The Chromatography Detective:
Troubleshooting Tips & Tools for LC & LCMS

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What do you do when.....

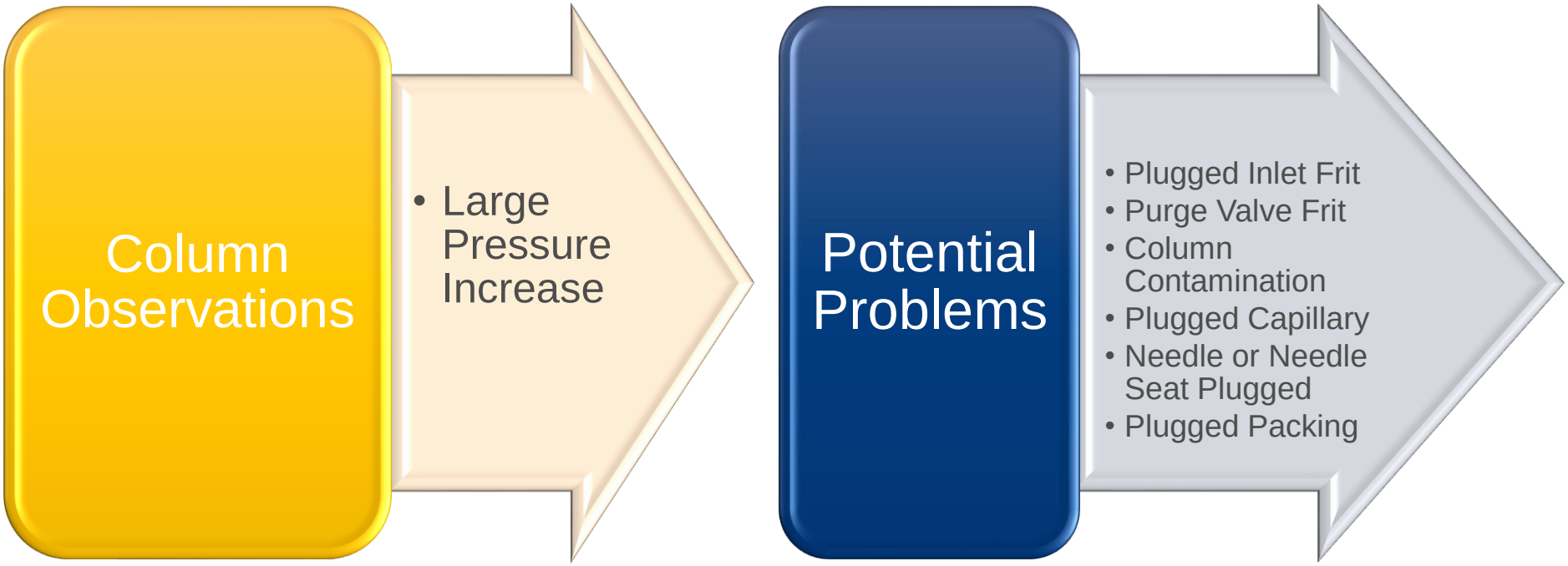
- Your chromatography changes
  - Peak shape
  - Retention

- You can't reproduce a method from
  - Pharmacopeia
  - Literature
  - Client
  - Colleague

Areas/Things to Investigate

- Separation
  - Pressure
  - Peak Shape
  - Retention
- Instrument
  - Components
  - Dwell volume & ECV
  - Tubing & Connections
- Column
  - Specifications
  - Characteristics
  - Tests
- Method Conditions
  - Mobile phase
  - Temperature
- Sample
  - Sample Prep
  - Injection

Pressure Issues



Note: Low pressure is typically a connection or LC issue; unless the column has been improperly used and disassembled or lost all its packing.

Correcting Overpressure

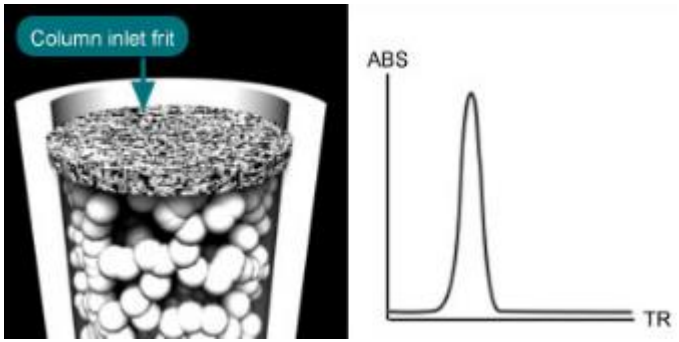
Determining the Cause and Correcting High Back Pressure

Many pressure problems relate to blockages in the system.
Check system pressure with / without column

If column pressure is high:

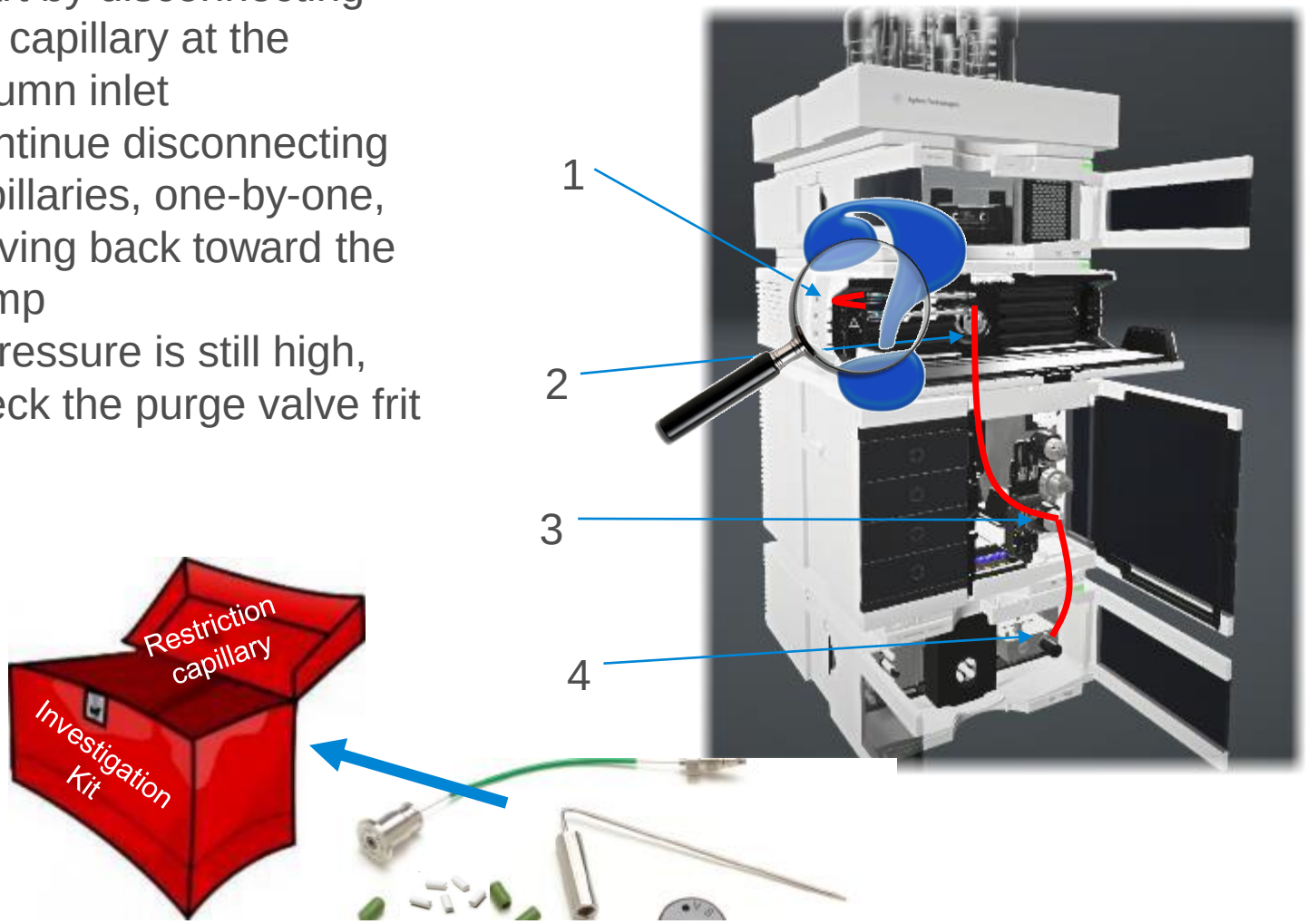
- Back flush column (care regarding future performance)
- Clear blocked frit (reverse flush with strong solvent)
- Wash column

  Eliminate column contamination and clear blocked packing
  Remove high M_w / adsorbed compounds
  Clear precipitate introduced from the sample or b



Investigating Pressure - Capillaries

- Start by disconnecting the capillary at the column inlet
- Continue disconnecting capillaries, one-by-one, moving back toward the pump
- If pressure is still high, check the purge valve frit



Tip: Prevent Column Pressure Problems

1. Filter mobile phase:
   - filter non-HPLC grade solvents
   - filter buffer solutions

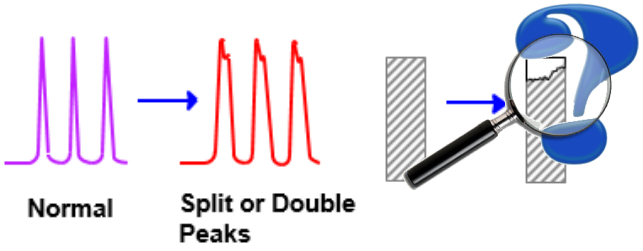
2. Filter all samples and standards
3. In-line filters
   - Install an in-line filter between auto-sampler and column (removes pump seal debris, injector rotor debris, and sample particulates). Use 2 um frit for 3.5 um/5um columns, use 0.5 um (or smaller) frit for 1.8 um/2.7 columns.
4. Perform sample clean-up (i.e. SPE, LLE) on dirty samples.
5. Appropriate column flushing – flush buffers from entire system with water/organic mobile phase
6. Replace buffers every 24-48 hours, never add to the bottle, always use a new one (see appendix, Avoid microbial growth)

Investigating Peak Shape Issues

Tailing, Broadening, Split, Loss of Efficiency (N, plates)

Split Peaks

1. Complex sample matrix or many samples analyzed - column contamination or partially plugged column frit.



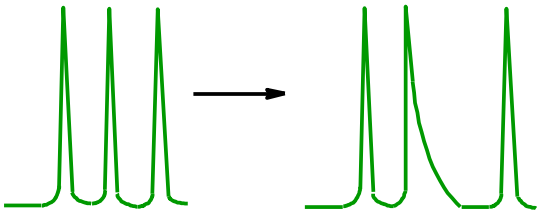
2. Mobile phase pH > 7 - column void due to silica dissolution. Use Poroshell 120 HpH, PLRP-S, Extend
3. Injection solvent stronger than mobile phase - likely split *and* broad peaks

Peak Tailing

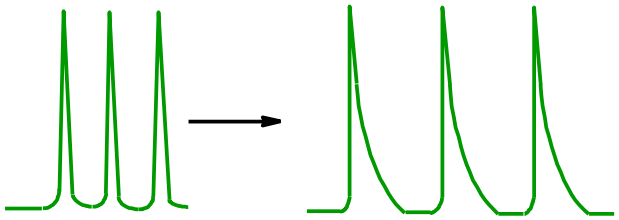
1. Mobile phase effects
2. Column choice; Reduce sample load – injection volume and concentration
3. Flush column and check for aging/void
4. Eliminate extra-column effects – tubing, fittings, UV cell
    a. ECV is volume in the sample flow path outside the column

Peak Shape: Tailing Peaks
First Question: All Peaks or Some Peaks?

Symmetry > 1.2



Normal Tailing



Normal Tailing

Causes

Some Peaks Tail:

- Secondary interactions
- Small peak eluting on tail of larger peak

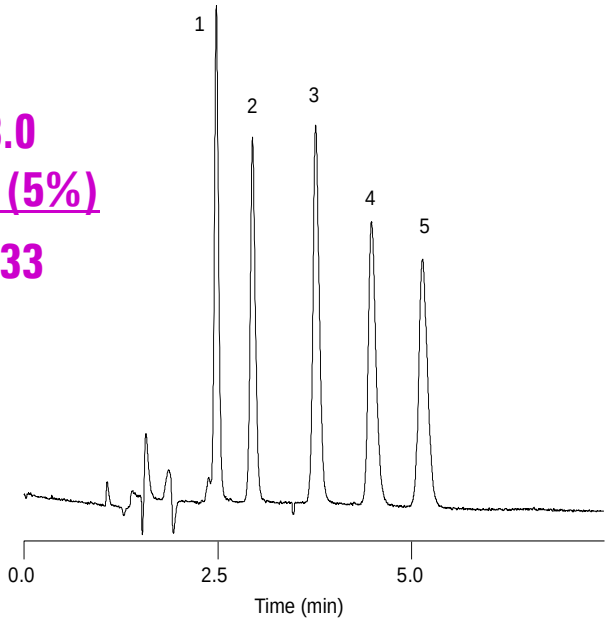
All Peaks Tail:

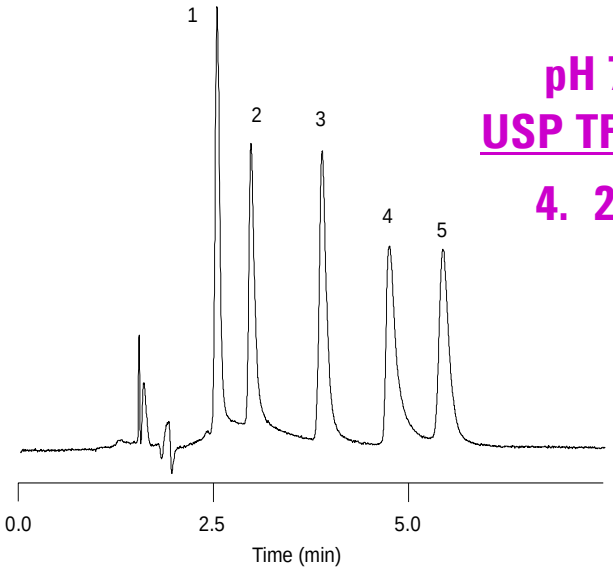
- Extra-column effects i.e. poor connections, too much volume
- Build up of contamination on column inlet (partially plugged frit)
- Bad column

Peak Tailing
Column “Secondary Interactions”

Column: C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

pH 3.0
USP TF (5%)
4. 1.33





pH 7.0
USP TF (5%)
4. 2.35

- Reducing the mobile phase pH reduces interactions with silanols that can cause peak tailing; No additional mobile phase modifiers required
- Consider bonded phase with more endcapping, designed for good pH 7 performance

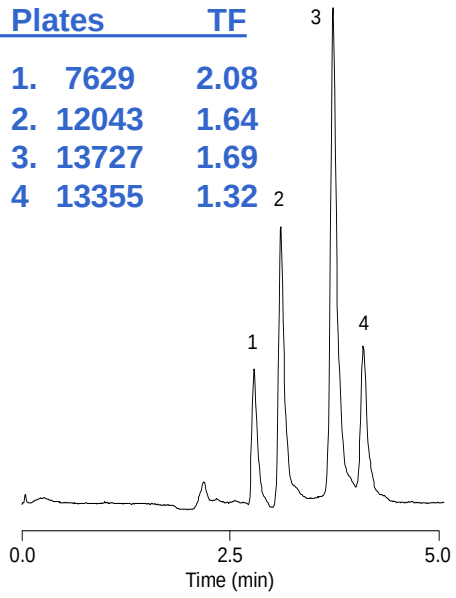
Peak Tailing - Column Contamination

Investigation – Determine if column is dirty or damaged

How? – Reverse column and run sample or standard

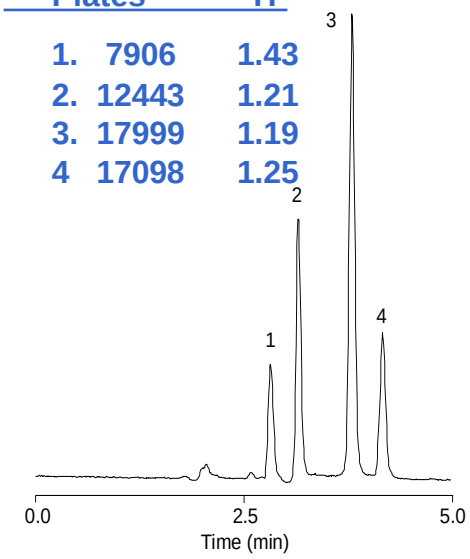
QC test forward direction

	Plates	TF
1.	7629	2.08
2.	12043	1.64
3.	13727	1.69
4	13355	1.32



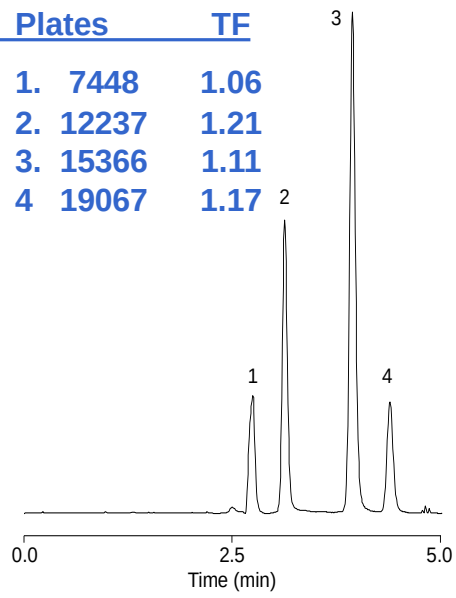
QC test reverse direction

	Plates	TF
1.	7906	1.43
2.	12443	1.21
3.	17999	1.19
4	17098	1.25



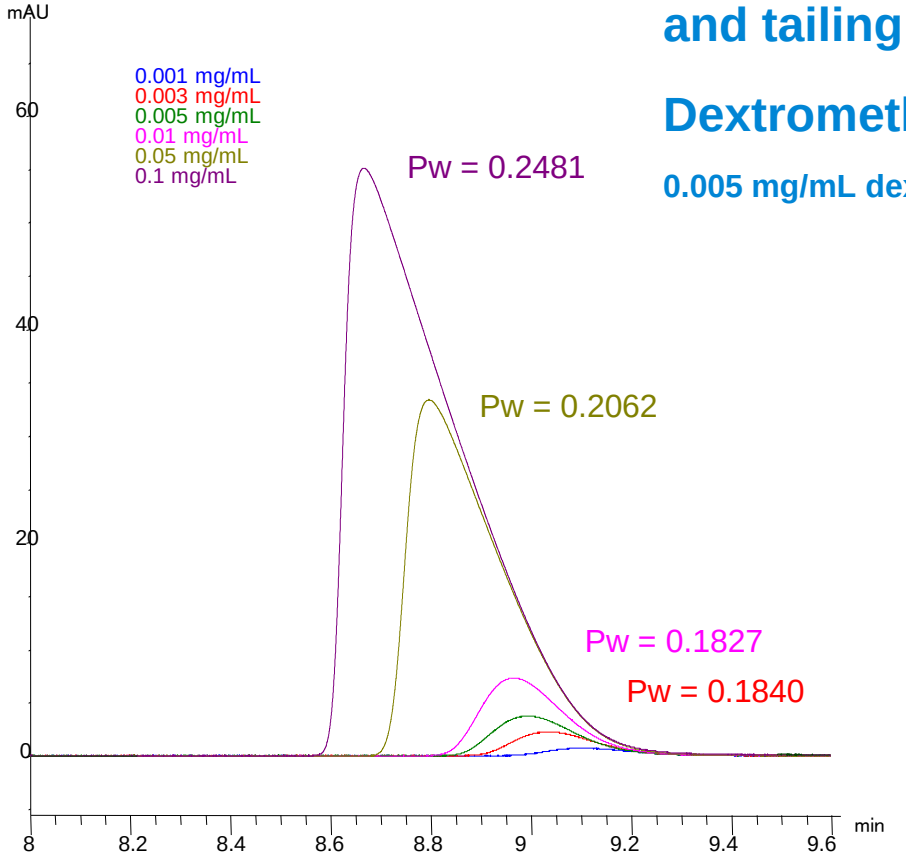
QC test after cleaning 100% IPA, 35°C

	Plates	TF
1.	7448	1.06
2.	12237	1.21
3.	15366	1.11
4	19067	1.17



Column: StableBond SB-C8, 4.6 x 250 mm, 5µm Mobile Phase: 20% H_2O : 80% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

Broadening and Tailing
Compare Peak Shape at Low and High Loads



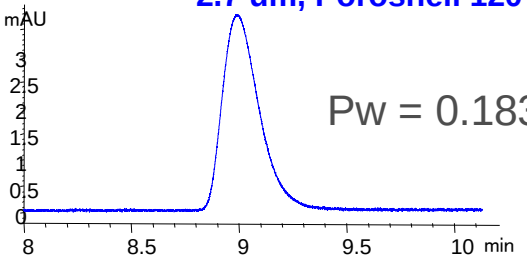
High Sample Loads can give broad or broad and tailing peaks

Dextromethorphan is 35% broader at high load

0.005 mg/mL dextromethorphan (4.1 uL injection volume)

Low sample loads provide symmetrical, non-tailing peaks with narrow peak widths

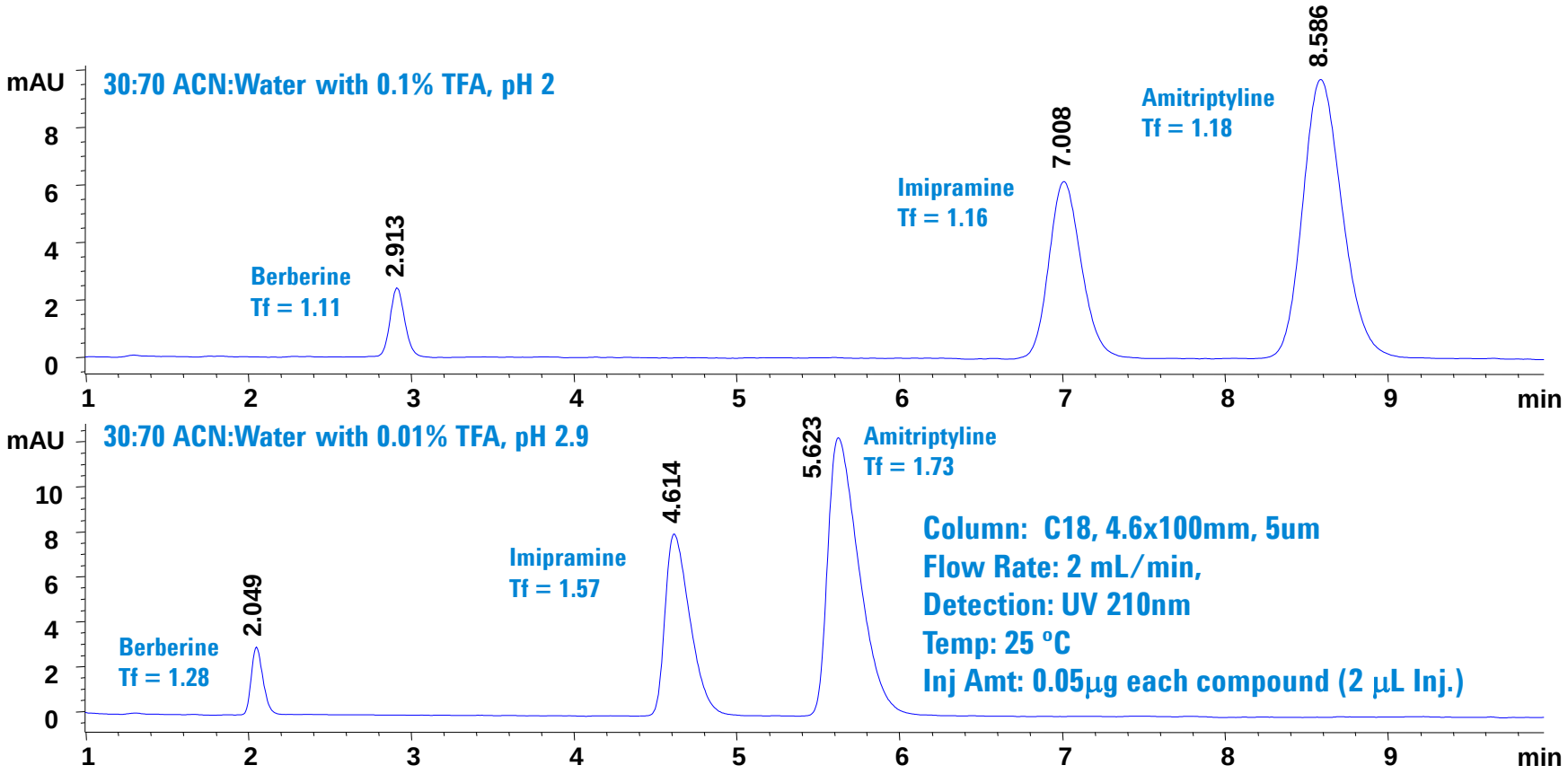
2.7 um, Poroshell 120 EC-C18



Retention Shifts

1. All Peaks Shift to Lower Retention (acids, bases, neutrals)
    a) Loss of Bonded-Phase
    b) Mobile Phase Unstable (less likely)
    c) Solvent Delivery System (flow rate)
2. All Peaks Shift to Greater Retention
    a) Loss of Organic Solvent in Aqueous / Organic Mix
    b) Column Change (less likely)
    c) Solvent Delivery System (flow rate)
3. Ionic Peaks Shift Retention
    a) Loss of Volatile MP Component (ionic strength, pH shift)
    b) Column Change (bonded phase or contamination)

Changes in Volatile Buffer Concentration
Shift in Retention Time and Peak Shape



Separation Conditions That Cause Changes in Retention*

Flow Rate	**+/- 1%**	**+/- 1% Tr**
Temp	**+/- 1 deg C**	**+/- 1 to 2% Tr**
%Organic	**+/- 1%**	**+/- 5 to 10% Tr**
pH	**+/- 0.01%**	**+/- 0 to 1% Tr**

*excerpted from “Troubleshooting HPLC Systems”, J. W. Dolan and L. R. Snyder, p 442.

Areas/Things to Investigate

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- Instrument
  - Components
  - Dwell volume & ECV
  - Tubing & Connections
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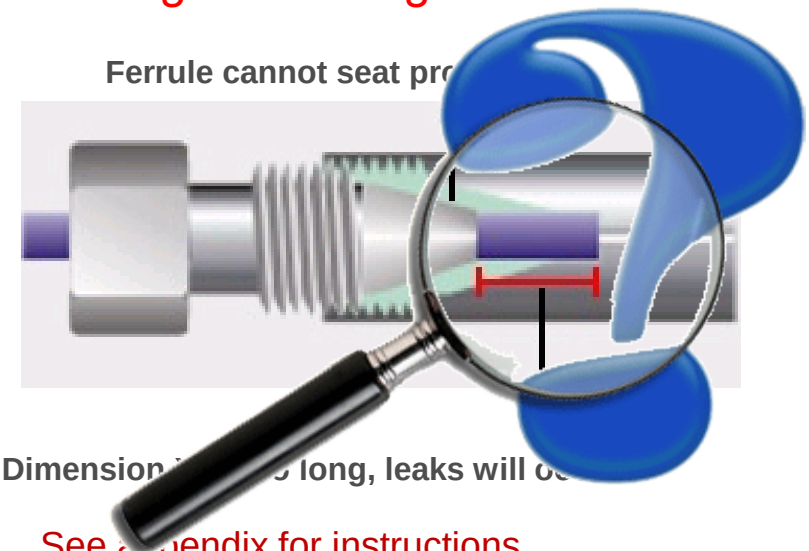
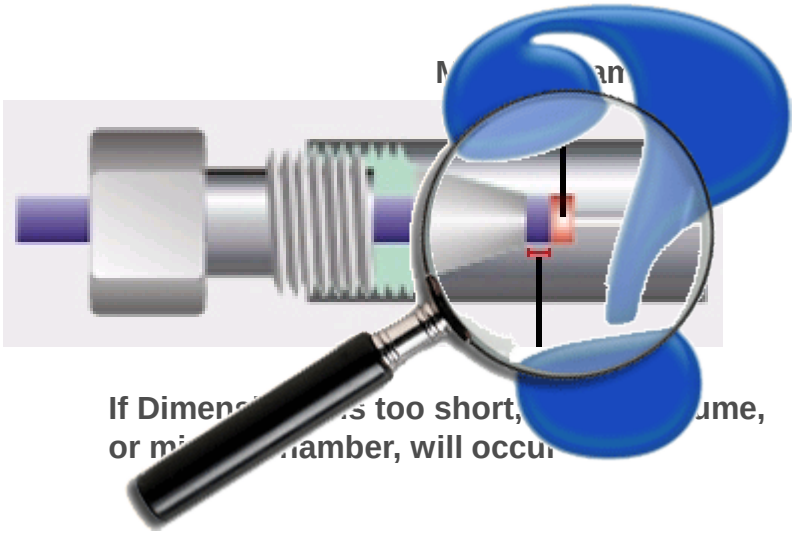
What Happens If the Connections Poorly Made ?

- Problems with improper connections
  - ✓ **Mistaken for chromatography issues**
- Making connections can vary with skill/technique
- Different manufacturers supply different types of fittings

Wrong … too long

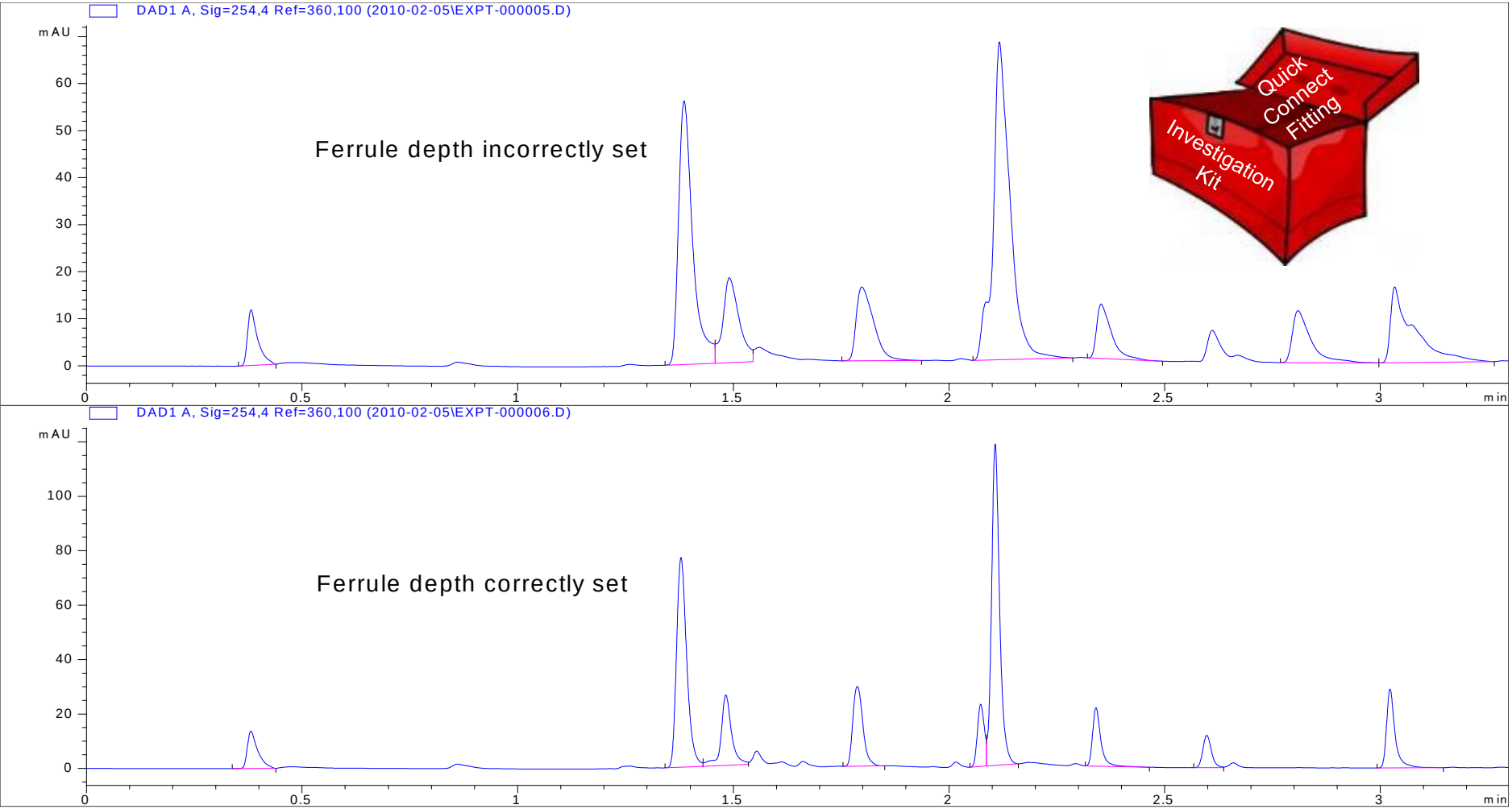


Wrong … too short



➢ See appendix for instructions

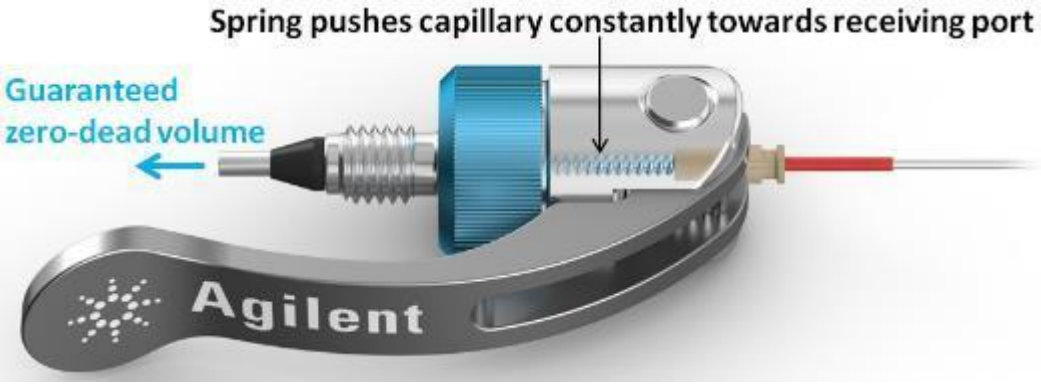
Effect of incorrectly setting the inlet tubing (1mm void)



Column : 50mm x 2.1mm x 1.8um Eclipse Plus C18

Simplify Column and Fitting Connections!

InfinityLab A-Line Quick Connect and Quick Turn



Quick Turn fitting

- **Ease of use functionality**
- **Quick Connect seals with a simple turn of the lever**

The spring-loaded design constantly pushes the tubing against the receiving port, delivering a reproducible connection with no dead volume for consistent chromatographic performance.

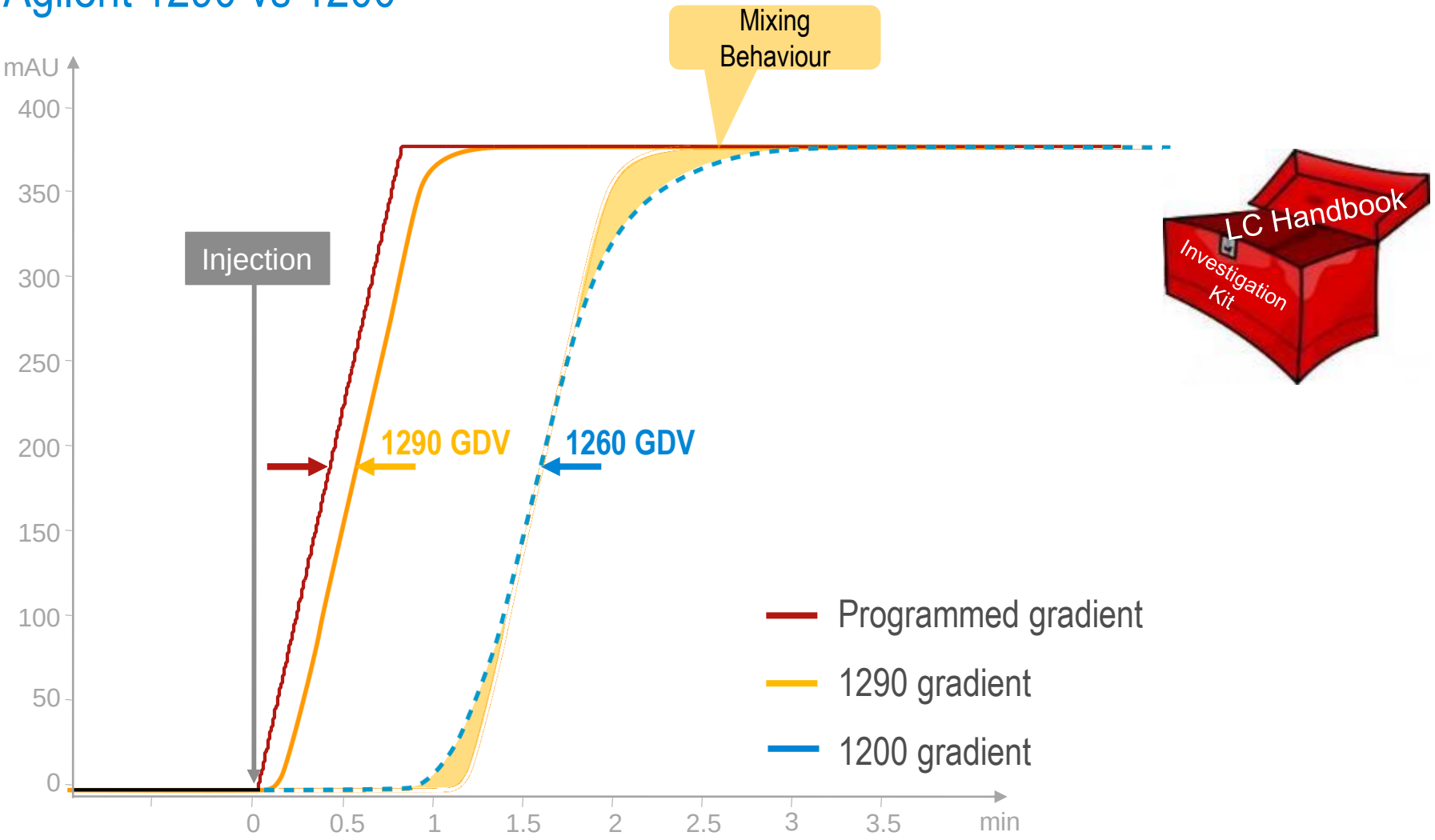
- **Quick Connect Finger-tight to 1300bar**
- **Quick Turn Finger-tight to 600bar, wrench-tight to1300bar**

Stem length is adjustable through the spring, which makes the fitting compatible with all types of LC columns

- See appendix for instructions

Delay Volume

Agilent 1290 vs 1200



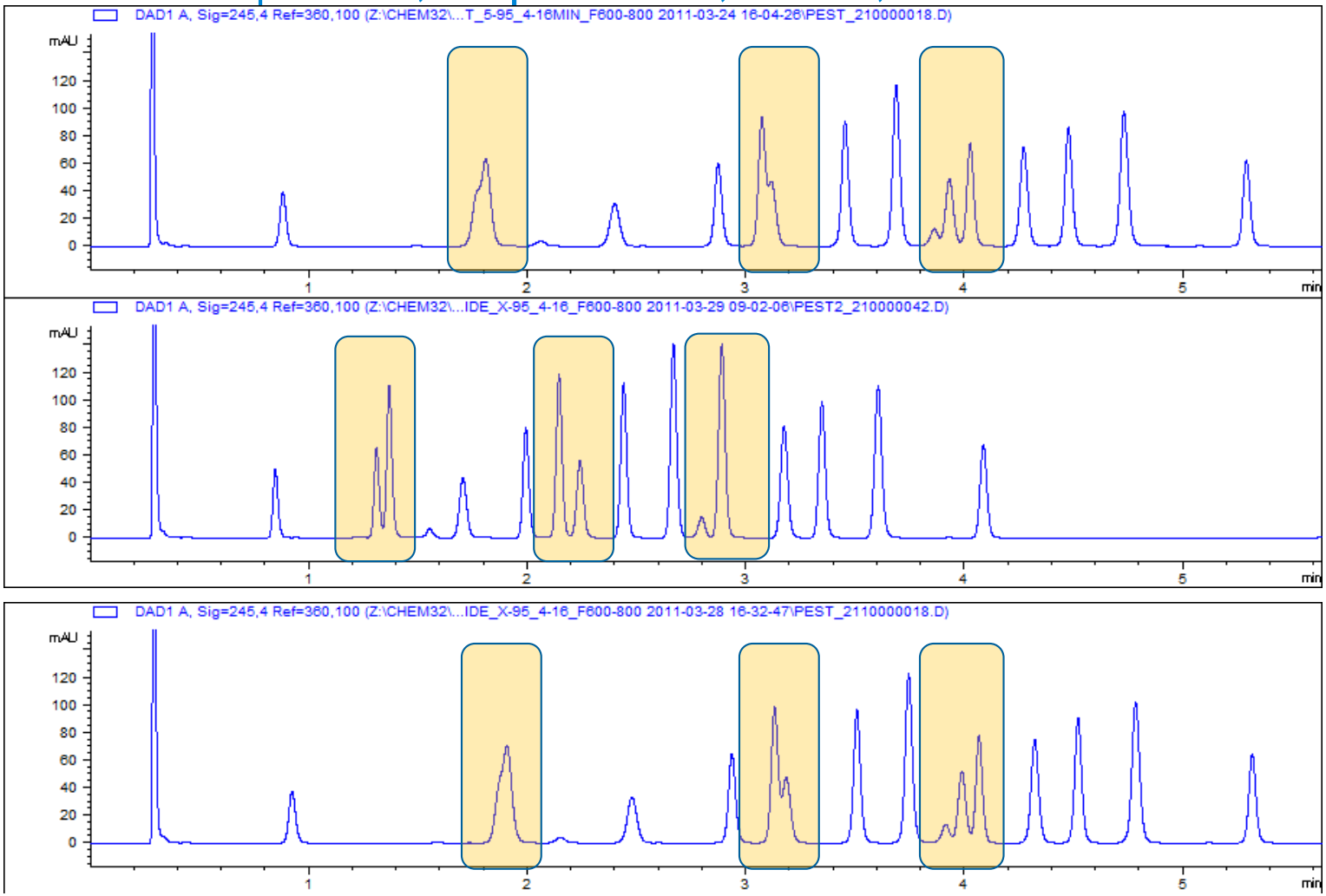
Instrument Dwell Volume Differences Can Cause Changes in Retention and Resolution

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

1100 Quaternary 400 bar

1290 Infinity II 1300 bar

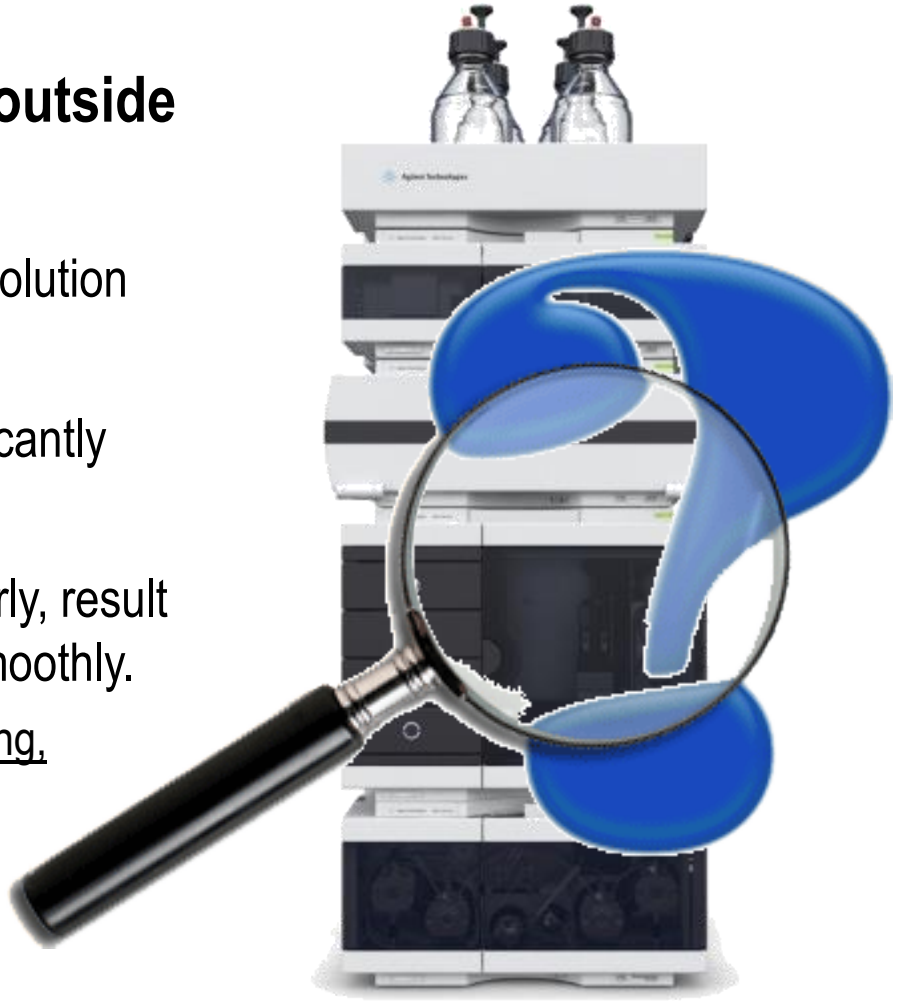
1290 Infinity II 1300 bar with ISET



Extra Column Volume

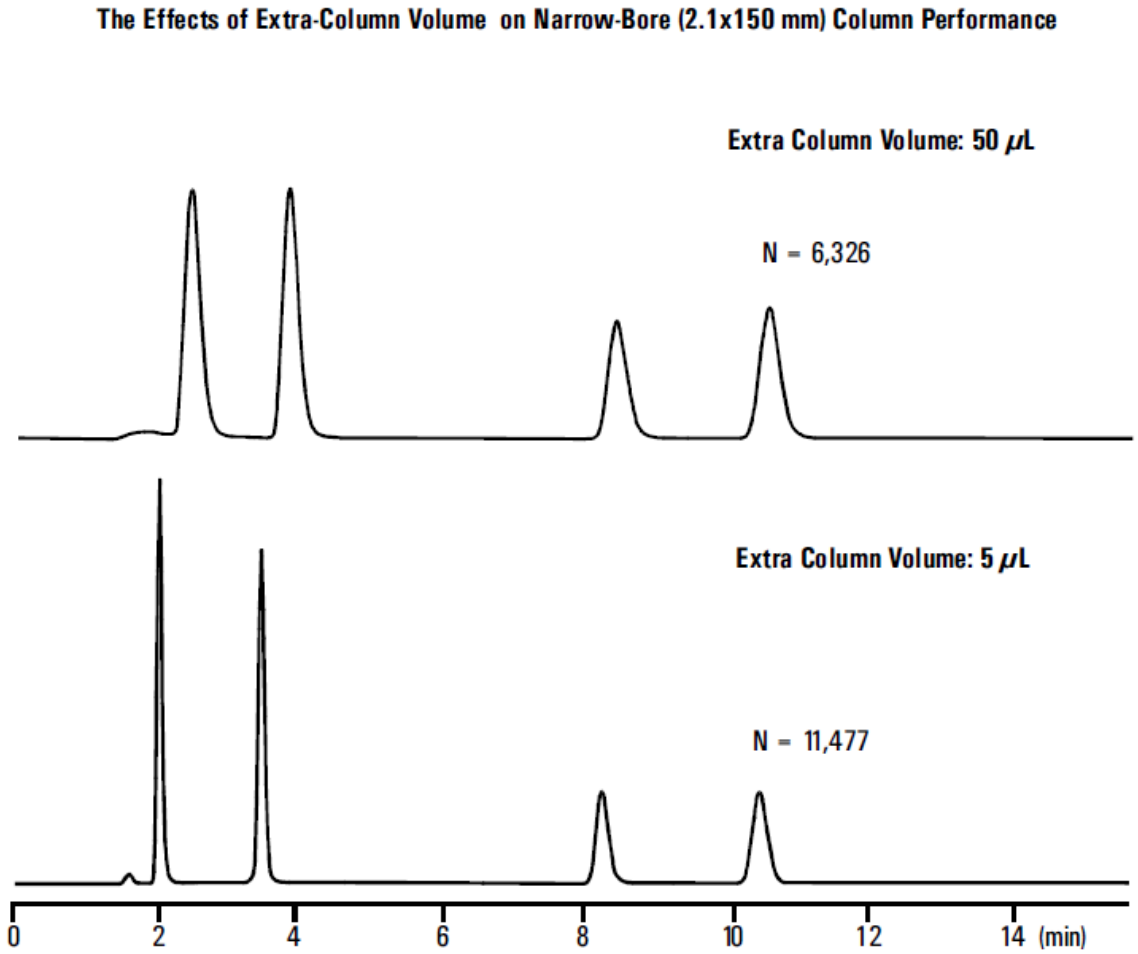
ECV is volume of sample flowpath outside of the column

- Extra column band broadening affects resolution and detection sensitivity
- 2.1mm ID columns and smaller are significantly affected by extra column volume effects!
- Connections and fittings, if made improperly, result in areas where the flow does not move smoothly.
  - Unswept or poorly swept areas will cause tailing, broadening and loss of column efficiency
  - Use Infinity Lab/A-Line fittings



Agilent 1290 Infinity II LC

System Design – Extra Column Volume Effects



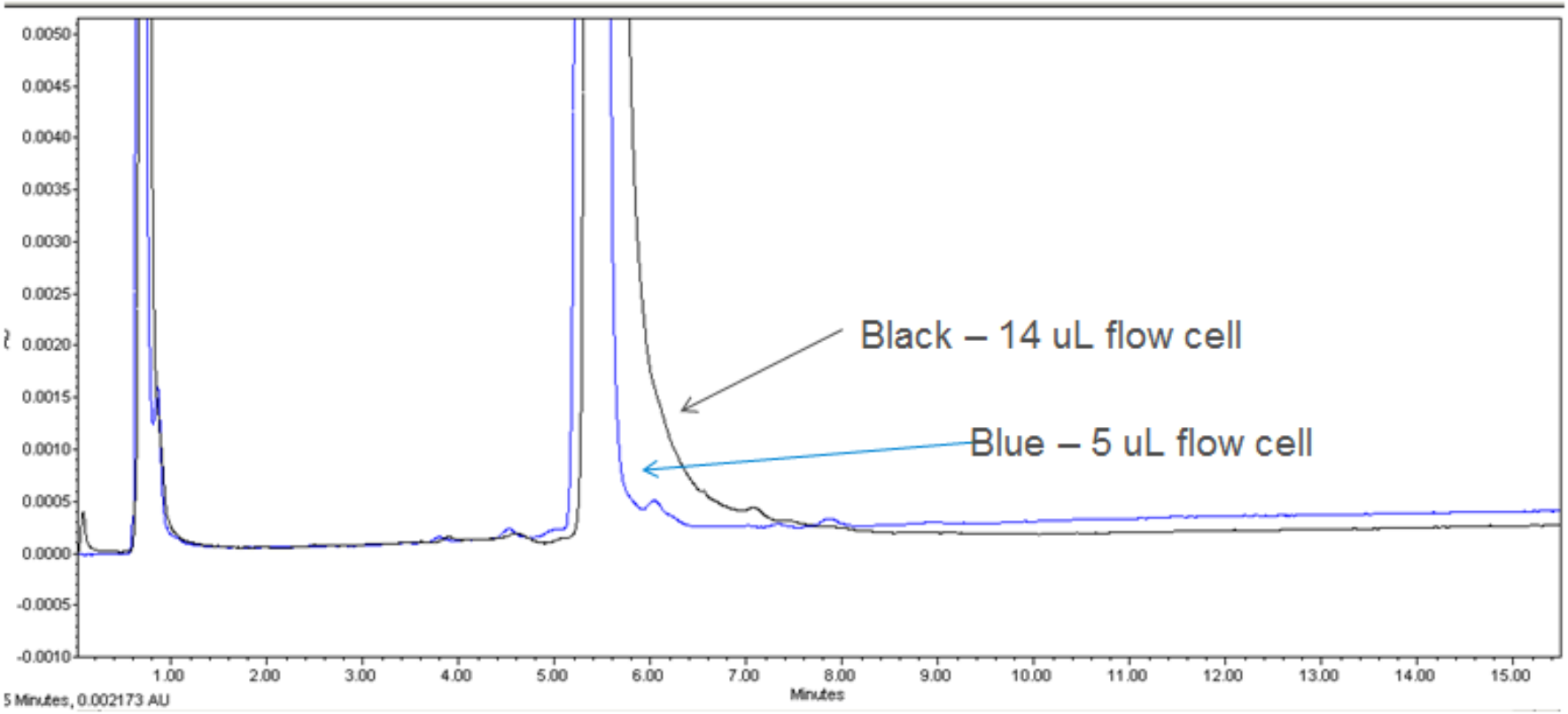
Volume Characteristics

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

Peak Volume Matters!

- Keep injector to column and column to detector tubing length and ID as small as possible.
- **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

Smaller Column id Needs a Smaller Flow Cell Volume



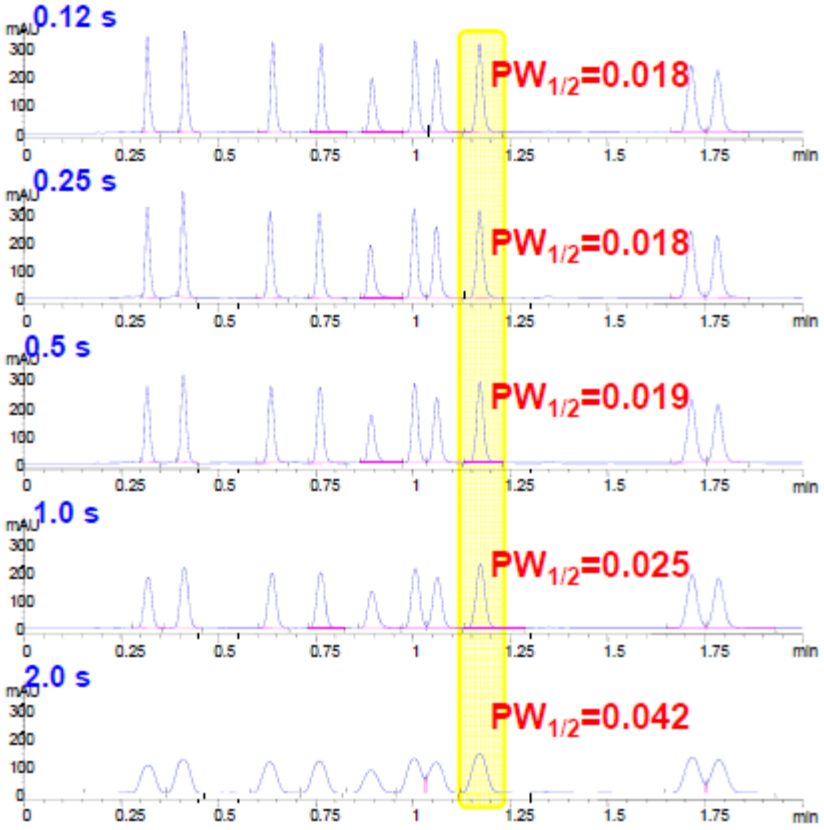
3 x 100mm Column
peak volume ~75 uL

Dimension	Sensitivity*	Resolution*
13 µl / 10mm	+++	+
5 µl / 6mm	++	++
2 µl / 3mm	+	+++

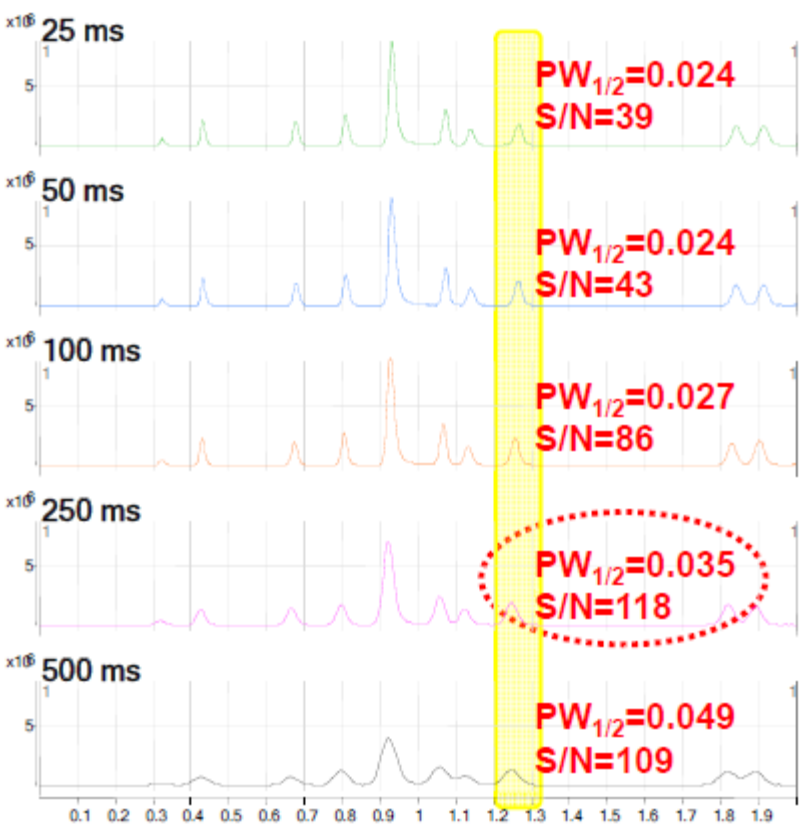
Investigate Your UV Data Collection Rate and MS Scan Rate in Scan Mode for Best Results

Column: ZORBAX RRHD SB-C18, 2.1 x 100mm, 1.8um, 1200 bar
Sample: Green Tea

UV Data Collection Rate



MS Scan Rate



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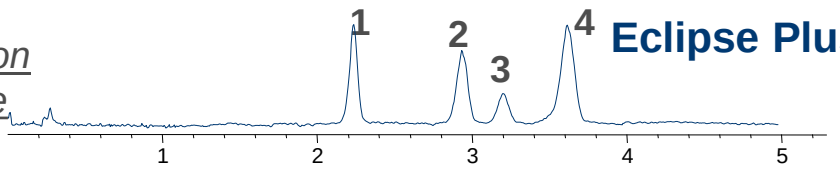
Poroshell “120” Chemistries

InfinityLab Poroshell Family		Pore Size	Temp. Limits	pH Range	Endcapped	Carbon Load	Surface Area
Best all around	EC-C18	120Å	60°C	2.0-8.0	Double	10%	130 m2/g
	EC-C8	120Å	60°C	2.0-8.0	Double	5%	130 m2/g
Best for low-pH mobile phases	SB-C18	120Å	90°C	1.0-8.0	No	9%	130 m2/g
	SB-C8	120Å	80°C	1.0-8.0	No	5.5%	130 m2/g
Best for high-pH mobile phases	HPH-C18	100Å	60°C	3.0-11.0	Double	Proprietary	95 m2/g
	HPH-C8	100Å	60°C	3.0-11.0	Double	Proprietary	95 m2/g
Best for basic compounds at low pH	CS-C18	120Å	60°C	2.0-8.0	Double	Proprietary	95 m2/g
Best for polar compounds (HILIC)	HILIC	120Å	60°C	0.0-8.0	N/A	N/A	130 m2/g
	HILIC-Z	120Å	80°C	3.0-11.0	Proprietary	Proprietary	130 m2/g
	HILIC-OH5	120Å	45°C	1.0-7.0	Double	Proprietary	130 m2/g
Best for alternative selectivity	Bonus-RP	120Å	60°C	2.0-9.0	Triple	9.5%	130 m2/g
	PFP	120Å	60°C	2.0-8.0	Double	5.1%	130 m2/g
	Phenyl-Hexyl	120Å	60°C	2.0-8.0	Double	9%	130 m2/g
	SB-Aq	120Å	80°C	1.0-8.0	No	Proprietary	130 m2/g
	EC-CN	120Å	60°C	2.0-8.0	Double	3.5%	130 m2/g
Best for Chiral separations	Chiral-T	120Å	45°C	2.5-7.0	Proprietary	Proprietary	130 m2/g
	Chiral-V	120Å	45°C	2.5-7.0	Proprietary	Proprietary	130 m2/g
	Chiral-CD	120Å	45°C	3.0-7.0	Proprietary	Proprietary	130 m2/g
	Chiral-CF	120Å	45°C	3.0-7.0	Proprietary	Proprietary	130 m2/g

Specifications represent typical values only

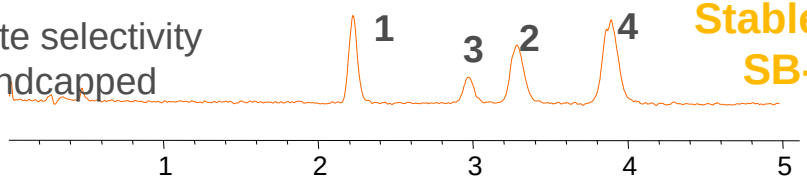
Not All C18s Are The Same

1st choice
Best Resolution & Peak Shape



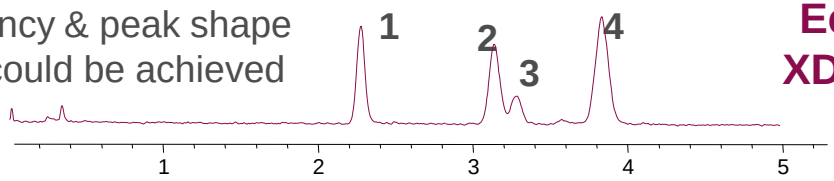
Eclipse Plus C18

2nd choice
Good alternate selectivity due to non-endcapped



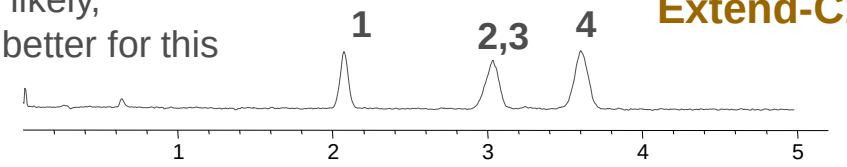
StableBond SB-C18

3rd choice
Good efficiency & peak shape
Resolution could be achieved



Eclipse XDB-C18

4th choice
Resolution not likely,
Other choices better for this separation.

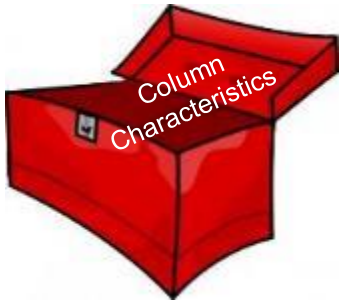


Extend-C18

Mobile phase: (69:31) ACN: water
Flow 1.5 mL/min.
Temp: 30 °C
Detector: Single Quad ESI positive mode scan
Columns: RRHT 4.6 x 50 mm 1.8 um

Sample:
1. anandamide (AEA)
2. Palmitoylethanolamide (PEA)
3. 2-arachinoylglycerol (2-AG)
4. Oleoylethanolamide (OEA)

- Multiple bonded phases for most effective method development
- Match to one you are currently using
- *Method development kits are available*



➢Don't assume every C18 will behave the same

Performance Report

SERIAL NUMBER: USDAZ01333

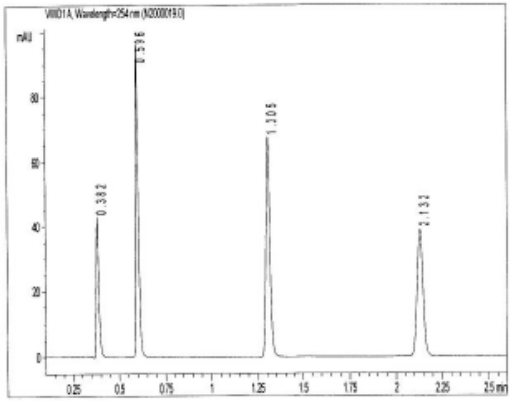
PART NUMBER: 959758-902
COLUMN TYPE: ZORBAX RRHD Eclipse Plus C18 2.1 x 100 mm, 1.8 µm
PACKING LOT #: B09089

TEST CONDITIONS

MOBILE PHASE	=	60% Acetonitrile / 40% Water
COLUMN PRESSURE	=	517.2 Bar
COLUMN FLOW	=	0.50 ml / min
LINEAR VELOCITY	=	0.436 cm / sec
TEMPERATURE	=	AMBIENT (Nominally 23 °C)
INJECTION VOLUME	=	1 µl

QUALITY CONTROL PERFORMANCE RESULTS FOR NAPHTHALENE

	TEST VALUES	SPECIFICATIONS
THEORETICAL PLATES =	22337	MIN = 21000
SELECTIVITY =	1.90	RANGE = 1.82 - 1.92
USP TAILING FACTOR = (@ 5% Peak Height)	1.08	RANGE = 0.98 - 1.20
k' =	4.58	



Sample components with concentrations diluted in mobile phase in the following elution order.

Peak #	Conc (ug/ml)	Sample Component
1	10	Uracil
2	400	Phenol
3	50	4-Chloro Nitrobenzene
4	80	Naphthalene

- Manufacturing test chromatogram is done on a modified LC system to minimize extra column volume and will differ from a typical lab instrument

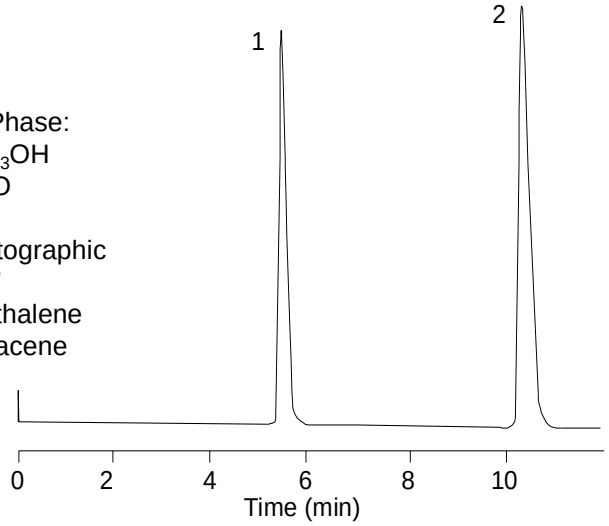
- Don't expect to get the exact same result as the performance report
- Test column performance on your instrument to have as a reference

Experimental Conditions for Classify Column Selectivity Changes

Bonded-Phase Test

Mobile Phase:
85% CH_3OH
15% H_2O

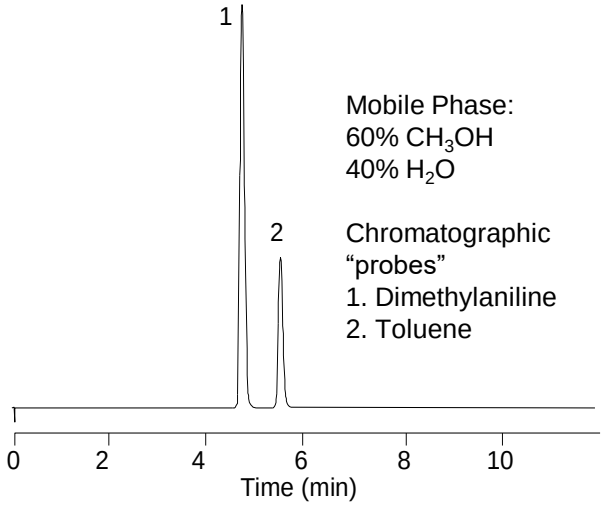
Chromatographic
"probes"
1. Naphthalene
2. Anthracene



Silanol Activity Test

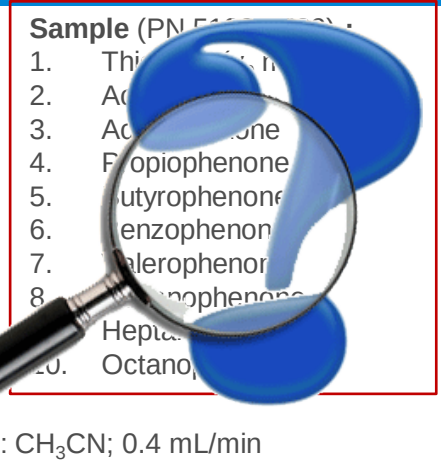
Mobile Phase:
60% CH_3OH
40% H_2O

Chromatographic
"probes"
1. Dimethylaniline
2. Toluene

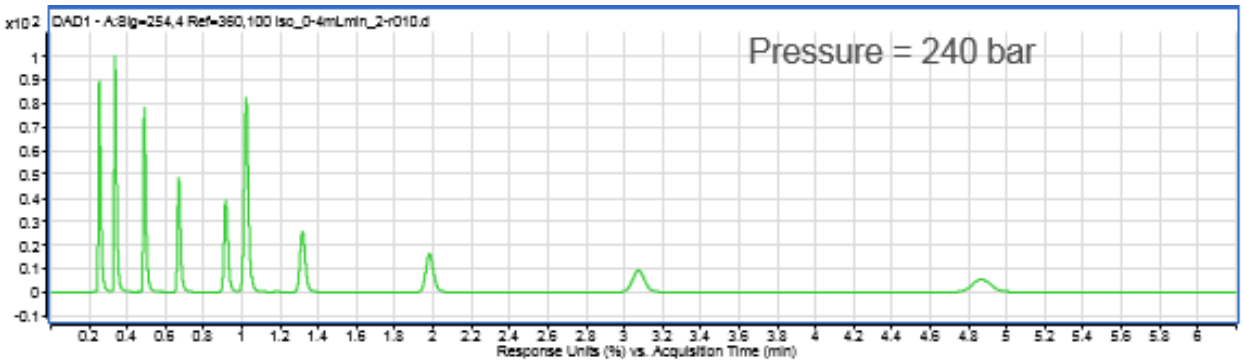


α value changes of >10% suggest changes in bonded-phase or silica

Investigation Kit
Column Test Mix - PN 5188-6529

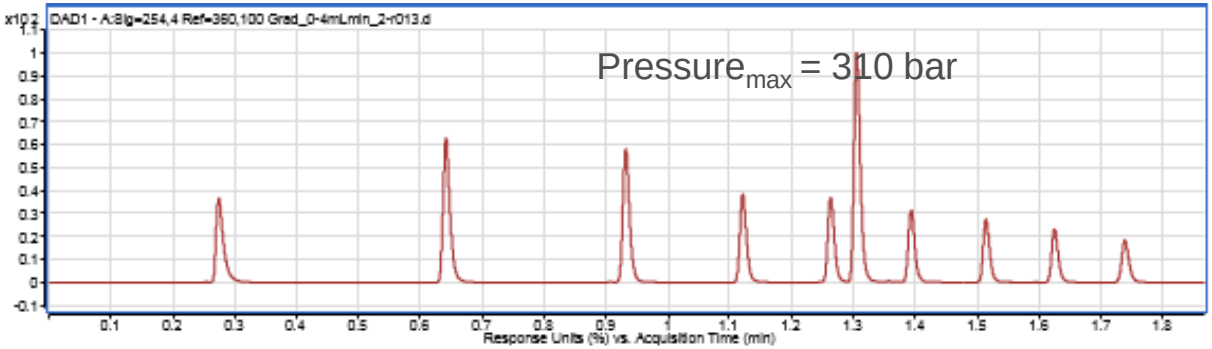


Isocratic
Optimized Agilent 1290 Infinity LC System, 3.9 µL Extra-Column Volume



A: H_2O; B: CH_3CN; 0.4 mL/min
Isocratic, 60% B
1 µL injection of RRLC Checkout Sample spiked w/ 50 µL 2 mg/mL thiourea in water/acetonitrile
TCC: 26 ºC
DAD: Sig = 254, 4 nm; Ref = Off
Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm x 50 mm, 1.8 µm

Gradient
Optimized Agilent 1290 Infinity LC System, 3.9 µL Extra-Column Volume



A: H_2O; B: CH_3CN; 0.4 mL/min

t (min)	0	1.2
%B	25	95

1 µL injection of RRLC Checkout Sample (PN 5188-6529) spiked w/ 50 µL 2 mg/mL thiourea in water/acetonitrile
TCC: 26 ºC
DAD: Sig = 254, 4 nm; Ref = Off
Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm x 50 mm, 1.8 µm

Why pH Matters
Column Lifetime

Low pH (pH <3)

Hydronium-catalyzed hydrolysis of bonded phase siloxane

- Loss of Bonded Phase
- Change in retention times (usually decrease)

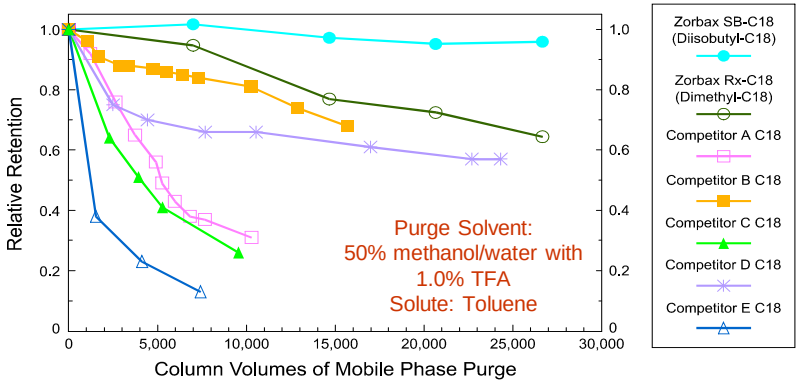
➢Choose column for low pH

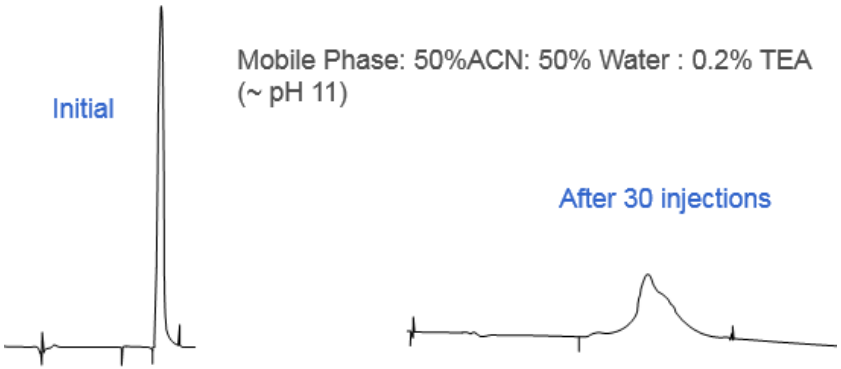
Intermediate to High pH (pH >7)

Dissolution of silica by the hydroxide ion

- Loss of silica, void development
- Loss of resolution

➢End-capped, high pH column, polymer





Areas/Things to Investigate

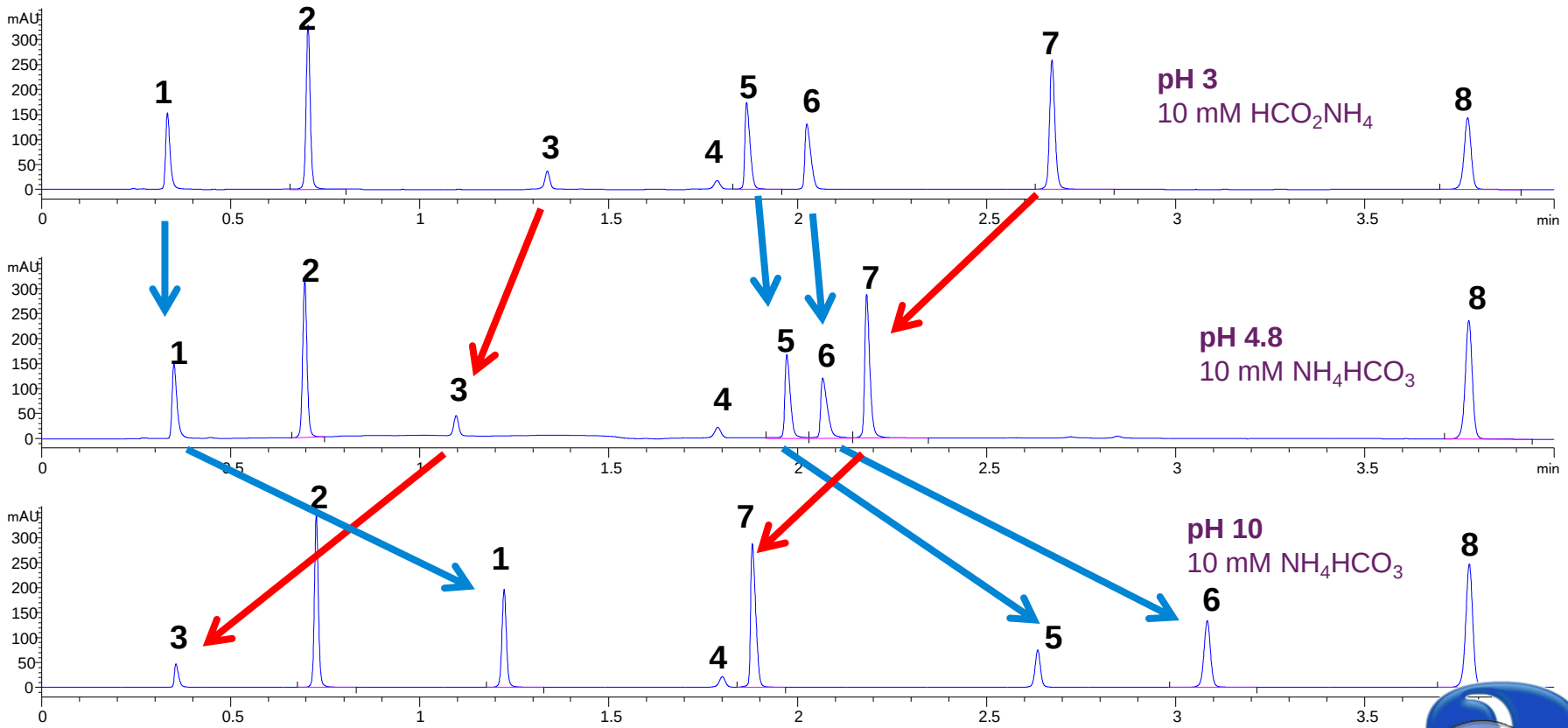
- Separation
  - Pressure
  - Peak Shape
  - Retention
- Instrument
  - Components
  - Dwell volume & ECV
  - Tubing & Connections
- Column
  - Specifications
  - Characteristics
  - Tests
- Method Conditions
  - Mobile phase
  - Temperature
- Sample
  - Sample Prep
  - Injection

pH Can Affect Your Separation

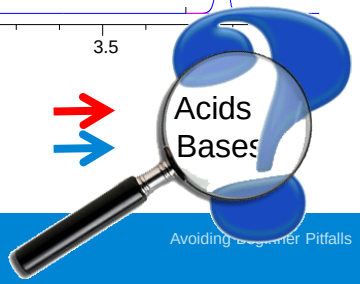
Time	% Buffer	% MeCN
0	10	90
5	90	10
7	10	90
2 ml/min		254 mn

1. Procainamide
2. Caffeine
3. Acetyl Salicylic Acid
4. Hexanophenone Deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone

Poroshell HPH-C18 4.6 x 50 mm, 2.7 µm

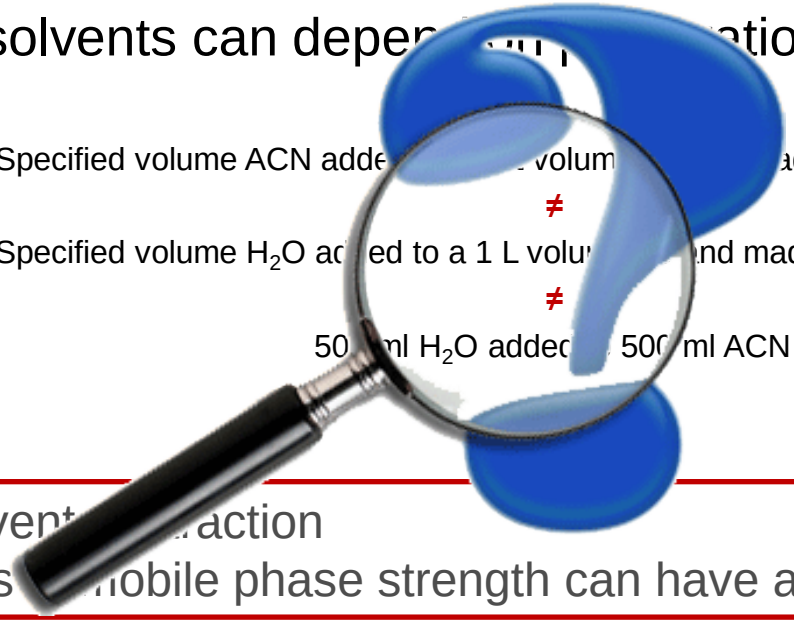


- Know if pH affects the retention of your analytes
- More retention for non-charged analytes
  - Acids at low pH and bases at high pH



Mobile Phase Preparation

- HPLC grade or better
- Buffer preparation procedure
  - Buffers usually contain insoluble material – filter
  - Buffer solubility decreases with increasing % organic* - Caution - 100%B with buffer salts
  - Be consistent – Document the process (see appendix)
- Volume % of solvents can depe[illegible]ation



Specified volume ACN adde[illegible] volum[illegible]ade to volume with H_2O

≠

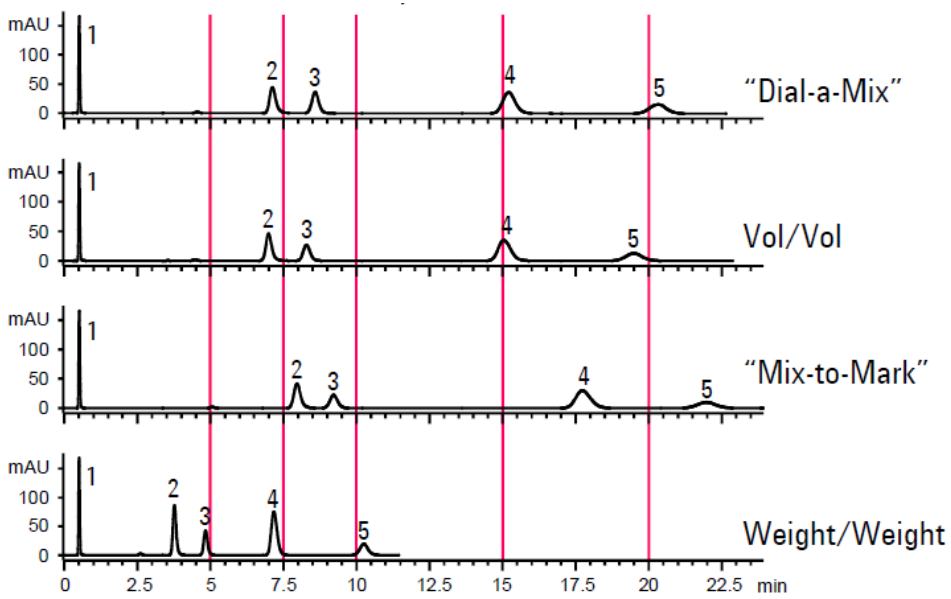
Specified volume H_2O ad[illegible]ed to a 1 L volu[illegible]nd made to volume with ACN

≠

50[illegible]ml H_2O added[illegible] 500 ml ACN

- Degree of solvent[illegible]raction
- Small changes[illegible]obile phase strength can have a large effect on retention

Mobile Phase Preparation
Effect on Chromatography



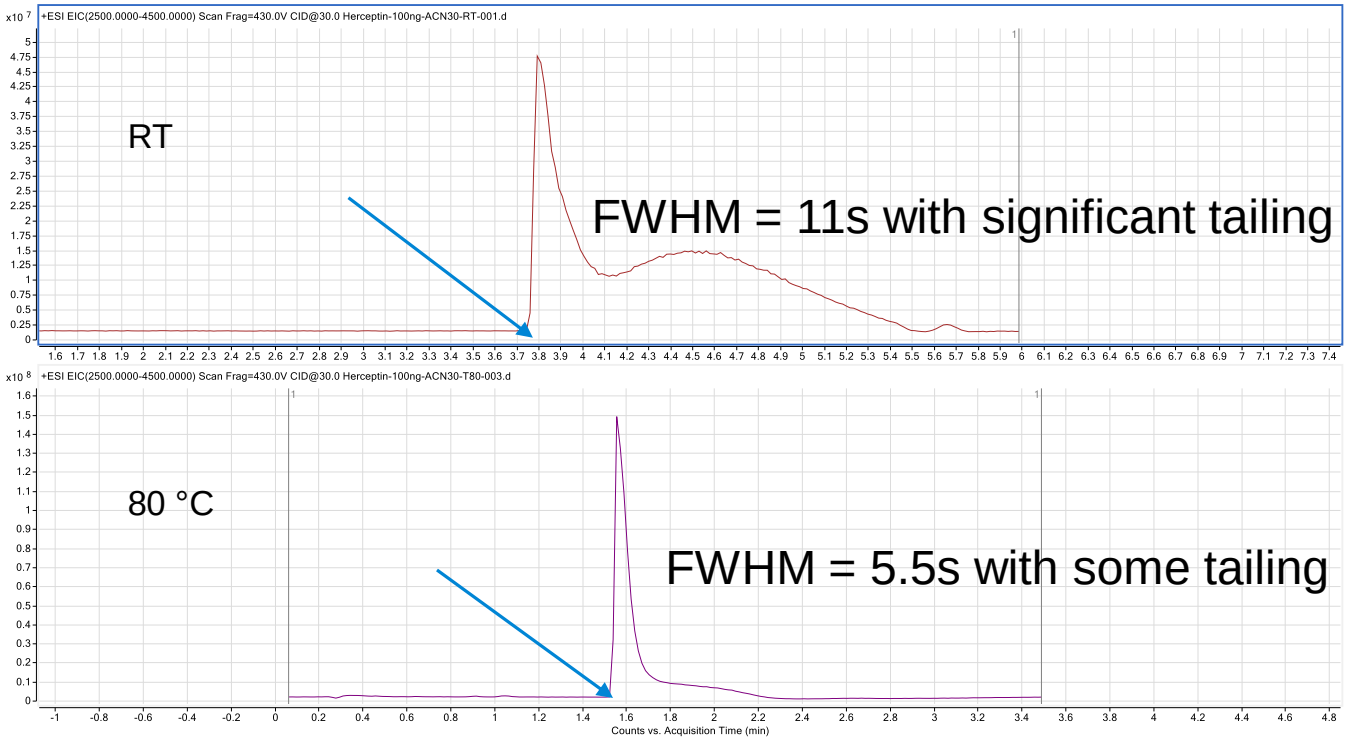
HPLC System:	Agilent 1100 with quaternary pump
Column:	ZORBAX Eclipse XDB-C8 Rapid-Resolution (3.5µm), 4.6 x 50 mm Agilent Part No. 935967-906
Mobile Phases:	Dial-a-Mix= A: water B: MeOH, pump 50% B Vol/Vol=250 mL water + 250 mL MeOH, pump 100% Mix-to-Mark = 250 mL MeOH, fill to 500 mL with water, pump 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

Chromatography Optimization: Column Temperature (Herceptin)



Zorbax 300 diphenyl Column, 2.1× 50 mm (or 100mm), 1.8µm, 0.5ml/min

Know if your sample is affected by temperature

Ghost Peaks
Where Do They Come From

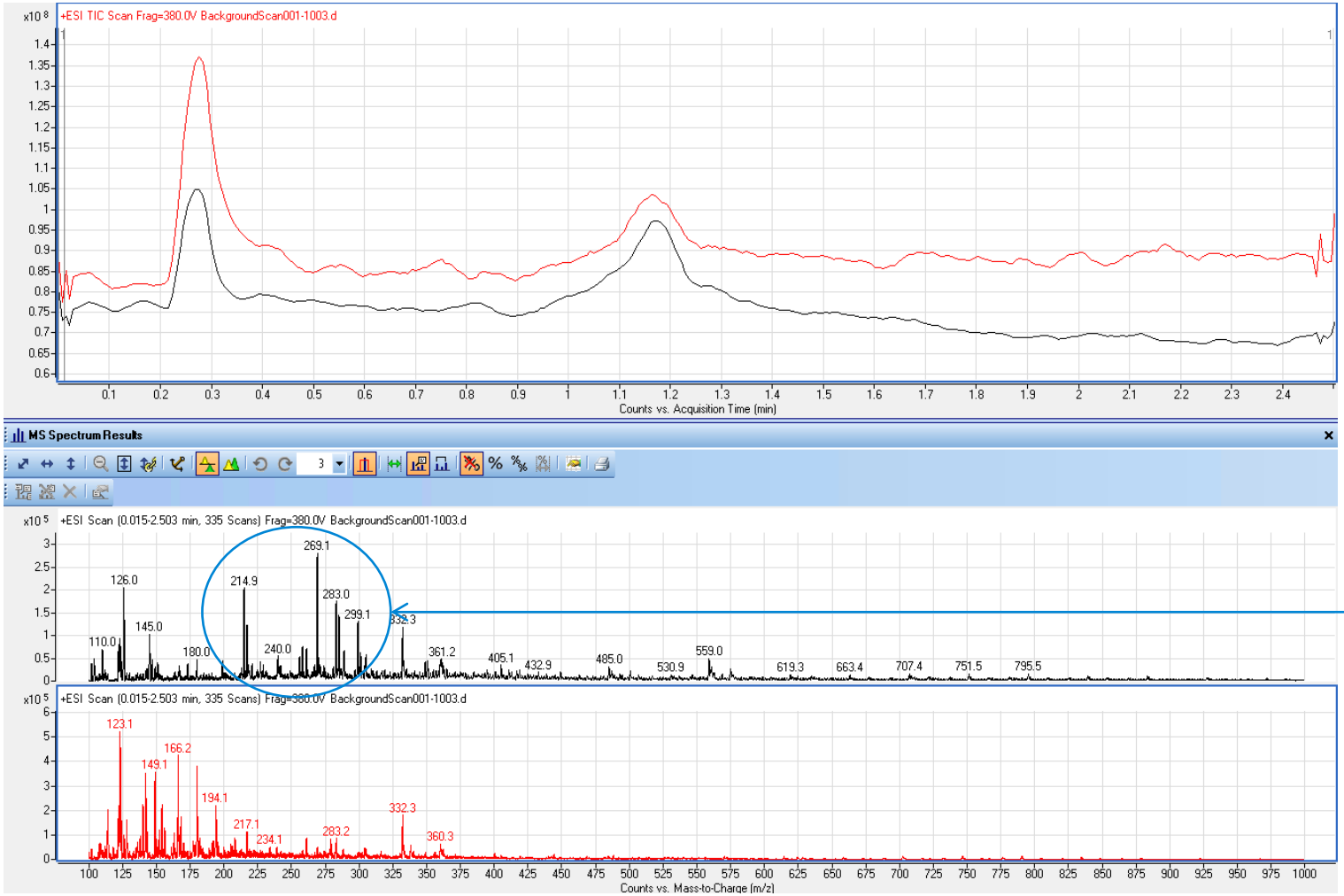
- Organic
- Additives
  - TFA
  - Salts
- H_2O
- Sample
- Other

Potential sources of contamination LC/MS

Main potential sources of contamination

- ✓ Solvent bottles: they are not flushed or specific for MS applications
- ✓ Solvents: use branded MS grade solvents from reliable suppliers
- ✓ Bottle head assembly, solvent inlet filters: don‘t touch with hands, consider stainless steel frits, wash before use, don‘t store them in non-MS solvents or plastic bags, consider using the filter for the bottle head assembly
- ✓ Degasser: flush all solvent channels with aqueous and organic solvents, switch channels for troubleshooting
- ✓ Packing materials for all parts

Impact of Water Quality



Black:
Water from supplier B

Red:
Milli Q water

Isocratic Method:
Channel A: water +0.1 formic acid
Channel B: Acetonitrile

Ions which seem to suppress the reserpine from the checkout sample

Isocratic method (Delta EMV (+): 150)

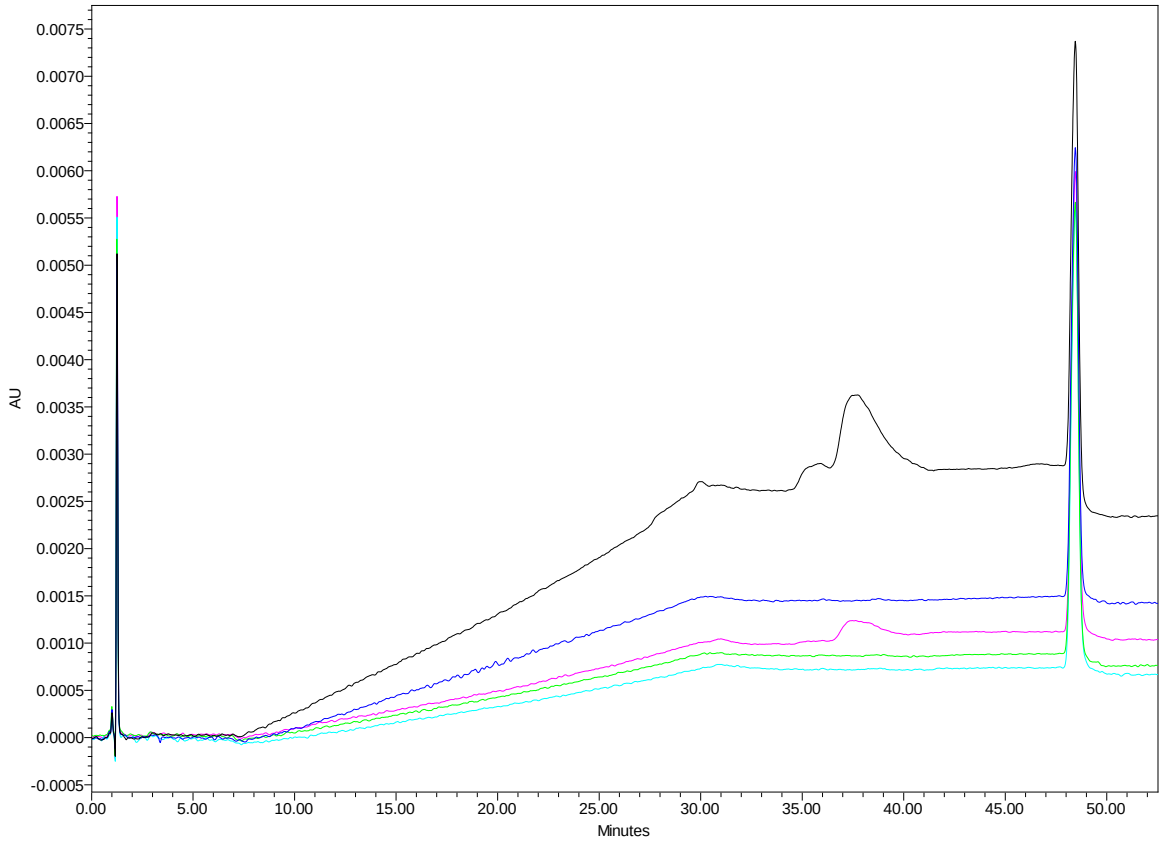
Results (specification with rms @ 0.1-0.35 min.) for the reserpine checkout with respect to ion supressing water

	T280		T281	
	Water from supplier B		Rwater changed (Milli Q)	
	s/n	height	s/n	height
1pg inj.	3292.8	214	12761	2572
	2846	213	13783.3	2502
	2906.7	223	36799.7	2590
	2068.3	210	21567.3	2583
	3100.5	227	18361.4	2657
Average	2843	217	20655	2581
	Option		Option	
50fg inj.	336.7	11	768.3	100
	83.4	7	1977.8	108
	76.9	9	958	97
	196.1	9	695	108
	370.2	8	1381.5	108
Average	213	9	1156	104

Using just a different type of water gives an improvement of 7.5 for the signal/noise ratio!

Water from supplier B/ Milli Q water

Acetonitrile Comparison



Multiple suppliers and lots of ACN tested

- Solvent - quality and consistency

Areas/Things to Investigate

- Separation
  - Pressure
  - Peak Shape
  - Retention
- Instrument
  - Components
  - Dwell volume & ECV
  - Tubing & Connections
- Column
  - Specifications
  - Characteristics
  - Tests
- Method Conditions
  - Mobile phase
  - Temperature
- Sample
  - Sample Prep
  - Injection

Tip: Prevention Techniques - A Better Choice!

- Filter samples
- Filter buffered mobile phases
- Use column protection
  - In-line filters
    - **Easy to Use and replace**
    - **Frits Available in 0.2,0.3, 0.5 and 2.0µ Porosity**
    - **Much Less expensive than a Column**
  - Guard columns

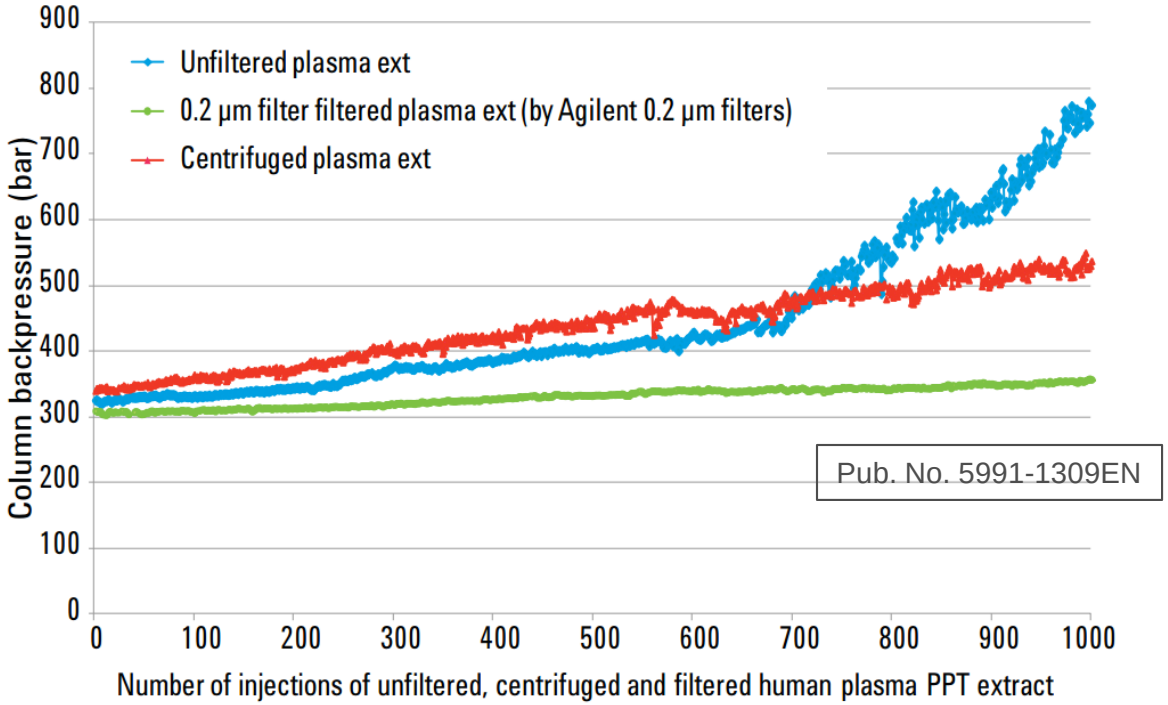
Easy

- Sample clean-up (i.e. SPE)
- Appropriate column flushing

Not As Easy

Sample

Consider the effects of your sample matrix



Unfiltered, centrifuged, and filtered plasma extracts
Zorbax RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm column, PN 959757-902

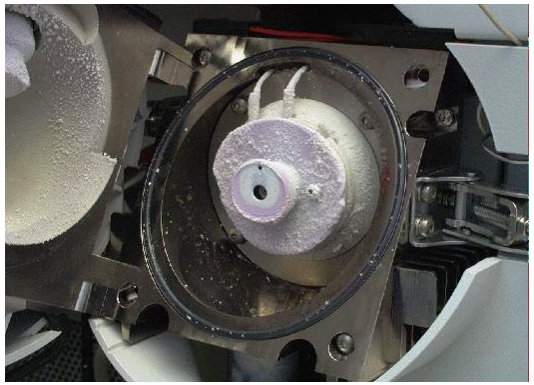
- Column plugging is one of the most common sources of LC column failure
- Especially with sub-2 µm columns, sample particulates can easily plug the column inlet frit

- Use an appropriate 0.2 µm filter with all samples prior to injection

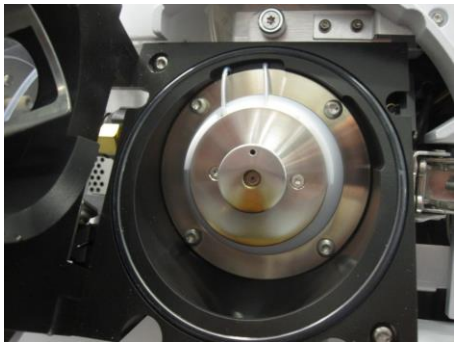
http://filtrationselectiontool.chem.agilent.com

Captiva Filtration Selection Guide: 5991-1230EN

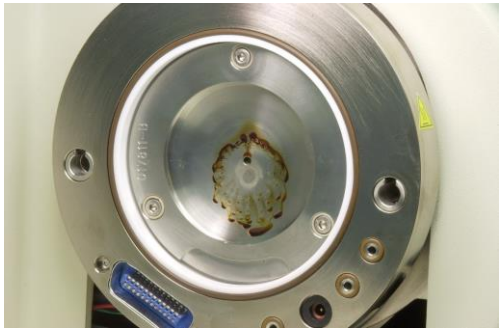
Examples of Instrument Contamination



Salt build-up in LC-MS ion source from unextracted salts



ESI Ion Source contamination after 3000x Urine Dilute/Shoot Injections



Curtain plate after injection of 25 samples with extractions from raisins without cleanup

Abrasive Mesh
Lint free cloth

Productivity Benefits with Sample Clean-Up

More Matrix Removal = Less Matrix Entering System = Time and Cost Savings!

- ✓ Less matrix build-up
  - – Less interferences
  - – Improved S/N
  - – Better reproducibility
- ✓ Better chromatography
  - – Less time spent on data analysis/manual integration
  - – Less time spent on re-runs/recalibrations
- ✓ Less maintenance
  - – Less instrument down-time
  - – Saves $$ on consumables/services
- ✓ Less troubleshooting
  - – “Is it my column or my MS”?
  - – Less instrument down-time

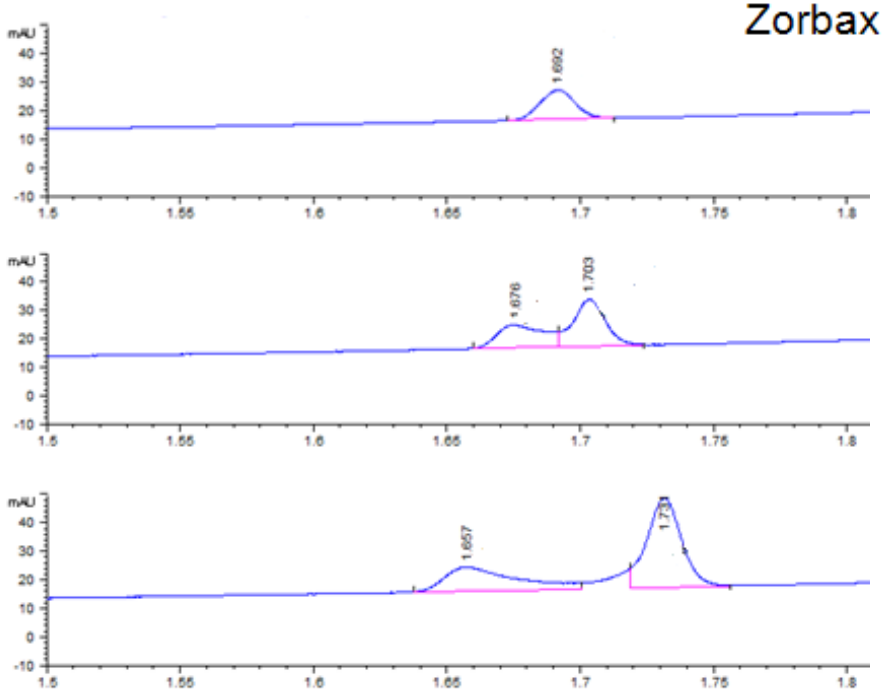
Injection Solvent Effects

Zorbax SB-C18, 1.8µm, 4.6 x 50 mm

20uL

50uL

100uL



Zorbax SB-C18, 4.6 x 50mm, 1.8um
MP: 80%H2O, 0.1% TFA;20% ACN
Inj. Solvent; 40%H2O:60% ACN

➢Peak splitting is often observed when injecting a large volume of sample in a solvent that is stronger than the mobile phase

➢Tip

- When injecting a sample in strong solvent, limit the size of the injection
- Inject the sample in a solvent that is no stronger than the starting conditions for the method

What Do We Troubleshoot…

Typical LC troubleshooting approach asks:

- What's wrong with the column?
- What's wrong with the instrument?

But… separations are controlled by more than just the column or instrument so the better question is

- Why doesn't my separation work as expected?

And… the answer could be a problem with the column, the instrument or something else (sample, mobile phase etc.).

Use your investigative skills to figure out what is wrong!

Investigation Kit

- ✓ Performance Report
- ✓ Spare column
- ✓ Column Test Mix – 5188-6529
- ✓ Isocratic Test Mix – 01080-68704
- ✓ Restriction Capillary – 5022-2159
- ✓ Quick Connect/Turn fittings
- ✓ Purge valve frits
- ✓ Spare solvent filters
- ✓ Capillary kit – LC dependent
- ✓ Checkout column for system
- ✓ *The LC Handbook*; 5990-7595EN

- ✓ Blanking nut (for pressure & leak tests) – 01080-83202
- ✓ LCMS
  - Tuning mix – MS dependent
  - Abrasive mesh – 8660-0827
  - Lint free cloth – 05980-60051
  - Spare capillary
- ✓ YouTube Maintenance Videos https://www.youtube.com/user/agilent
- ✓ Troubleshooting Videos https://www.agilent.com/en-us/products/liquid-chromatography/lctroubleshootingvideos

Contact Agilent Chemistries and Supplies Technical Support

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

Option 1 for GC/GCMS Columns and Supplies

Option 2 for LC/LCMS 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

Appendix

In-line Filters

RRLC in-line filter
0.2 µm pore filter, connecting capillary, max 600 bar
4.6 mm - 5067-1553
2.1mm - 5067-1551

1290 Infinity in-line filter
0.2 µm pore filter, connecting capillary, max 1200 bar
5067-4638

Column Cleaning

Flush with stronger solvents than your mobile phase
Make sure detector is taken out of flow path

Reversed-Phase Solvent Choices in Order of Increasing Strength
Use at least 10 x V_m of each solvent for analytical columns

1. Mobile phase without buffer salts (water/organic)
2. 100% Organic (MeOH 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*

* When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.

Standard LC fittings

Step 1

Select a nut that is the right length for the fitting.

Step 2

Slide the nut over the end of the tubing.

Step 3

Carefully slide the ferrule components on after the nut. Finger-tighten the assembly while making sure the tubing is completely seated in the bottom of the fitting.

Step 4

Use a wrench to gently tighten the fitting by 1/4 to 1/2 turn where you want to connect it; this will force the ferrule to seat onto the tubing. Do not over-tighten!

¼ in wrench

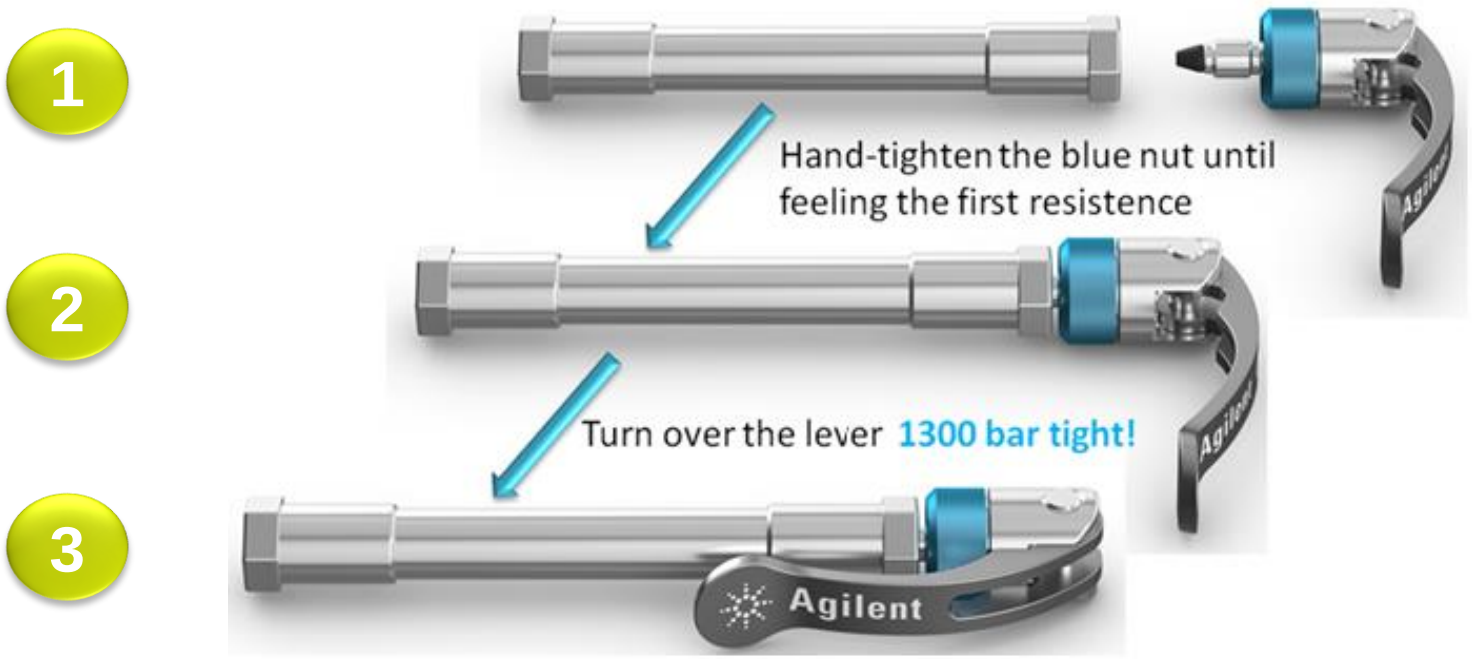
Step 5

Once you are sure your fitting is complete, loosen the nut and inspect the ferrule for correct position on the tubing.

- Investigate – Agilent on YouTube
  https://www.youtube.com/user/agilent

Rapid column changes for rapid method development

The Agilent A-Line Quick Connect Fitting



- A simple lever tightened fitting for rapid, leak free column changes
- Compatible with all columns (need Quick Connect capillaries)
- Enables quick change of columns, with minimal worry of leaking or performance issues

Separation Ruggedness
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

Using Buffers Successfully
Initial Column and System Equilibration

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

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 stable, or for gradient methods, precede former with 1 or 2 blank gradients.

Using Buffers Successfully
Shutdown State and Instrument Flushing

Shutdown State

Next day use—using 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.

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

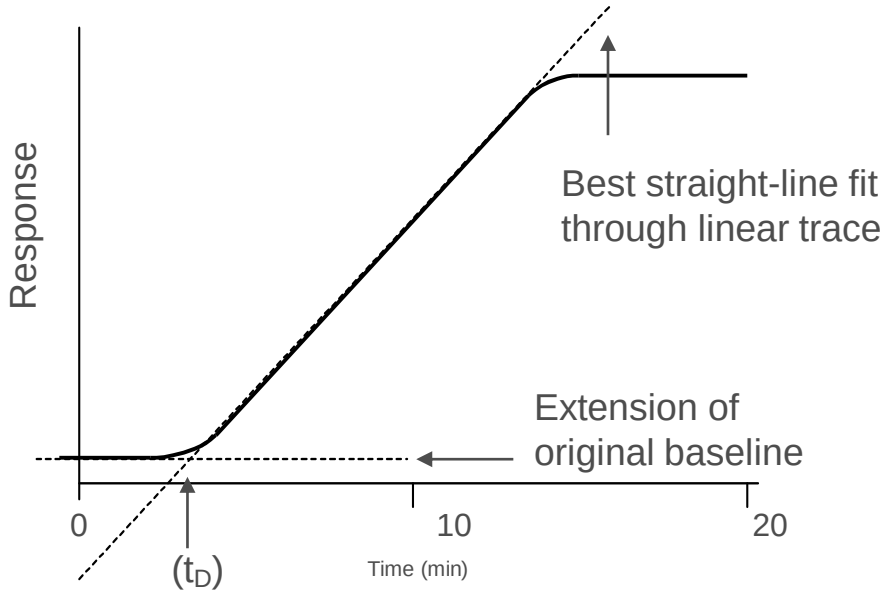
Monitor at 265 nm

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

Measuring Dwell Volume



Intersection indentifies
dwell time (t_D)

$V_D = t_D \times F$

V_D = Dwell Volume

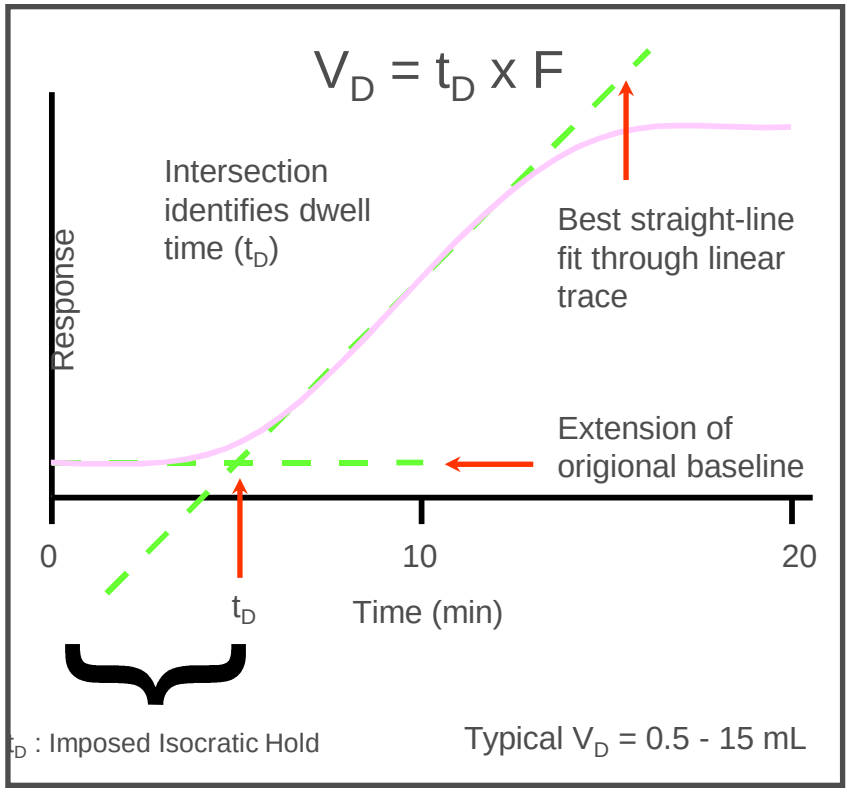
001815S1.PPT

Measuring Dwell Volume

Dwell Volume Impact

A chromatogram generated on one instrument (V_{D1}) can have a very different profile if generated on another instrument (V_{D2})

If using gradient conditions - report dwell volume (V_D)
V_D varies from instrument to instrument

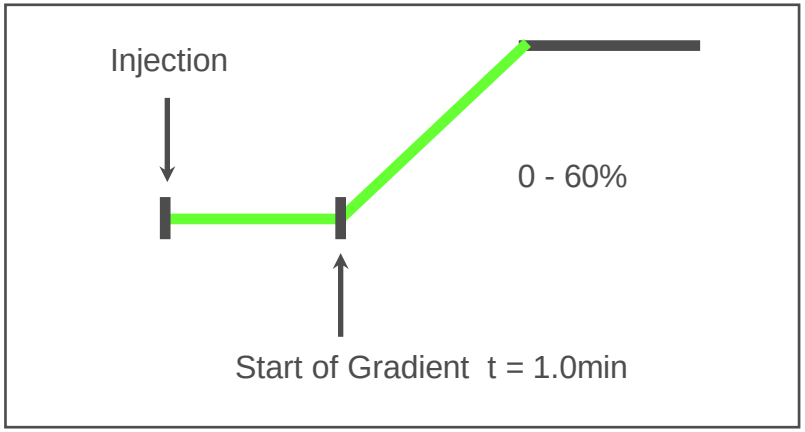


High Pressure Mixing: V_D = mixing chamber + connecting tubing + injector

Low Pressure Mixing: V_D = the above + pump heads + associated plumbing

Correcting for Dwell Volume

1. Measure the Dwell Volume of your HPLC System
   V_D = 1.0 mL
2. Draw Effective Gradient Profile at First Flow Rate
   Calculate the time delay (imposed isocratic hold) caused by dwell volume
   $V_D = t_D \bullet F$ 1.0 mL = $t_D \bullet$ 1.0 mL / min
   where F = 1.0 mL / min for 4.6 x 150 mm column
   V_D = 1.0 mL
   $t_D = F/V_D$ t_D = 1.0 mL / min / 1.0 mL
   t_D = 1.0 min



Correcting for Dwell Volume

If $V_{D1} > V_{D2}$

Compensate for longer V_{D1} by adding an isocratic hold to V_{D2}, such that Hold + $V_{D2} = V_{D1}$

If $V_{D1} < V_{D2}$

Delay injection, such that V_{D2} - delay = V_{D1}

(very difficult to accomplish in practice)

How to Estimate the Extra Column Volume of an HPLC System

One Way:

Remove HPLC column from instrument

Join injector and detector tubing with zero-dead-volume (ZDV) union

Inject (0.5 - 2 μL) of toluene in 100% acetonitrile

Make conc. about 1-5 mg/mL

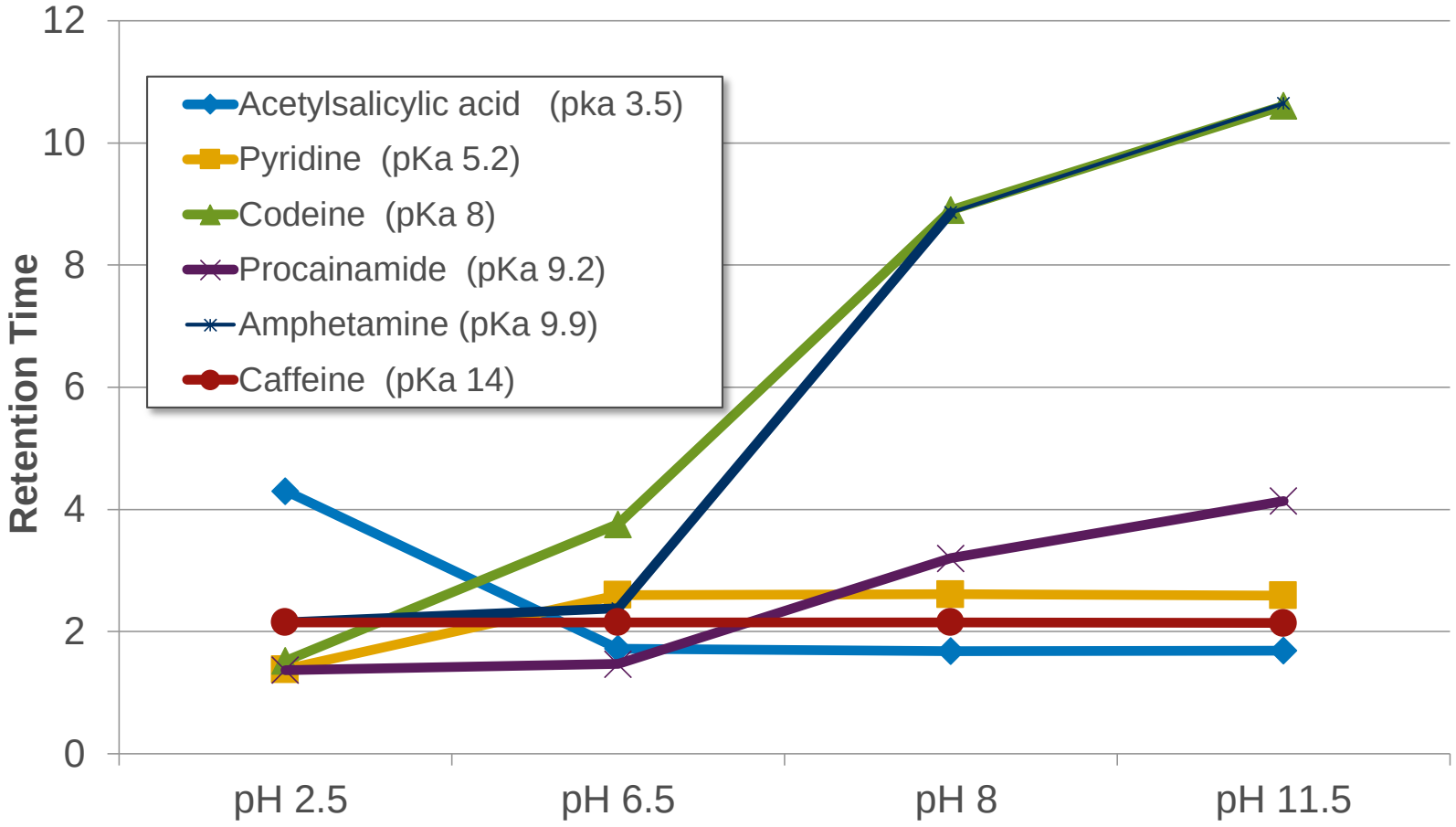
Determine width of peak at base ($w_{instrument}$)

Peak bandwidth follows:

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

Change in Retention with pH for Ionizable Compounds is Compound-Dependent

More retention for non-charged analytes (i.e. acids at low pH and bases at high pH)



Mobile Phase: 45% MeOH, 55% 20 mM Phosphate Buffer

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