pub. No. 5990-7595EN
- LC Troubleshooting Poster, Pub. No. 5994-0709EN
- Agilent University http://www.agilent.com/crosslab/university
- Tech support http://www.agilent.com/chem/techsupport
- Resource page http://www.agilent.com/chem/agilentresources
  – Quick reference guides
  – Catalogs, column user guides
  – Online selection tools, how-to videos
- InfinityLab Supplies catalog (5991-8031EN)
- Your local FSE and specialists
- YouTube – Agilent Channel
- Agilent Service Contracts

Appendix

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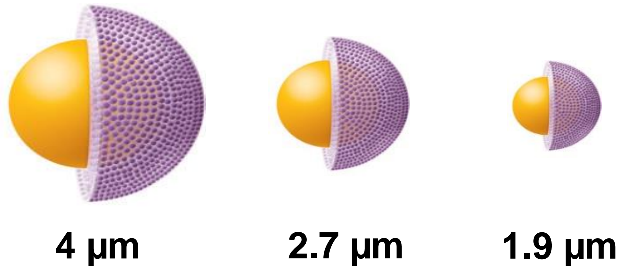
Agilent InfinityLab Poroshell 120 phases

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



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Buffer Preparation

1. Dissolve salt in organic-free water in 1- or 2-L beaker. Use appropriate volume to leave room for pH adjustment solution. Equilibrate solution to room temperature for maximum accuracy.

2. Calibrate pH meter. Use 2-level calibration and bracket desired pH. Use appropriate audit solution to monitor statistical control (for example, potassium hydrogen tartrate, saturated solution, pH = 3.56).

3. Adjust salt solution to desired pH. Minimize amount of time electrode spends in buffer solution (contamination). Avoid overshoot and readjustment (ionic strength differences can arise).

4. Transfer pH-adjusted buffer solution quantitatively to volumetric flask, dilute to volume, and mix.

5. Filter through 0.45 µm filter. Discard first 50 – 100 mL filtrate. Rinse solvent reservoir with small volume of filtrate and discard. Fill reservoir with remaining filtrate or prepare premix with organic modifier.
   - Agilent Solvent Filtration Kit, 250-mL reservoir, 1000-mL flask, p/n 3150-0577
   - Nylon filter membranes, 47 mm, 0.45 µm pore size, p/n 9301-0895

Determining the Dwell Volume of Your System

Look it up in the LC manual or follow the procedure below

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

Run gradient profile 0 - 100% B/10 min at 1.0 ml/min

Record

Expected Dwell Volume in UHPLC's – uL range!

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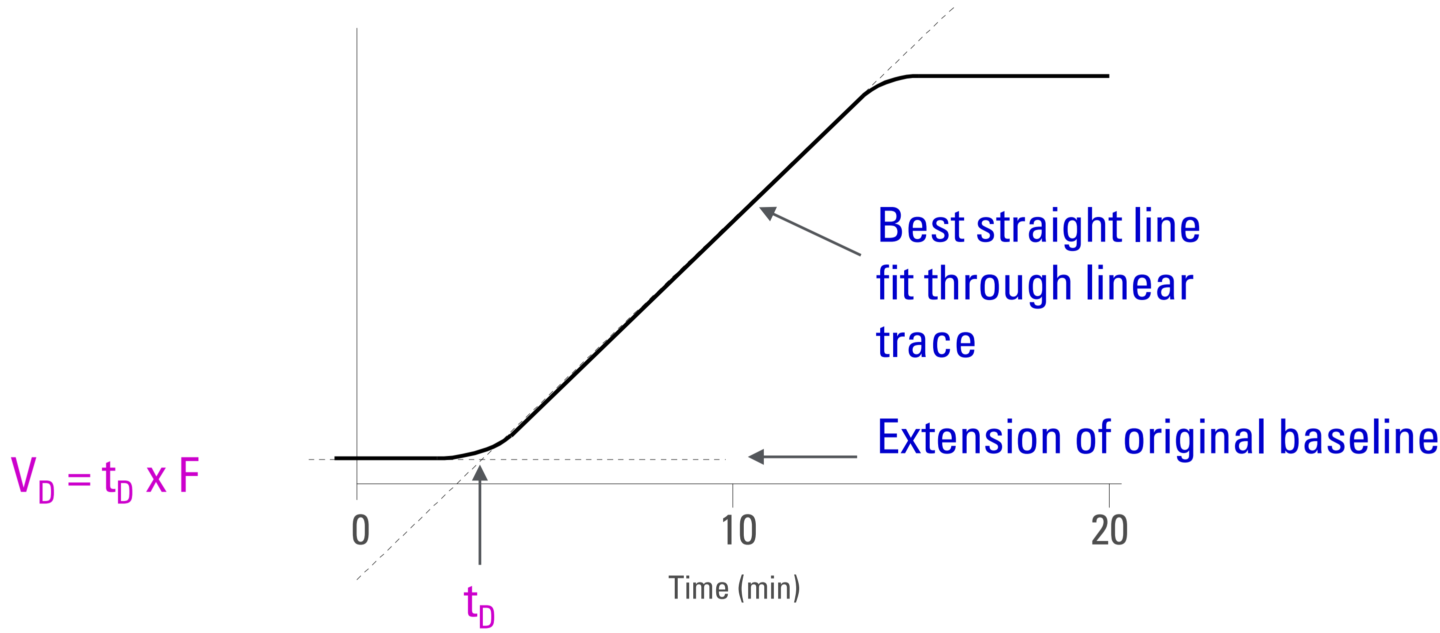
Measuring Dwell Volume (V_D)



- Intersection of the two lines identifies dwell time (t_D)
- Dwell volume is equal to product of the flow rate and the dwell time.

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