Surviving Chromatography

Part 2: Corrective Action

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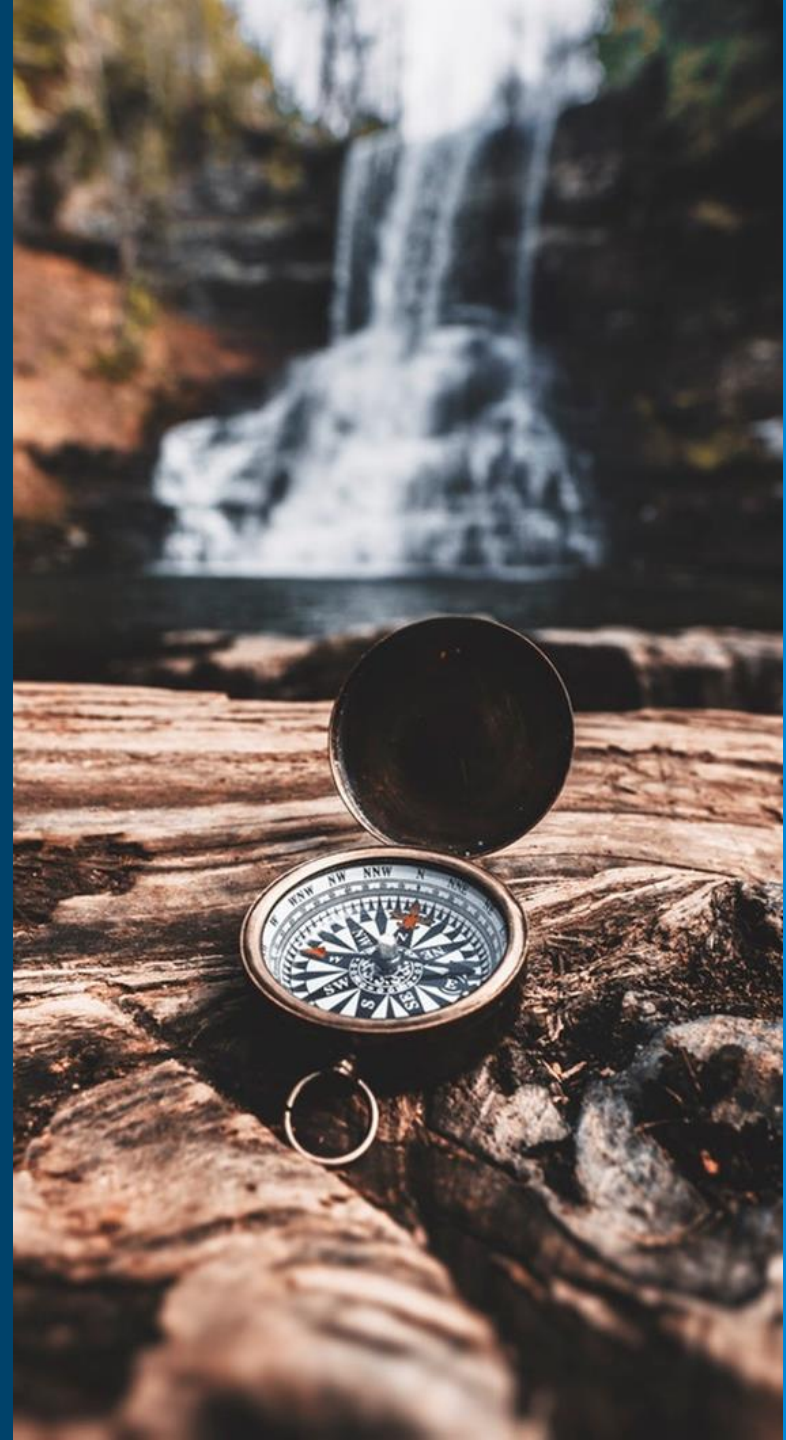
October 11, 2018



“My Chromatography is Not Where it Should be”

– Points for Discussion for chromatography that has ‘gone off track’

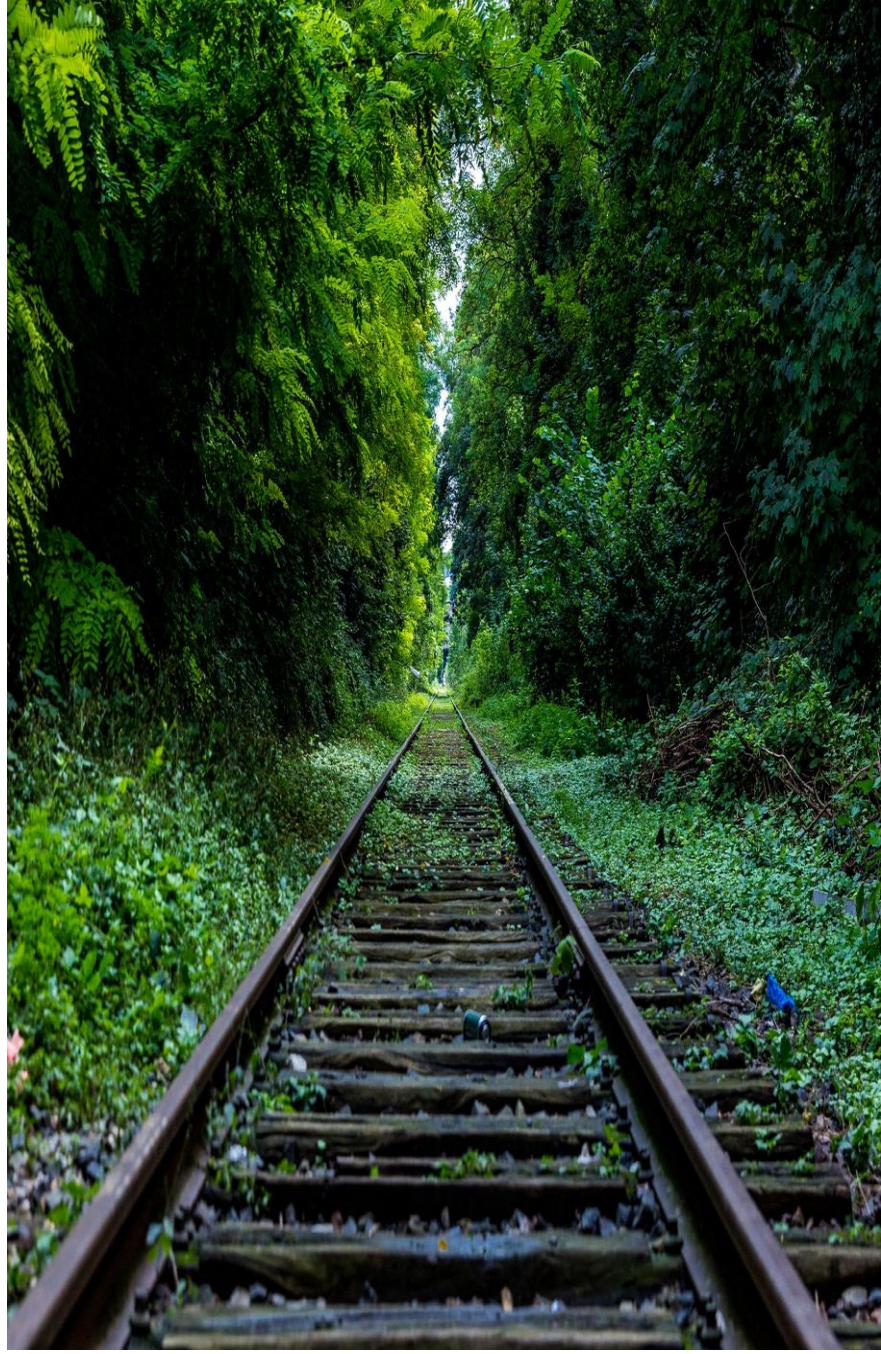
- Common observed problems in HPLC
  (method, column, or instrument related -????)

  Baseline Issues
  Pressure
  Peak Shapes
  Changes in Retention/Selectivity

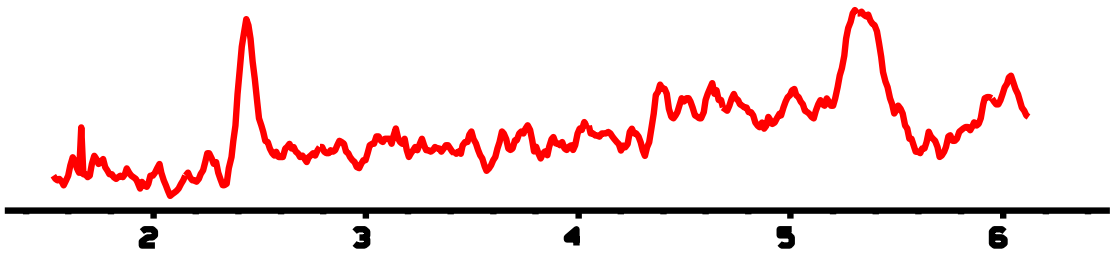
- ❖ Examine/Look into the reasons for problems
- ❖ Corrective Action/Preventive Maintenance/ Good Practices

Definition:

Corrective Action - action taken to eliminate causes of non-conformities or other undesirable situations.



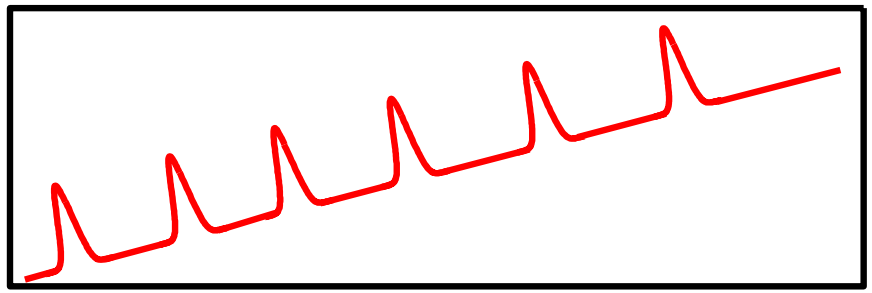
Common Problems
Noisy Baselines



Possible Causes

- Dirty flow cell
- Lamp failing
- Pulses from pump (if periodic)
- Temperature effects on detector
- Air bubbles passing through detector

Drifting Baseline



Possible Causes

- Gradient elution
  - Mobile phase component
- Temperature unstable (RID)
- Contamination in mobile phase
- Mobile phase not in equilibrium with column
- Contaminant bleed in system
  - Hardware chemical compatibility

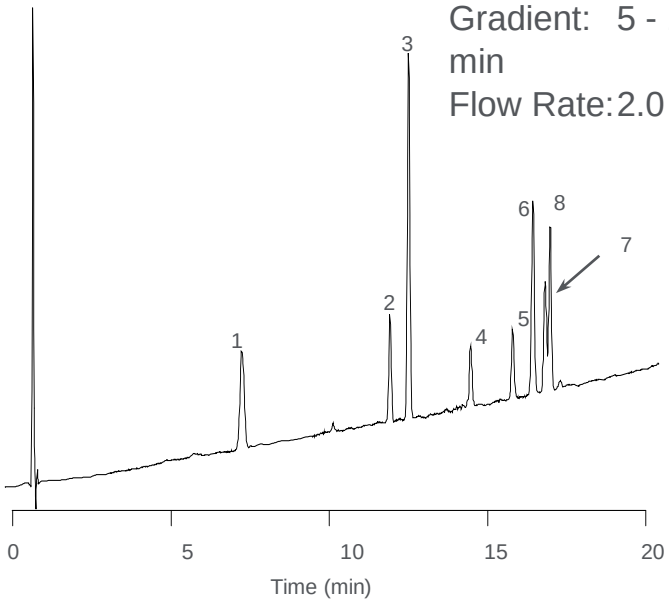
Drifting Baseline: Effect of TFA

A: 0.1% TFA in H_2O B: 0.1% TFA in ACN Temperature: 35°C

Sample: 1. Phenacetin 2. Tolmetin 3. Ketoprofen 4. Fenoprofen
5. Ibuprofen 6. Phenylbutazone 7. Mefenamic acid 8. Flufenamic acid

Eclipse XDB-C8
4.6 x 150 mm, 5 µm

Gradient: 5 - 100% B in 30 min
Flow Rate: 2.0 mL/min



Corrective Action:

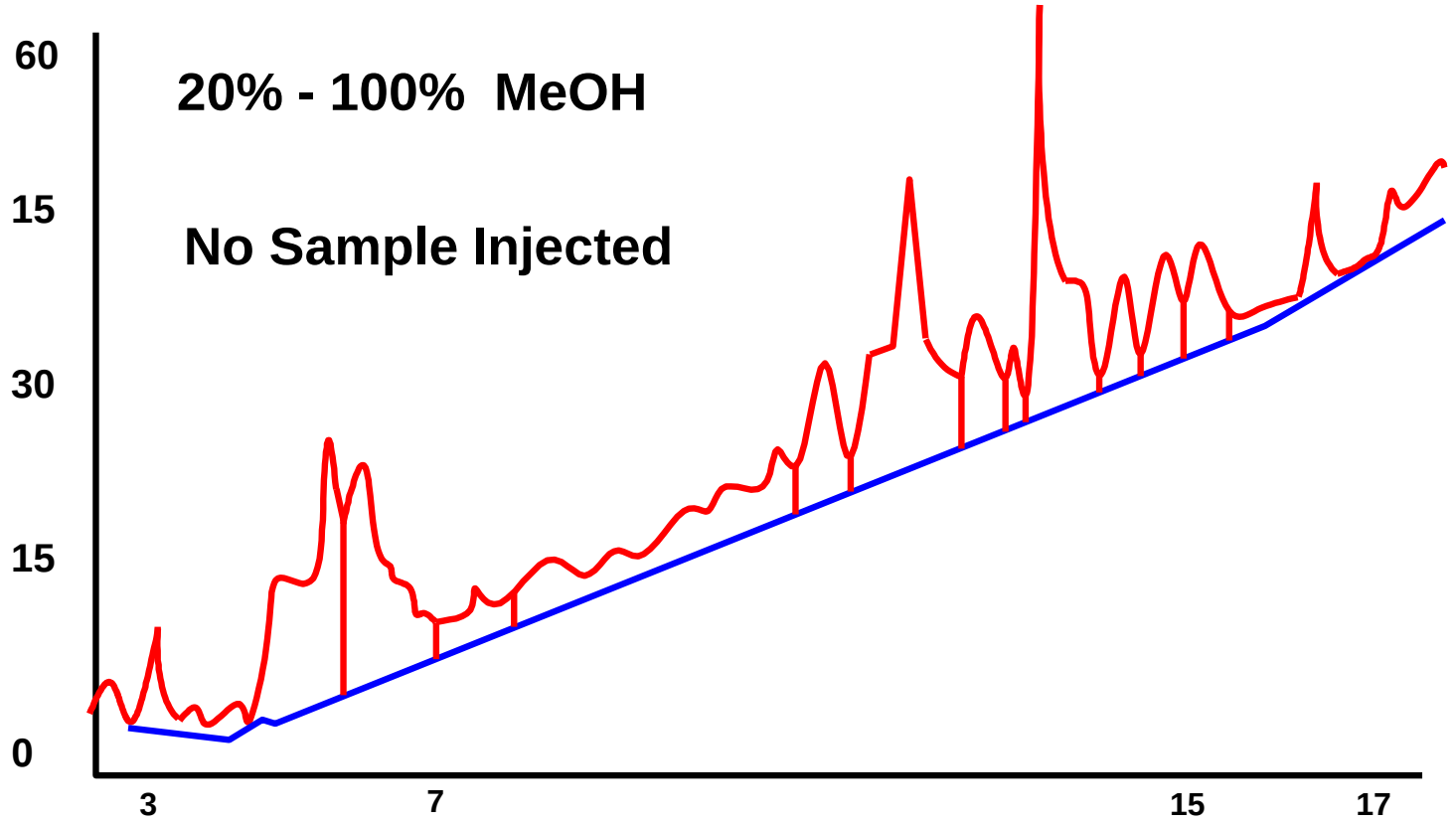
✓Wavelength @215nm
- run at 254nm less impact

✓Adjust TFA concentration in Solvent B to level baseline; 0.8%-0.9% TFA

Important: Know the UV Cutoff of your mobile phase components

Unstable Baseline- Ghost Peaks

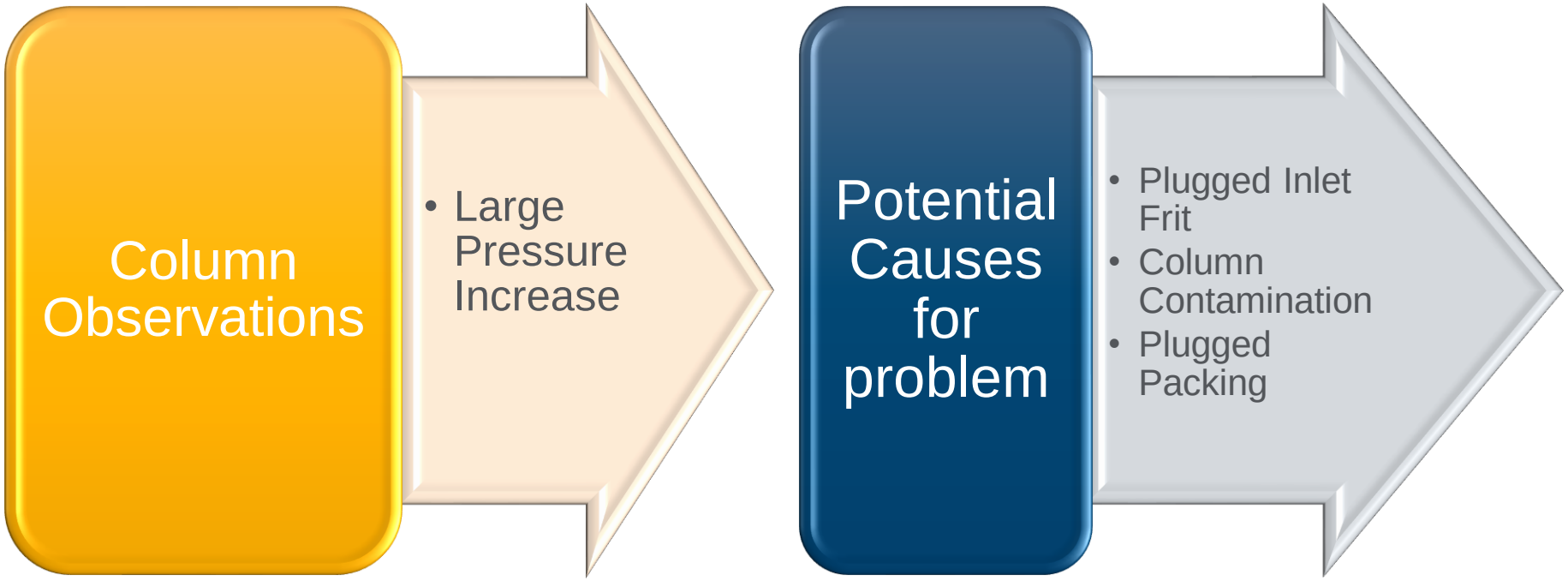
No Sample is Injected but peaks appear –



Possible Causes:

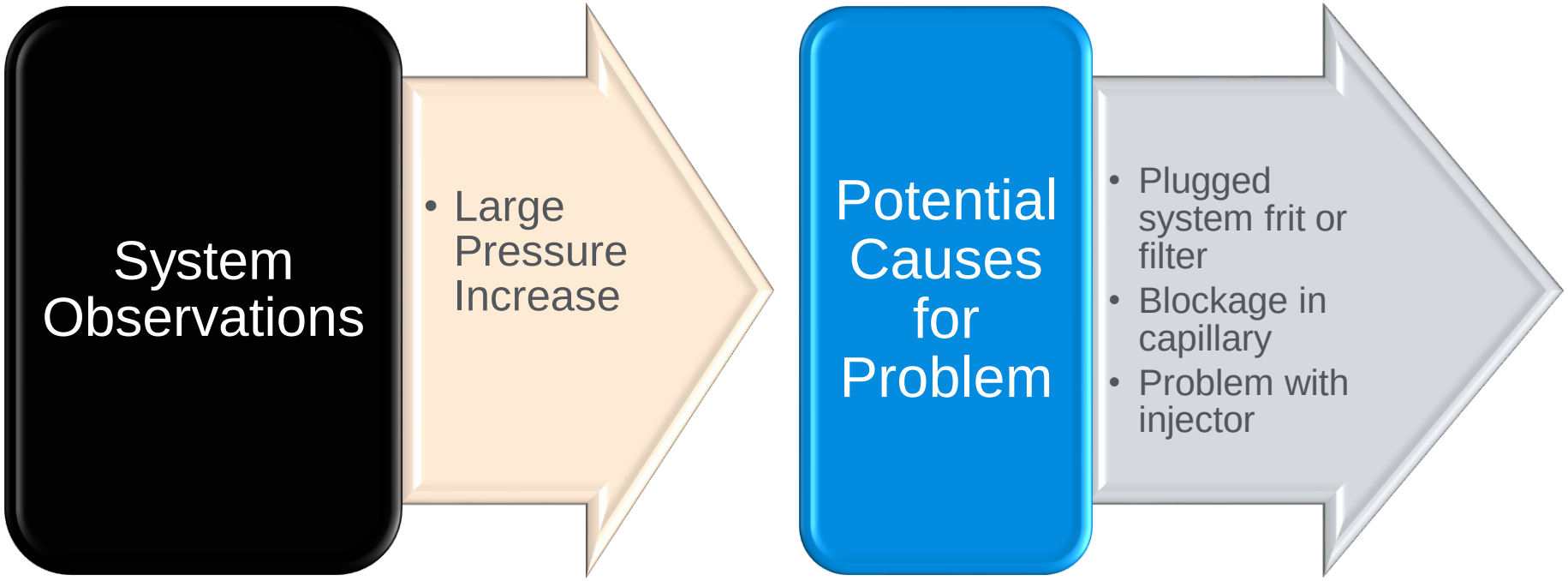
- Dirty mobile phase
- Sample carryover
  - May imply poor recovery
- Peak from an early run (isocratic)

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.

Pressure Issues



Note: if Low pressure is observed, it is typically a connection or another possible system issue;

Determine the Cause and Correct

Possible Causes

- Column inlet frit contaminated/plugged
- Frit in purge valve contaminated/plugged
- Column contaminated
- Blockage in a capillary, particularly needle seat capillary
- Rotor seal in injection valve plugged
- Guard or in-line filter
- **Check pressure with/without column**

Back Pressure: Determining the Cause and Correcting

Steps to take:

Check the pressure with and without the column - many pressure problems are due to blockages in the system, at other points before the column.

Remove the column in steps ;

- Detach at column outlet – pressure still high?
- Detach at column inlet – pressure still high?

Remove the Guard Column in steps:

- Detach at g. column outlet – pressure still high?
- Detach at g column inlet – pressure still high?

Check also any inline filters – pressure still high?

Determine the Cause and Correct

If Column pressure is found to be high –

Corrective Action

- Wash column (see Appendix)
  - ✓ Eliminate column contamination and plugged packing
  - ✓ high molecular weight/adsorbed compounds
  - ✓ precipitate from sample or buffer
- Back flush column
  - ✓ Clear plugged frit (check column manufacturer user guide)
  - ✓ Replace frits (not usually recommended)
- Replace Column

Determine the Cause and Correct

If backpressure is found to be system related:	**Corrective Action:**
Guard Cartridge: cartridge b.pressure high	Replace cartridge in holder
Inline Filter: frit is blocked	Replace Inline Filter Frit
Autosampler: plugged needle	Replace Needle Assembly
blockage @ needle seat	Replace Needle Seat
Pump: plugged purge valve frit	Replace purge valve frit

Pressure : Sample Considerations

Pub. No. 5991-1309EN

Sample not ‘clean’/ dirty sample:

Filtration is basic sample preparation method for all kinds of samples

Physically removes particulates from the sample

Removes unwanted matrix

Prevents blocking of capillaries, frits, and column inlet (especially for UHPLC)

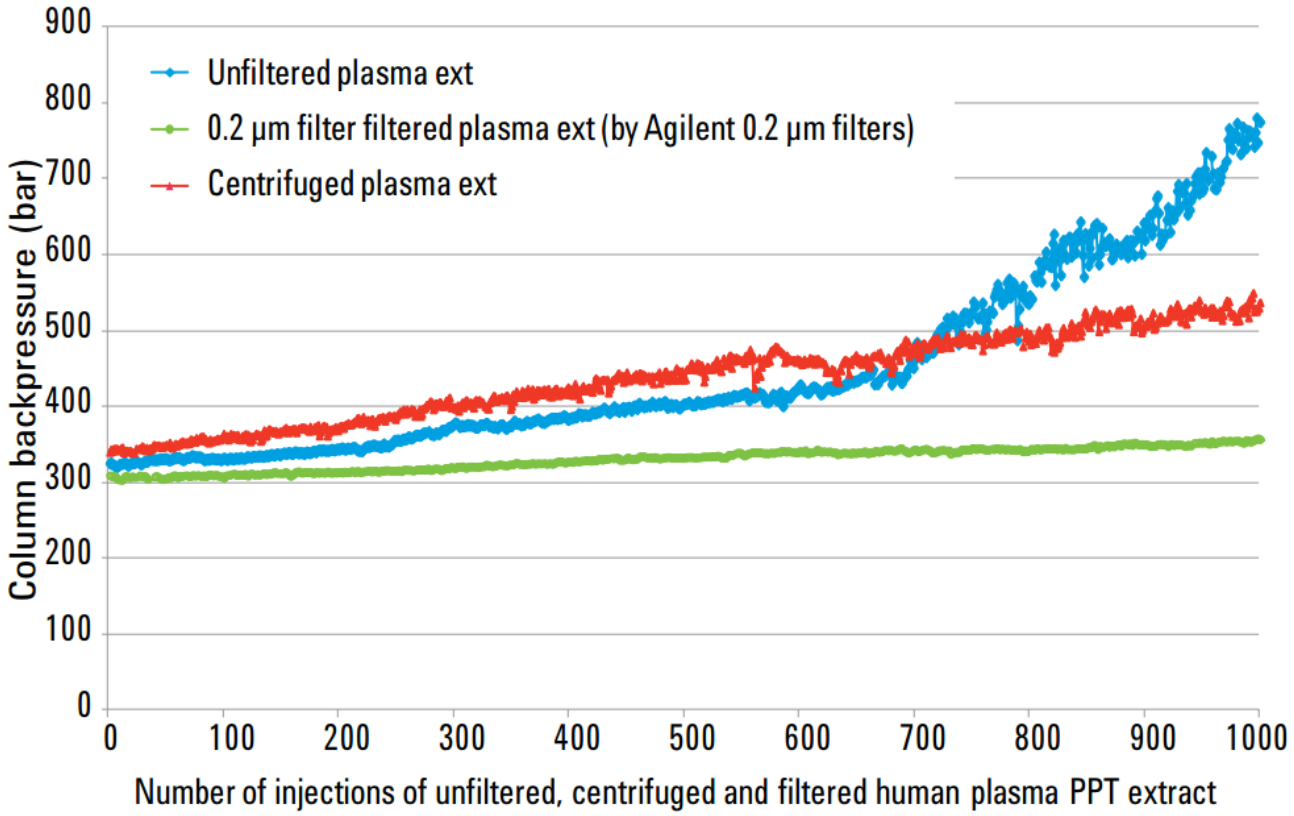
Results in less downtime of the instrument or for need for cleaning or replacing a column

Results in less wear and tear on the critical moving parts of the injection valves

Individual syringe filters, 96-well plate formats available also

Captiva Syringe Filters Guide 5991-1230EN

Pressure : Sample Considerations

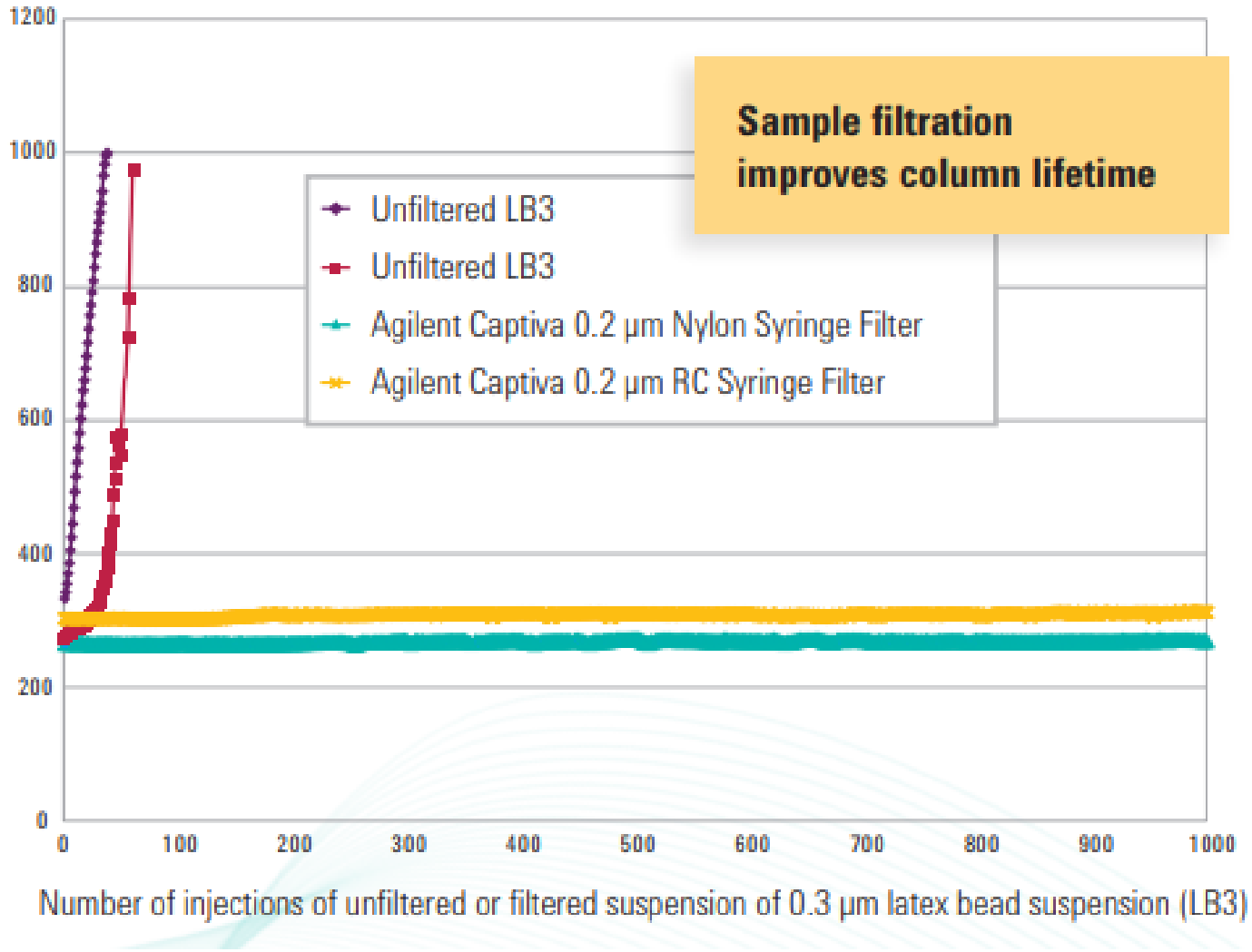


Unfiltered, centrifuged, and filtered plasma extracts
Zorbax RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm column, PN 959757-902

[Syringe Filter Online Selection Tool](#) @ Agilent.com

Captiva Syringe Filters Guide 5991-1230EN

Pressure: Sample Considerations



Zorbax RRHD Eclipse Plus C18,
2.1 x 50 mm, 1.8 µm column, PN 959757-902

Corrective Action:

Keep column backpressures reduced or a minimum by simply filtering your sample prior to injection or perform another type of Sample Clean Up

Captiva Syringe Filters Guide 5991-1230EN

Pressure: Mobile Phase Considerations

What is in your Solvent Bottle?

Potential Symptoms and Problems that might be noted:

- **Increased system pressure or pressure fluctuations**
- **Increased column pressure, premature column failure**
- Can mimic application problems
- Gradient inaccuracies
- Ghost peaks

Pressure: Mobile Phase Considerations

Corrective Action:

Eliminate/ Reduce Microbial Growth

Things to do:

- Use freshly prepared mobile phase
- Filter & use Solvent Inlet 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

Growth in a solvent bottle over 1 week
Solvent : water in clear bottle

Corrective Action: reduce/minimize Pressure Problems

MORE to do:

- Appropriate system flushing – flush buffers from entire system with water/organic mobile phase.
- Replace buffers every 24-48 hours

Infinity Lab Supplies
Pub. No. 5991-8031EN

TroubleShooting Pressure

How do we see that the backpressure is changing??

1. Continuously increasing pressure even *with no* injections
   - Pump Seals
   - Mobile Phase Particulates
   - Mobile Phase Solubility
   - Mobile Phase Unstable (polymerization)
   - Column Void Formation (use condition dependent)
   - Microbial Growth
2. Increasing pressure *with* sample injections
   - Sample Particulates
   - Sample Not Soluble in Mobile Phase
   - Sample Components Irreversibly Bound to Stationary Phase

Peak Shape Issues

What are the Common Problems relating to Peak Shape:

Peak Tailing

Doublet peaks

Split Peaks

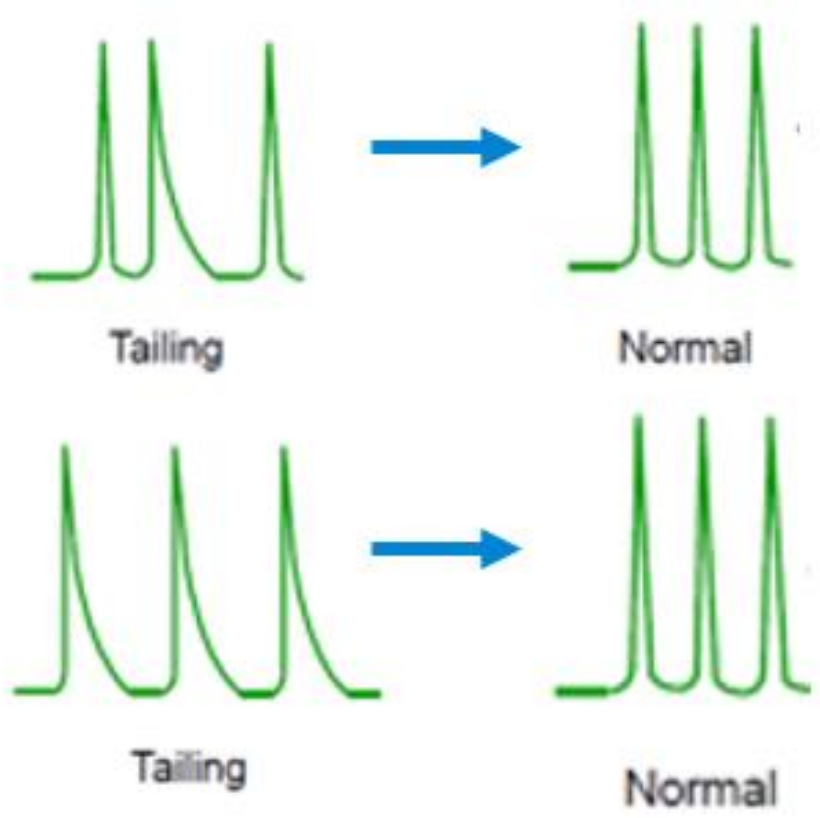
Broad Peaks

- **Dispersion**
- **ECV – extra column volume**

Peak Shape - Tailing

All peaks or some peaks????

Symmetry >1.2



Causes

Some Peaks Tail:

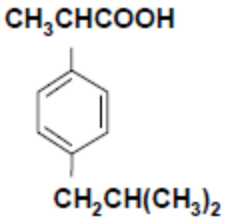
- Secondary - retention effects.
  - Residual silanol interaction
- Small peak eluting on tail of larger peak

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 or bad choice of column

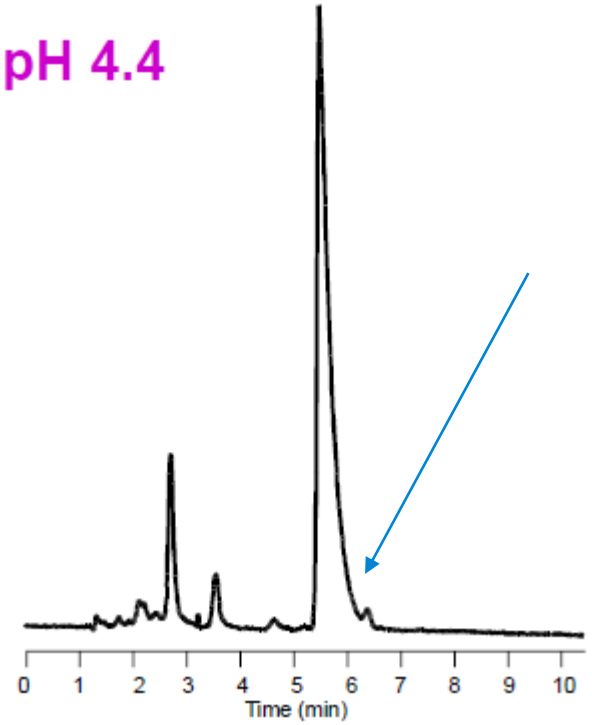
Peak Shape - Tailing: effect of pH

Column: ZORBAX SB-C8 4.6 x 150 mm, 5 µm Mobile Phase: 40% 5 mM KH_2PO_4: 60% ACN
Flow Rate: 1.0 mL/min. Temperature: RT

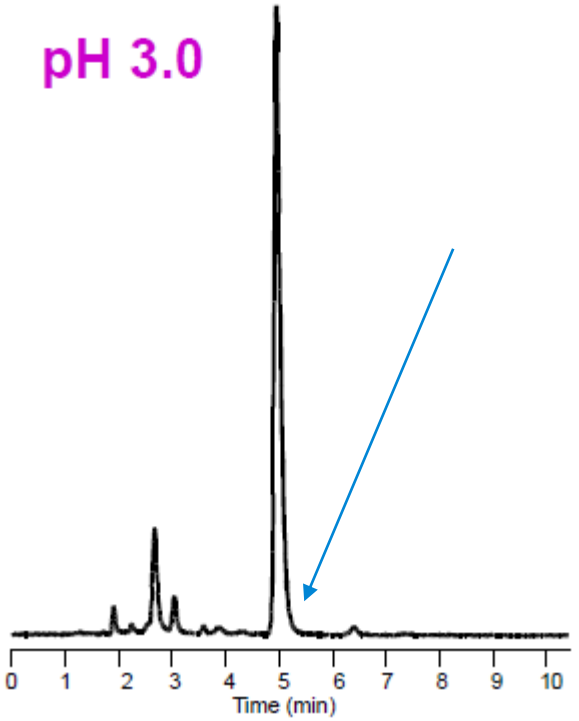


Ibuprofen
pK_a = 4.4

pH 4.4



pH 3.0

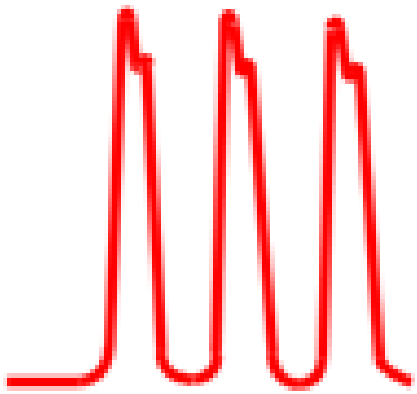


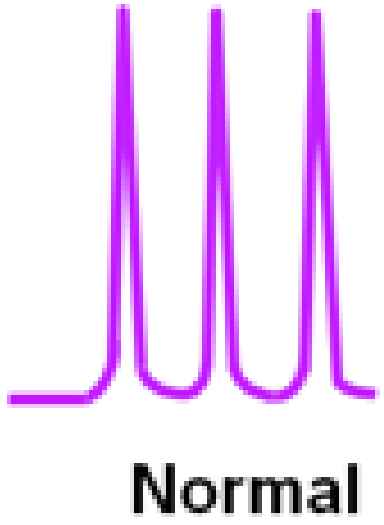
Peak Shape: Determining the Cause

Other points of consideration:

1. **Complex sample matrix or many samples analyzed**
   i. Likely column contamination or partially plugged column frit
2. **Mobile phase pH>7 – may have a column void due to silica dissolution**
   i. Unless specialty column used; Poroshell120 HpH, Zorbax Extend C18, or polymer-based, like PLRP-S
3. **Injection solvent stronger than mobile phase**
   i. Likely split and broad peaks, shape dependent on injection volume and k value

Peak Shape: Double Peaks

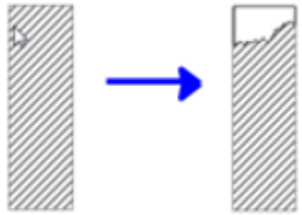




Possible Causes

- Void in column
- Partially plugged frit
- Only one peak
- Sample solvent mismatch

Ex: void in column

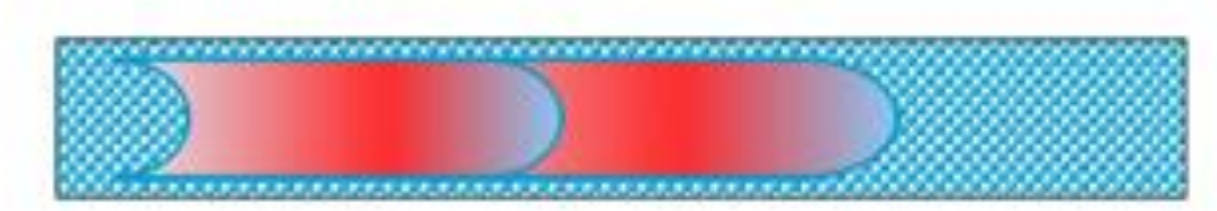


Corrective Action:

- Replace the column
- Rev. flush and/or clean the column
- Remake sample
- Make up sample if different solvent

Peak Shape: Split Peaks

- Using a Strong Sample Solvent Can Cause peaks to move too fast; can Compromise Peak Shape



- Solvent Stronger than Mobile Phase prevents a significant portion of Sample from adsorbing onto packing
- Bands become spread out relatively far into column
- By the time the Mobile Phase flushes the strong solvent the bands are very broad and may form split (two) peaks

Peak Shape: Split Peaks

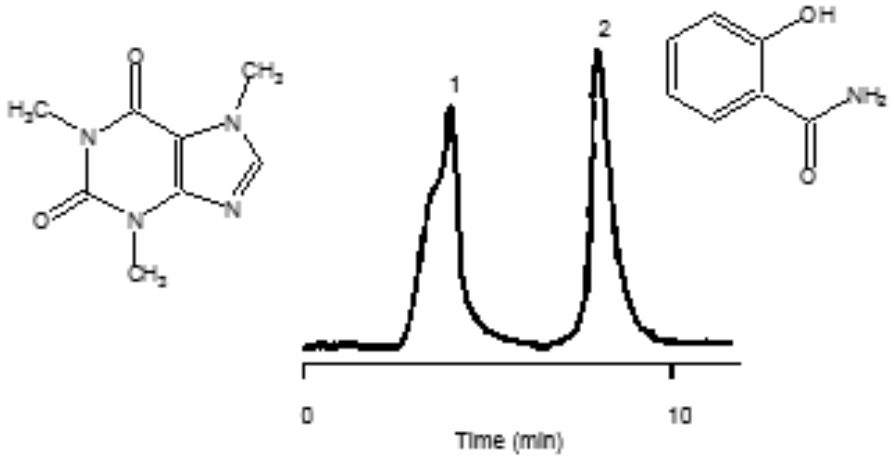
Injection Solvent Effects

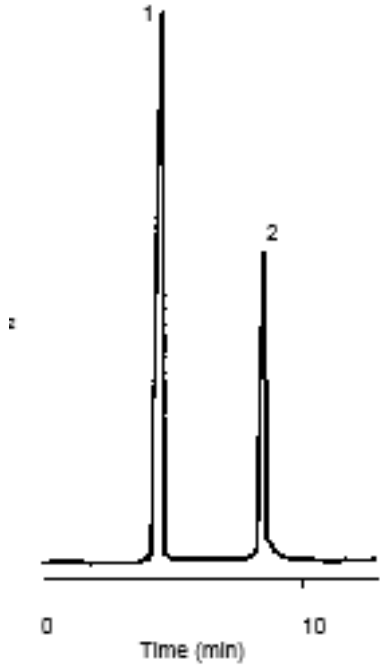
* Strong Sample Solvent Can Compromise Peak Shape

Column: Zorbax StableBond SB-C8, 4.6 x 150 mm, 5 mm
Mobile Phase: 82% H_2O : 18% ACN
Injection Volume: 30 uL

Sample: 1. Caffeine 2. Salicylamide

A. Injection Solvent
100% Acetonitrile





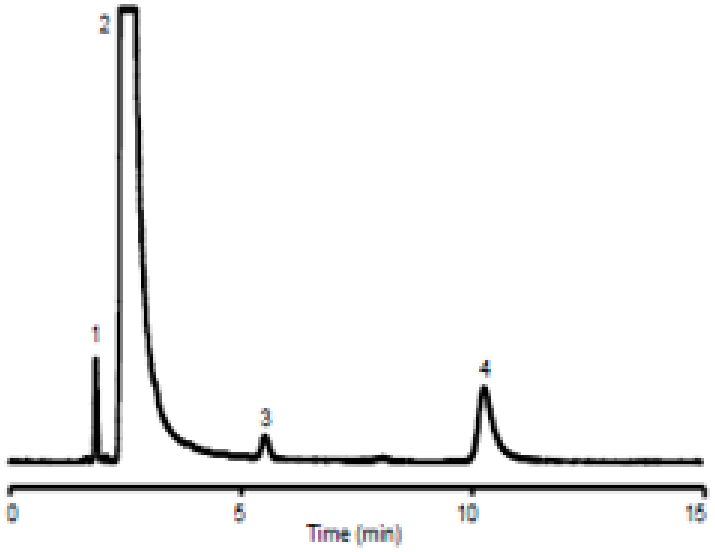
Correct By:

B. Injection Solvent
= Mobile Phase

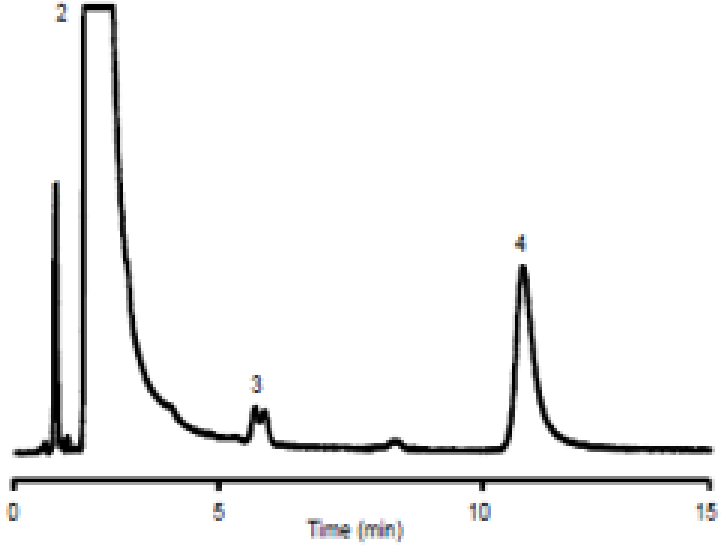
82% H20: 18% ACN

Split Peaks: Column Contamination

Injection 1

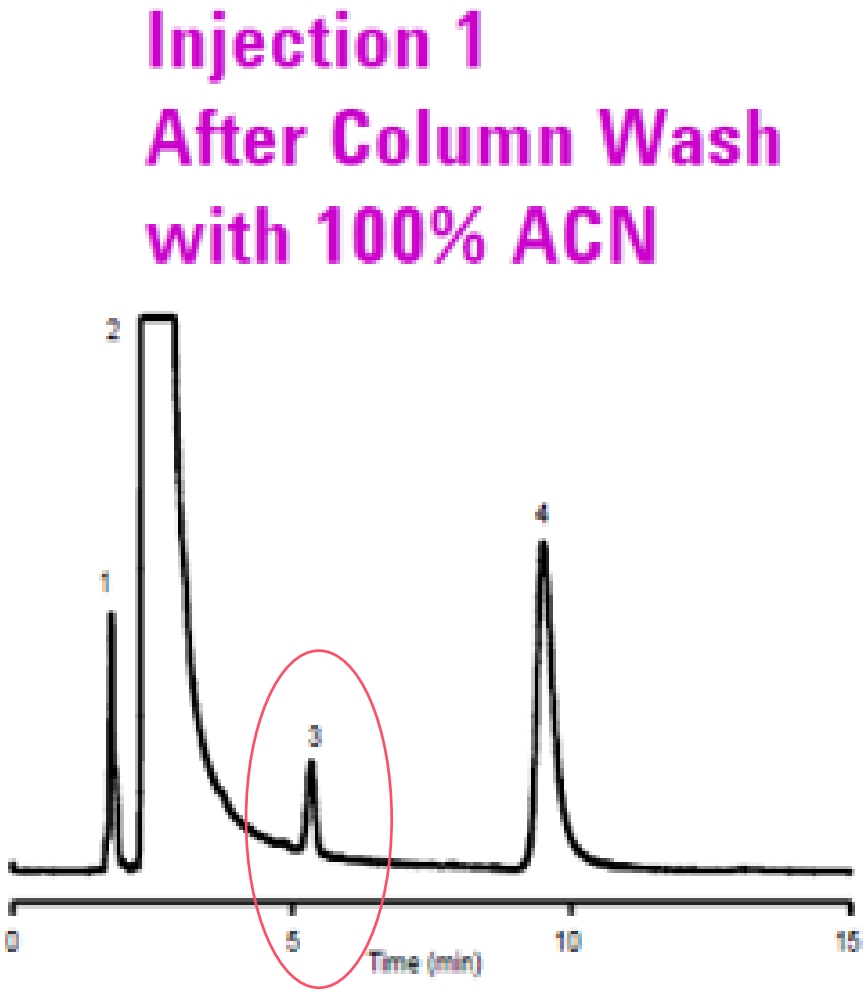


Injection 30



Column: StableBond SB-C8, 4.6 x 150 mm, 5 mm Mobile Phase: 60% 25 mM Na_2HPO_4, pH 3.0 : 40% MeOH Flow Rate: 1.0 mL/min
Temperature: 35°C Detection: UV 254 nm Sample: Filtered OTC Cold Medication: 1. Pseudoephedrine 2. APAP 3. Unknown 4. Chlorpheniramine

Split Peaks: Column Contamination



Corrective Action:

Column washing eliminates peak splitting, which resulted from a contaminant in the column

Column: StableBond SB-C8, 4.6 x 150 mm, 5 mm

Mobile Phase: 60% 25 mM Na_2HPO_4, pH 3.0 : 40% MeOH Flow Rate: 1.0 mL/min

Example of recommendations for Column cleaning

Flush with a stronger solvents than that in your mobile phase
Make sure to take the detector out of the flow path.

Reversed Phase Solvent Choices
In order of increasing strength

Use at least 10 x Vm of each solvent for analytical columns

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

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

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

Peak Shape: Broadening, Dispersion & ECV

Non-column Sources of Dispersion

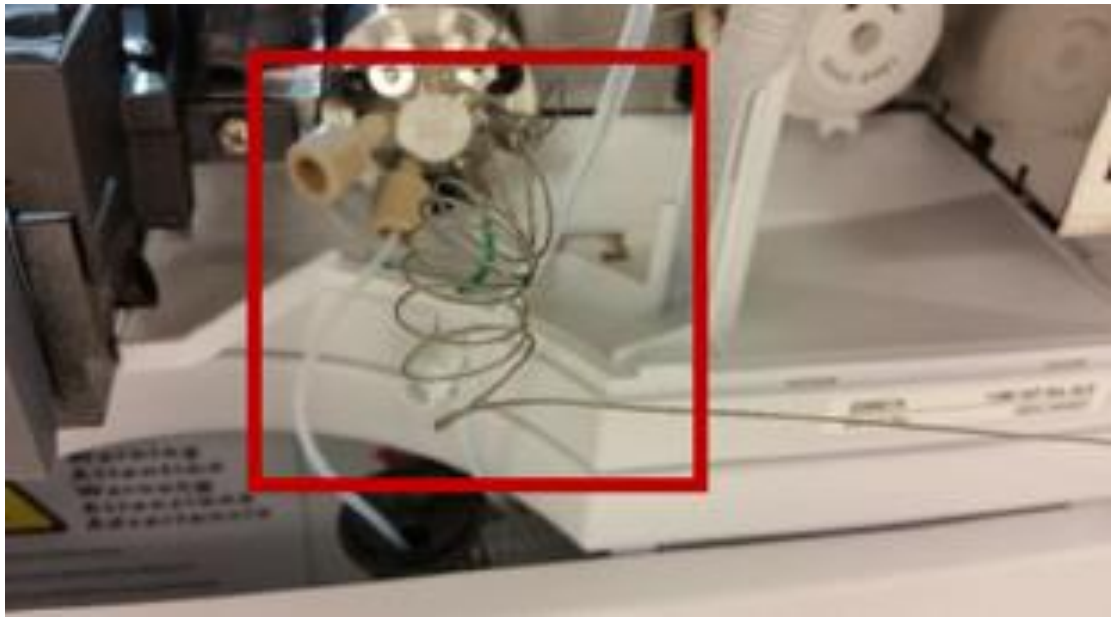
- General:
  - Interconnecting tubing (i.d., length, internal surface)
  - Connectors (unions, tees, bulkhead fittings)
  - Guard columns and/or inline particle filters
  - Switching valves for autoSPE, column selection, column regeneration
- Sampler:
  - Diluent strength and injection volume
  - Sample aspirating needle and loading/transfer port
  - Sampler switching valve(s) contacting sample
- Detection:
  - Inlet heat exchangers, flow cell volume and geometry
- Data filtering effects in high speed applications

ECV – extra column volume

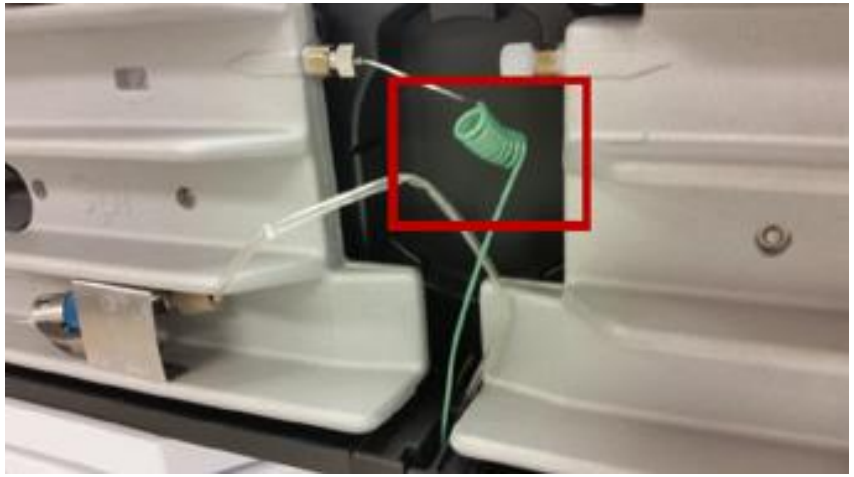
Peak Shape: Broadening, Dispersion & ECV

ECV is the volume in the LC system outside of the column

@ Detector



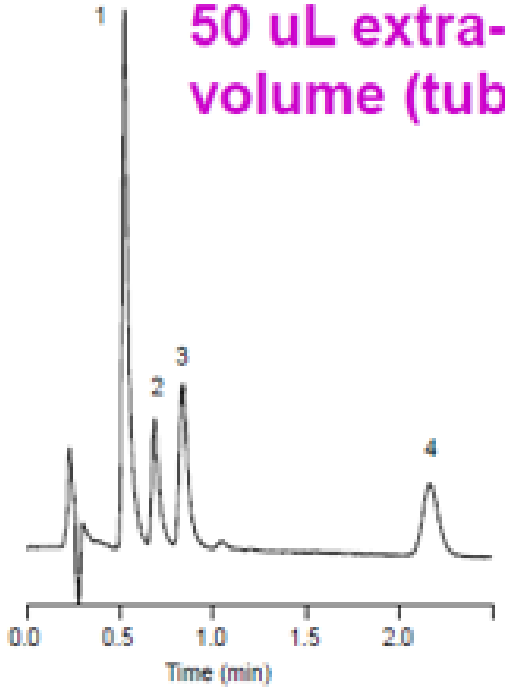
@ Autosampler



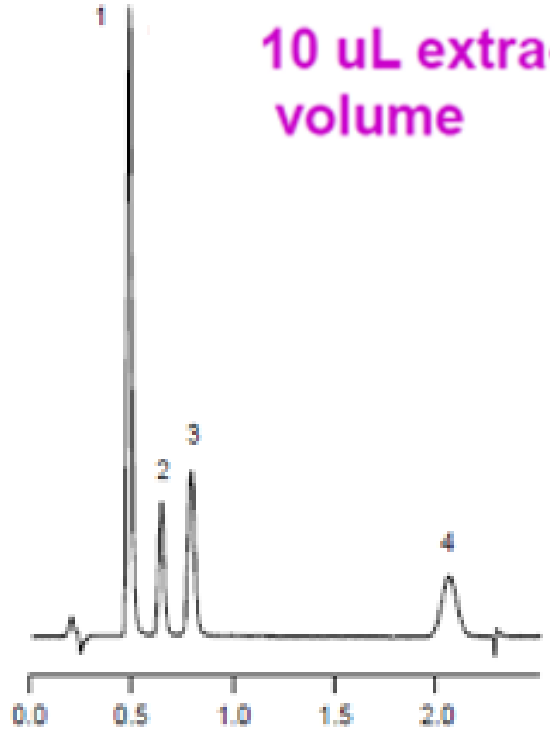
@ Column Compartment

Peak Shape: Broadening & Extra Column volume

50 uL extra-column volume (tubing)



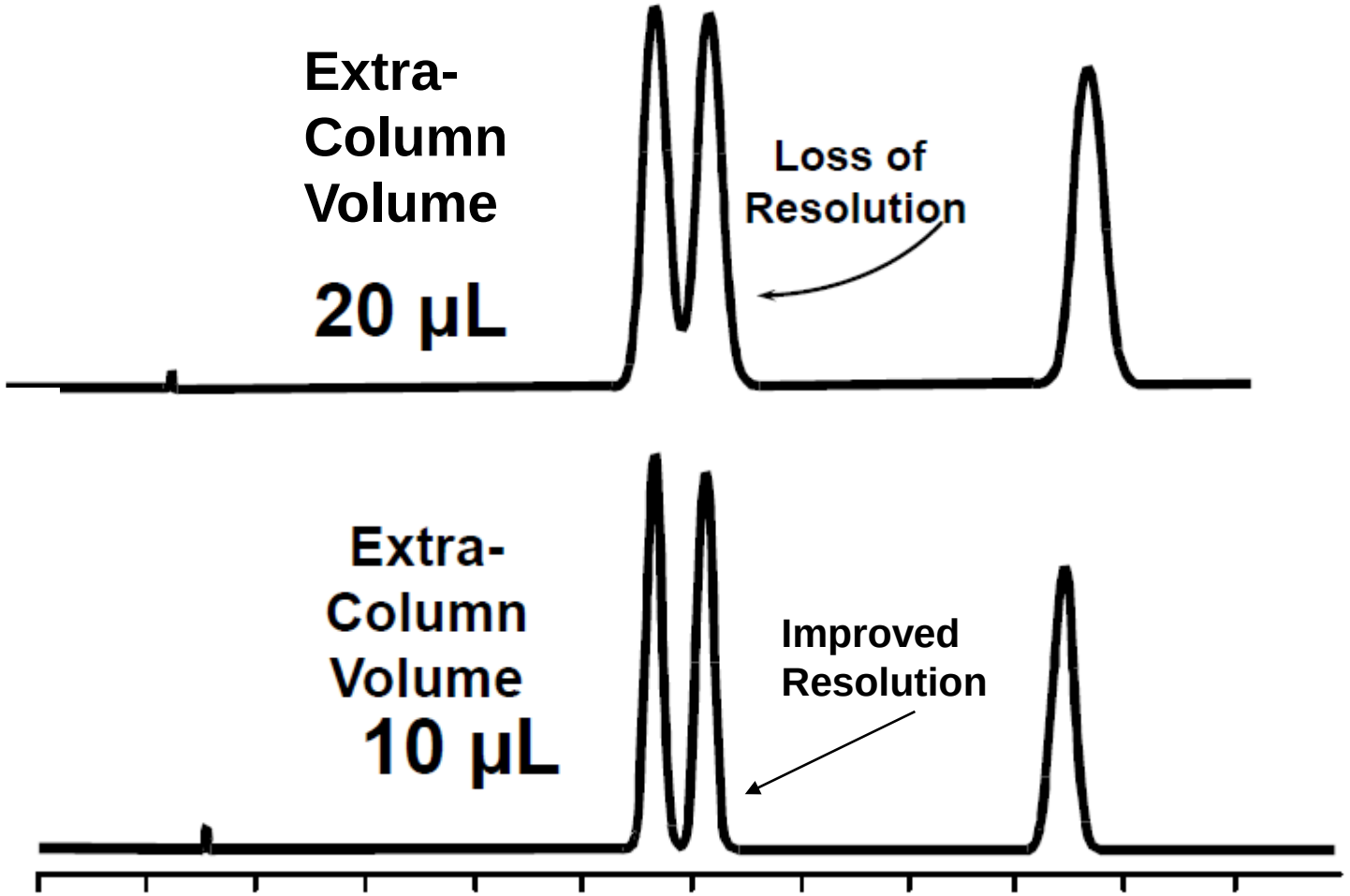
10 uL extra-column volume



Column: StableBond SB-C18, 4.6 x 30 mm, 3.5 µm Mobile Phase: 85% H_2O with 0.1% TFA : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylalanine 2. 5-benzyl-3,6-dioxo-2-piperazine acetic acid 3. Asp-phe 4. Aspartame

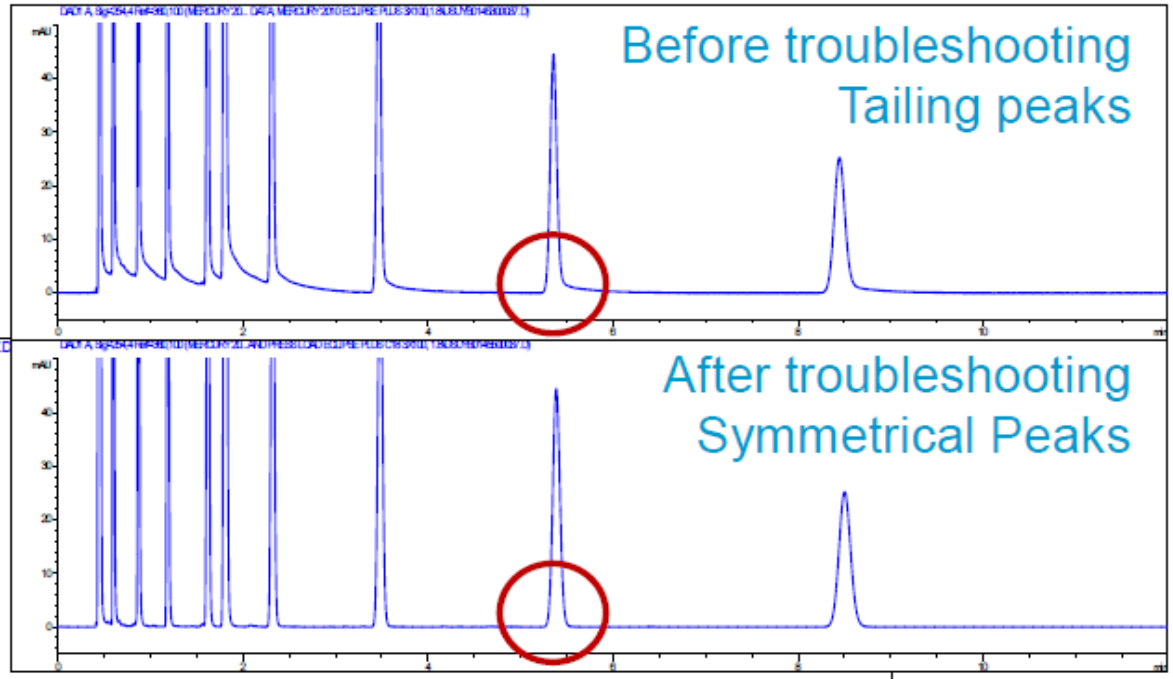
Peak Shape: Effect of Extra Column Volume

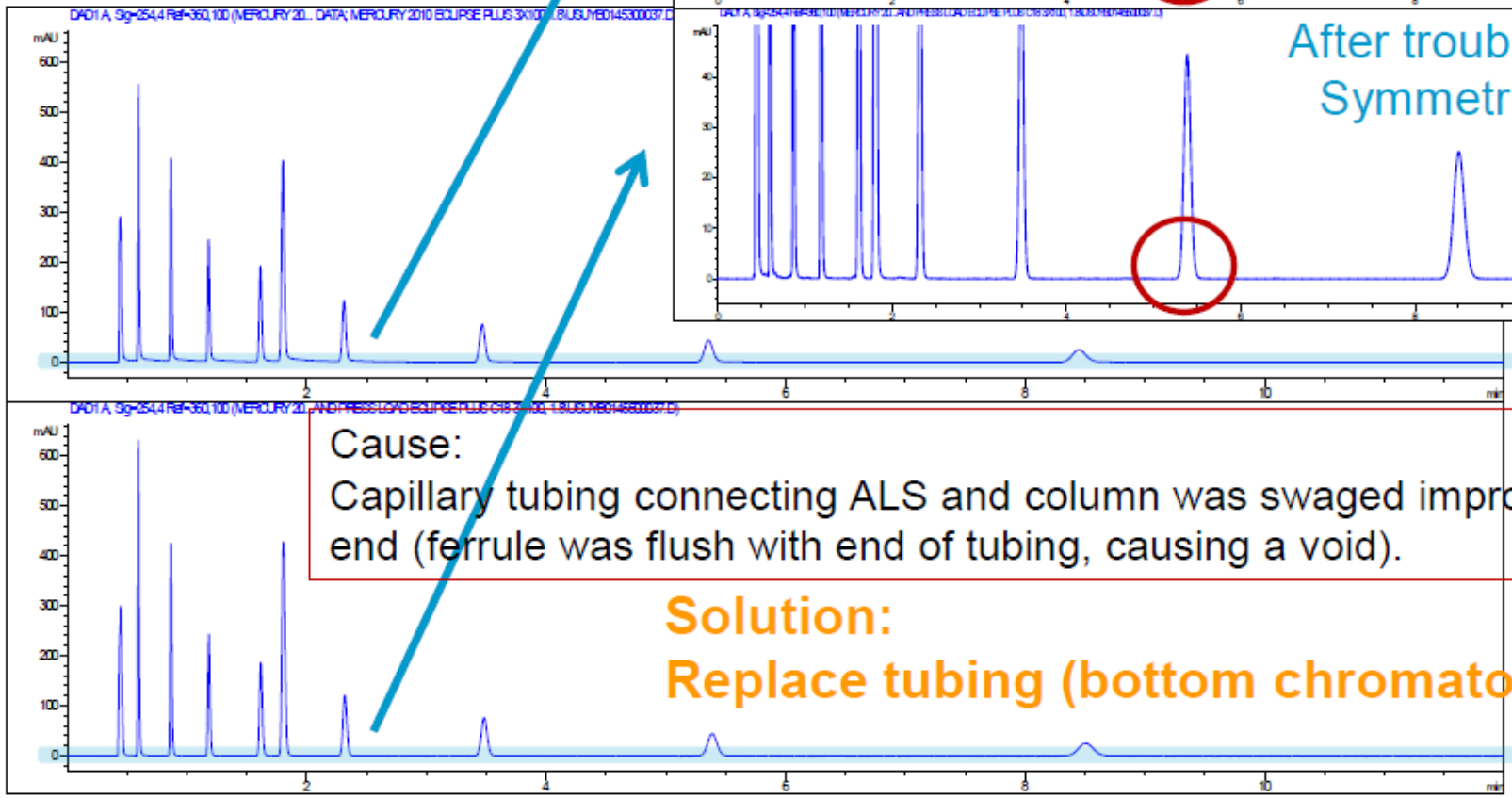
2.1 x 150mm columns
Flow rate: 0.2ml/min



Peak Shape - Peak Tailing, Extra Column Effects

Problem:
All peaks tail
(top chromatogram).

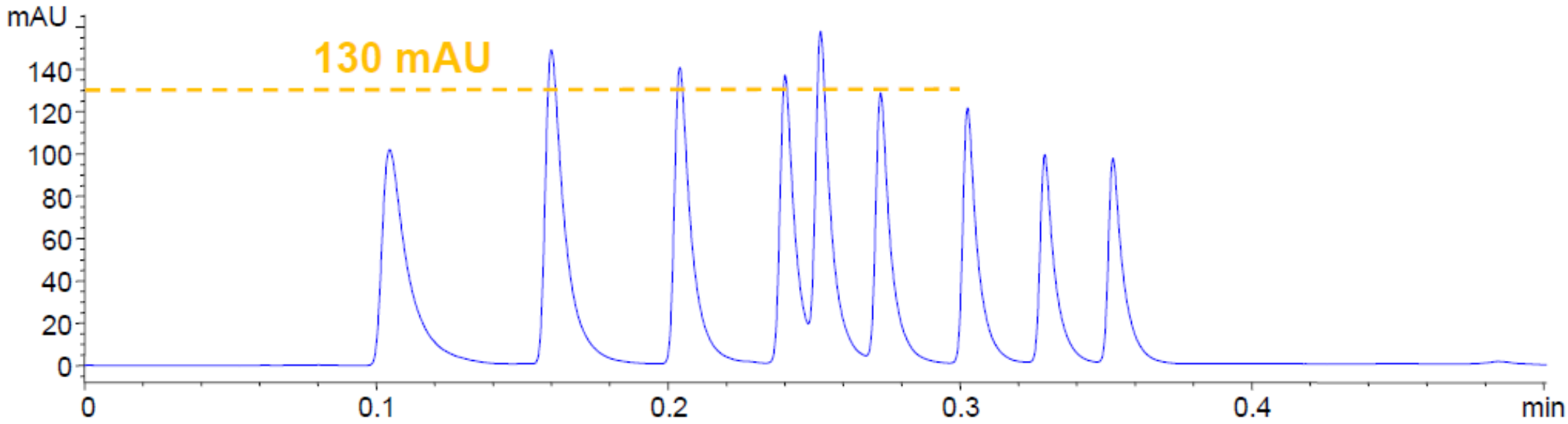




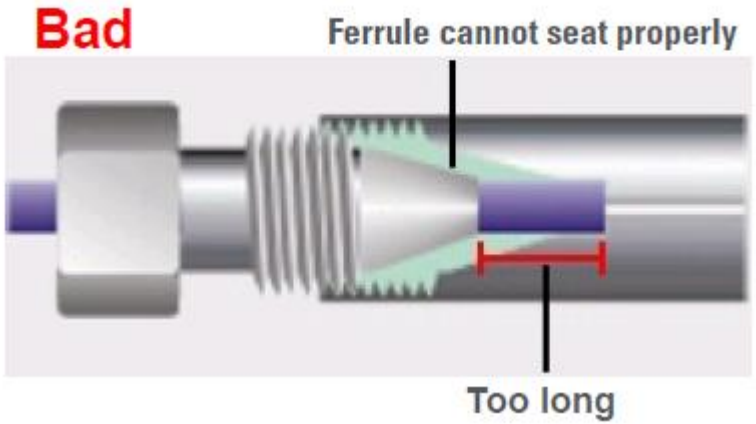
Cause:
Capillary tubing connecting ALS and column was swaged improperly on the ALS end (ferrule was flush with end of tubing, causing a void).

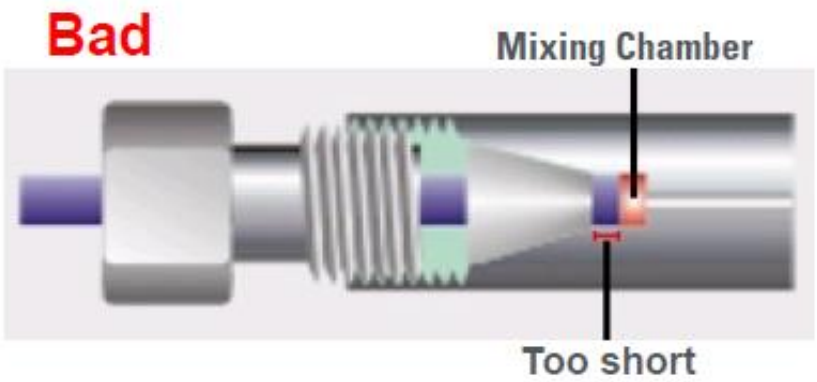
Solution:
Replace tubing (bottom chromatogram).

Peak Shape: importance of correct connections



Corrective Action: Making Correct Connections



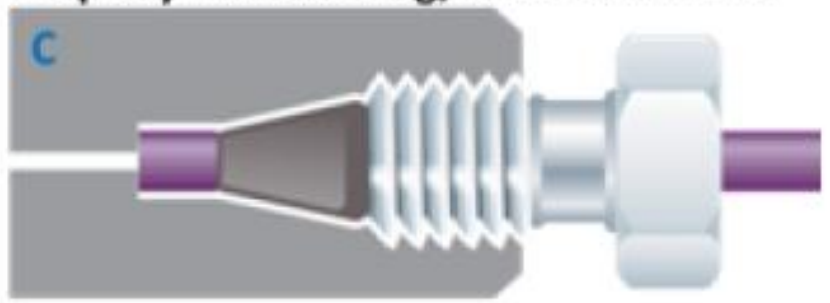


Poor Fitting Connections

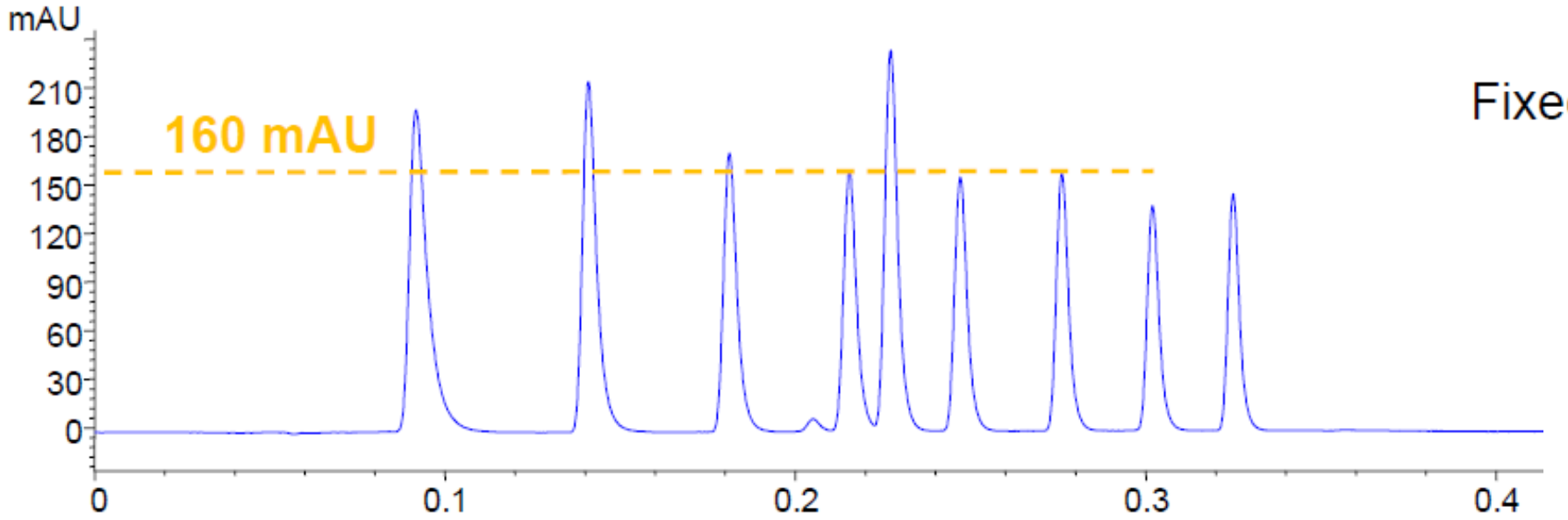
- Will broaden or split peaks or cause tailing
- Will typically affect all peaks, but especially early eluting peaks
- Can cause of carry-over

Good

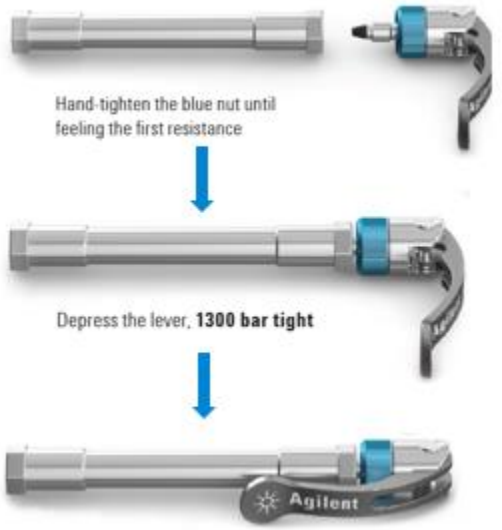
Properly fitted tubing, no dead volume



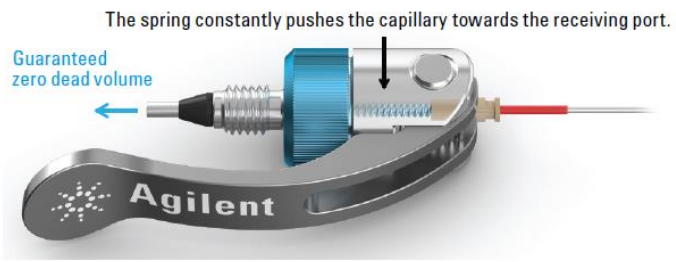
Peak Shape: Correct Connections



Fixed!



Correct connection every time



Quick Connect

Quick Turn

Compatible to 1300 bar

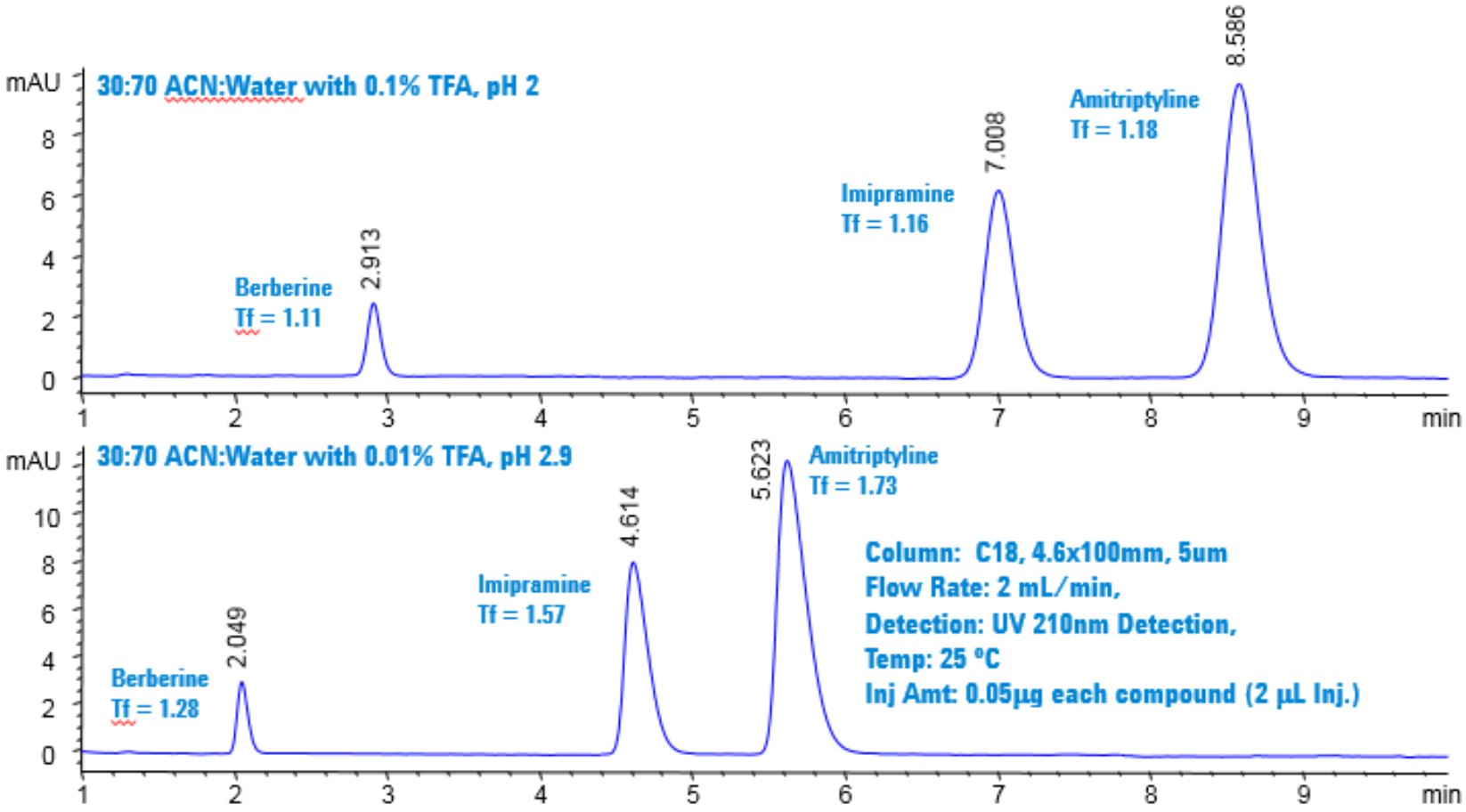
Agilent Technical Note: Agilent InfinityLab UHPLC Fittings
Pub No 5991-5525EN

Retention Time Shifts / Selectivity Differences

1. All Peaks Shift to Lower Retention (acids, bases, neutrals)
   - Loss of bonded phase
   - Mobile phase unstable
   - Solvent delivery system (flow rate or mixing)
2. All Peaks Shift to Greater Retention
   - Loss of organic solvent in aqueous/organic mix
   - Column change (less likely)
   - Solvent delivery system (flow rate or mixing)
3. Ionic Peaks Shift Retention
   - Loss of volatile MP component (ionic strength, pH shift)

Retention Time Shifts and Peak Shape

Change in Volatile Buffer Concentration



Retention
Separation Conditions That Can Cause Changes*

Condition	Change	Retention	Change
Flow Rate	+/- 1%	t_R	+/- 1%
Temperature	+/- 1 deg C	t_R	+/- 1 to 2%
% Organic	+/- 1%	t_R	+/- 5 to 10%
pH	+/- 0.01%	t_R	+/- 0 to 1%

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

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

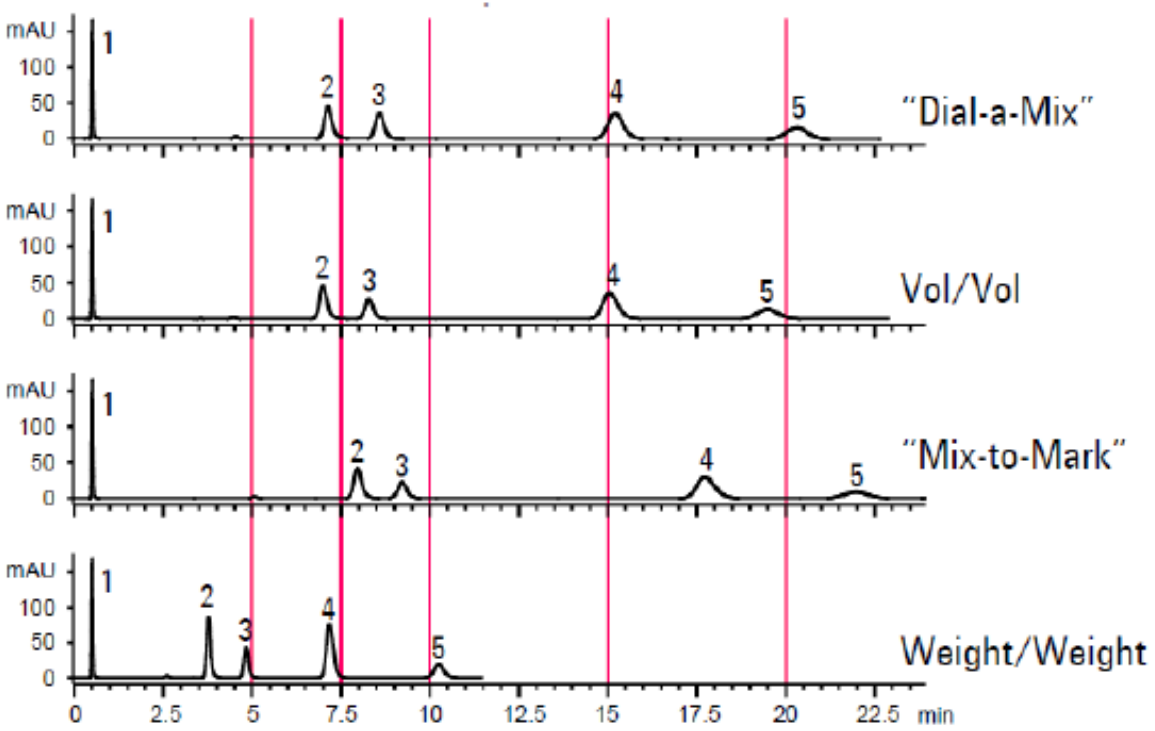
When preparing mobile phases:

- ✓ HPLC grade or better
- ✓ Buffer prep procedure

➢ **Be Consistent**

➢ **Document the Process**

Mobile Phase Preparation: Effect on Chromatography

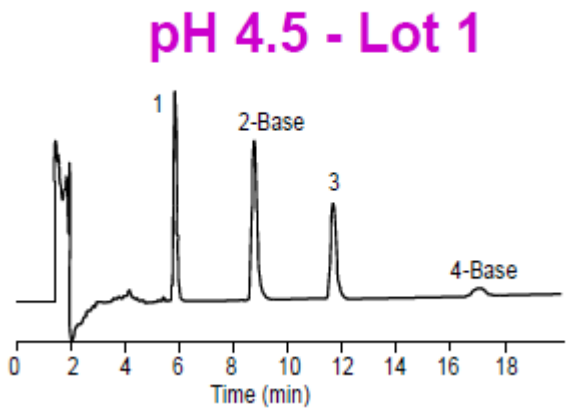


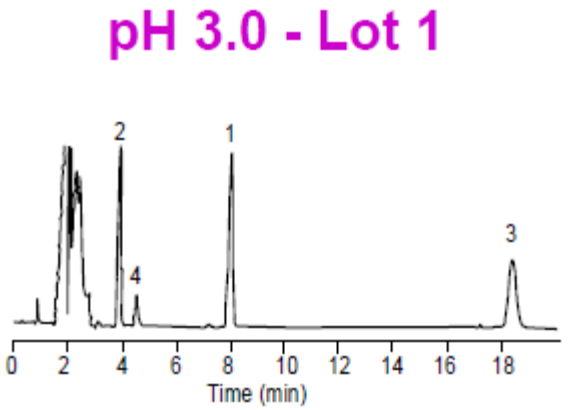
HPLC System:	Agilent 1100 with quaternary pump
Column:	ZORBAX Eclipse XDB-C8 Rapid-Resolution (3.5µm), 4.6 x 50 mm Agilent Part No. 935967-906
Mobile Phases:	Dial-a-Mix= A: water B: MeOH, pump 50% B Vol/Vol=250 mL water + 250 mL MeOH, pump 100% Mix-to-Mark = 250 mL MeOH, fill to 500 mL with water, pump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

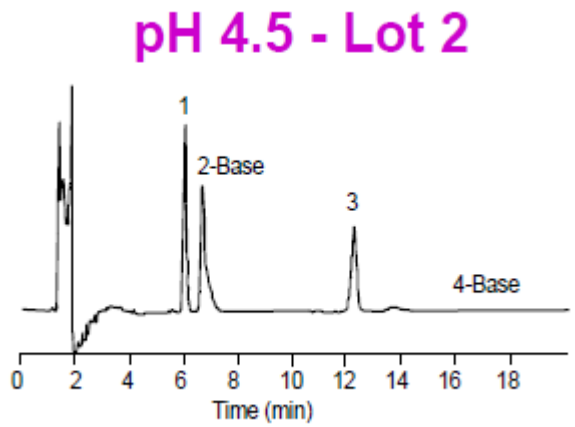
1. Uracil
2. Butylparaben
3. Napthalene
4. Dipropylphthalate
5. Acenaphthene

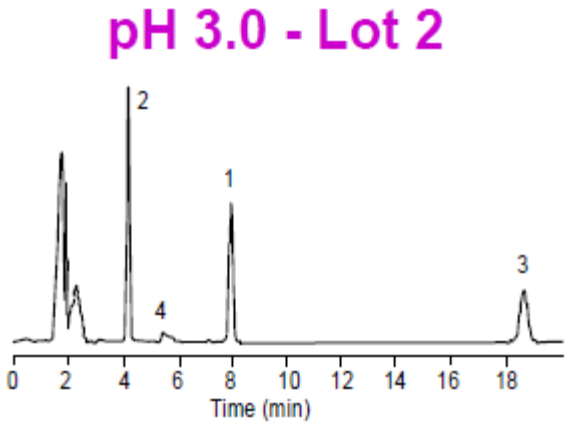
- Method used to prepare MP can significantly affect the elution pattern
- Be consistent
  - w/w is more accurate than v/v

Retention Time Shift – Selectivity differences due to incorrect pH





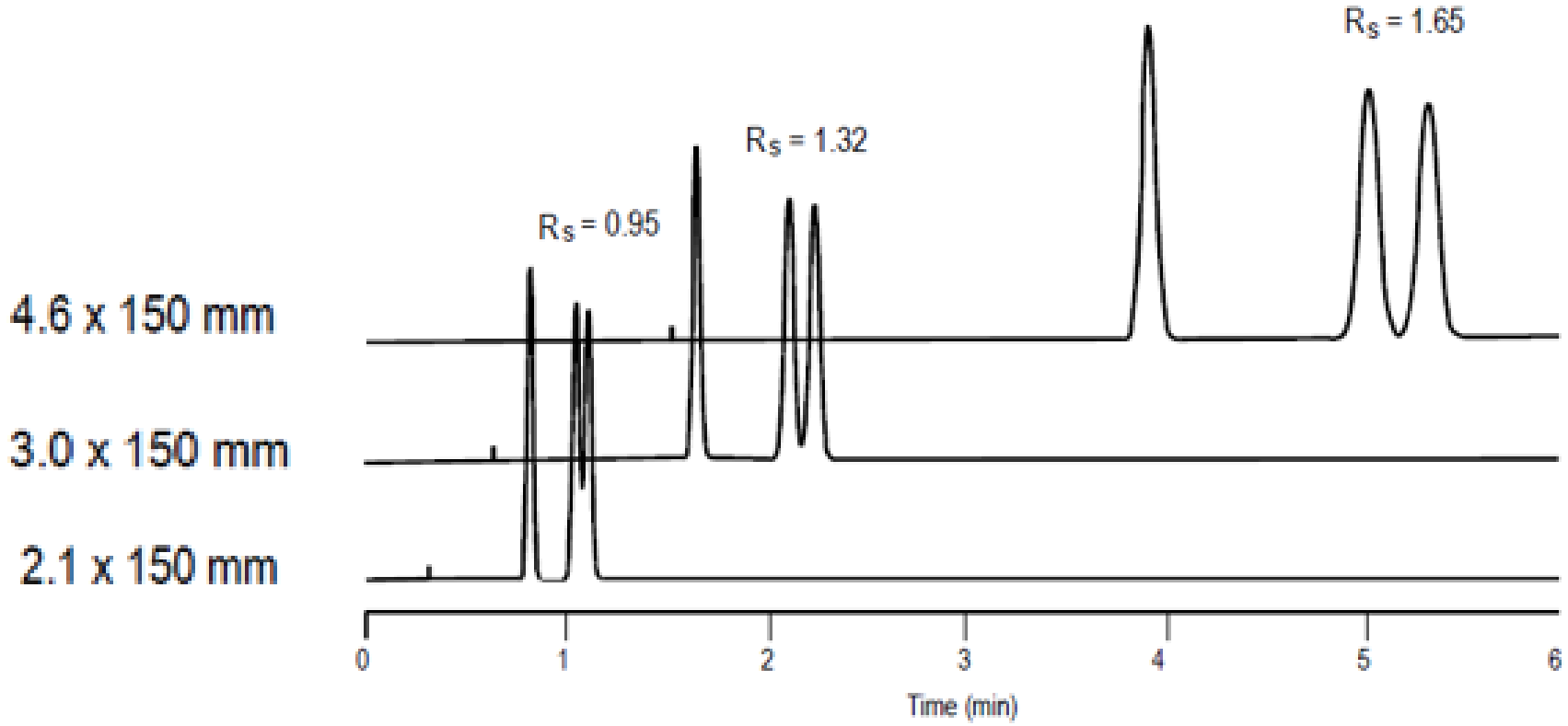




pH 4.5 shows selectivity change from lot to lot for basic compounds

pH 3.0 shows no selectivity change from lot to lot ; indicates silanol activity at pH 4.5

Retention Time Shift: change of column ID



Flow Rate: 1.0 mL/min

Corrective Action:

0.4ml/min

0.33ml/min

Retention Time Shifts

Mobile Phase Related Problems

- Make fresh, compare to aged
  - · pH
  - · conductivity
  - · chromatographic test

Corrective actions:

- For the sample, are the MP conditions correct for the column

Column Related Problems

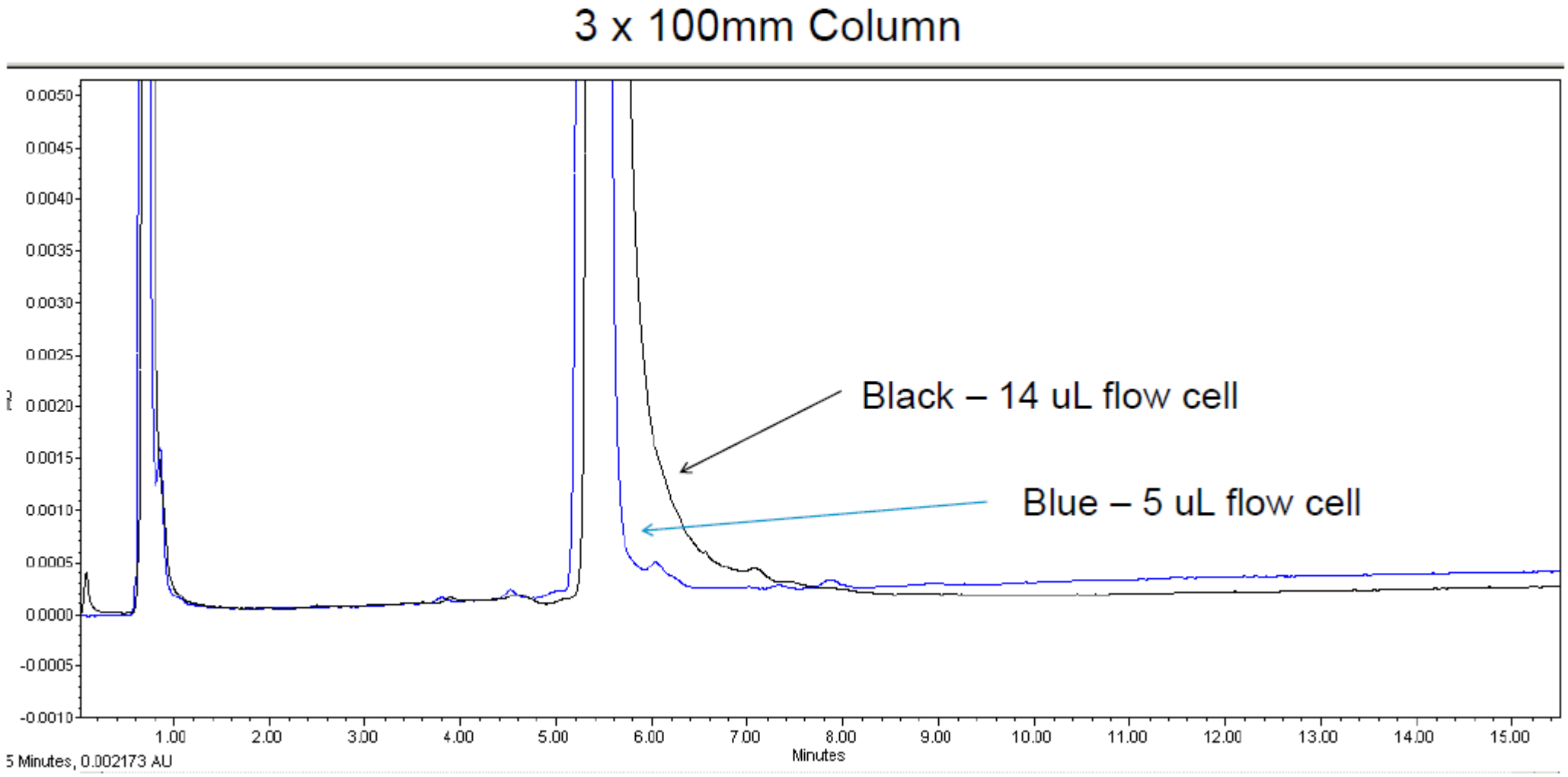
- Test new column, Are you working with the correct column?
- Test current column with test mixture or e.g., Toluene
- "Wash" column and retest
- Consider effect of sample matrix

OTHER LC Instrumentation Considerations

Possible causes: reg issues with baseline & peak shape

- Flow cell volume – consideration of the size of the flow cell to use
- Lamps – selecting correctly to ensure optimal performance
- Data Collection Rate Setting - too low, too high

Flow Cell Volume: importance for smaller ID columns



Flow Cell Volume: effect can be peak broadening

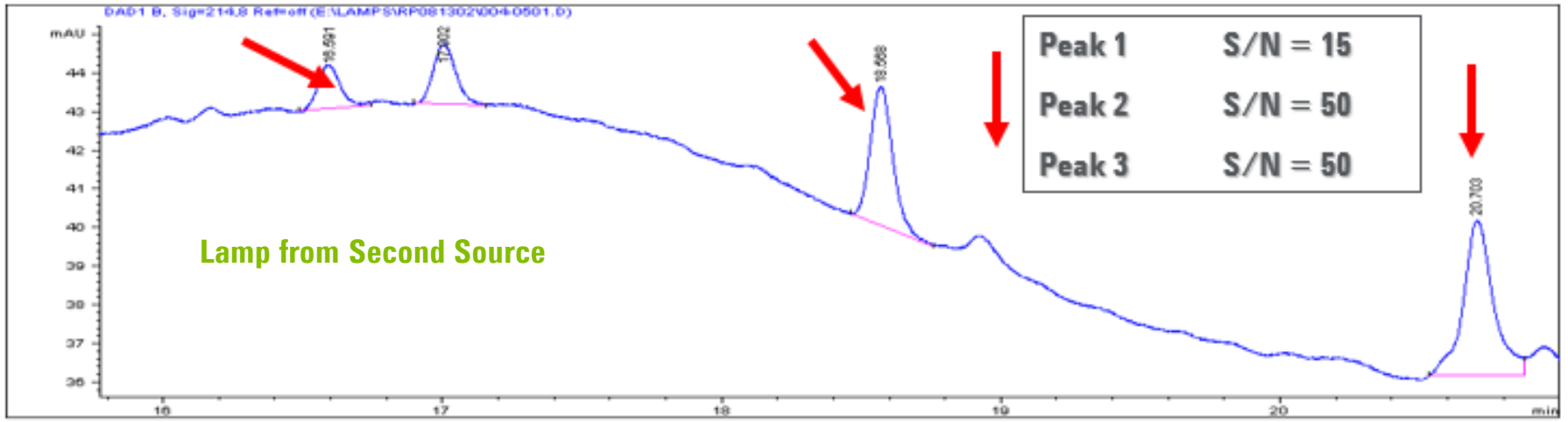
Differences in Detector Flow Cell Volume Can Affect N and R_s

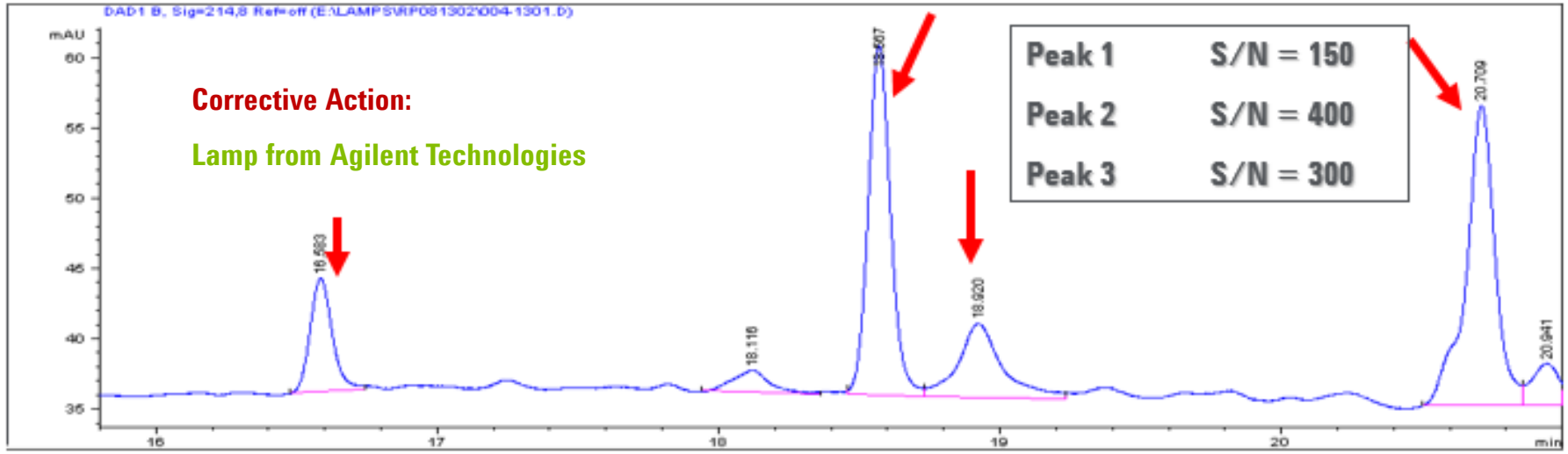
Scenario: ZORBAX Rapid Resolution Column: 75 mm, 3.5-µm; Flow Rate: 1mL/min; k = 3

Flow Cell Volume	Band Broadening* (4.6 mm)	Band Broadening* (2.1 mm**)
1.7 µL	0.3%	6%
8 µL	6%	138%
14 µL	19%	423%

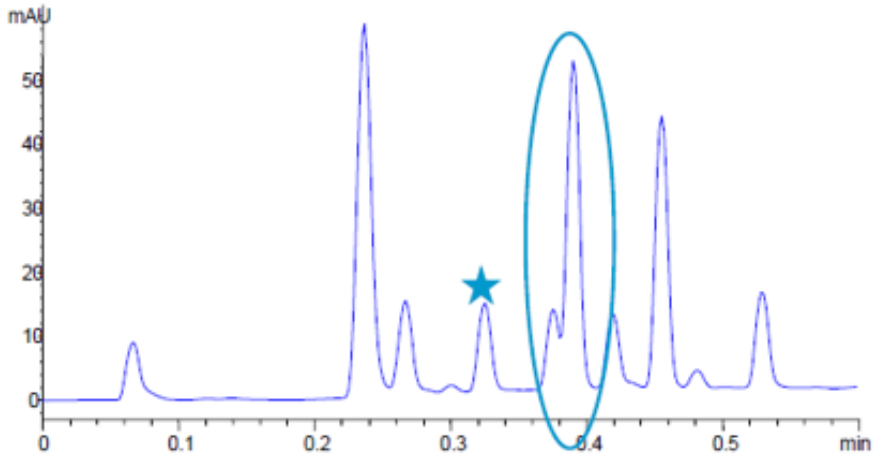
*Versus 8571 theoretical plates (HPLC Calculations Assistant, Version 2.1, Savant Audiovisuals)
**Flow Rate, 0.2 mL/min

Non Spec lamp: instability of baseline, response reduced

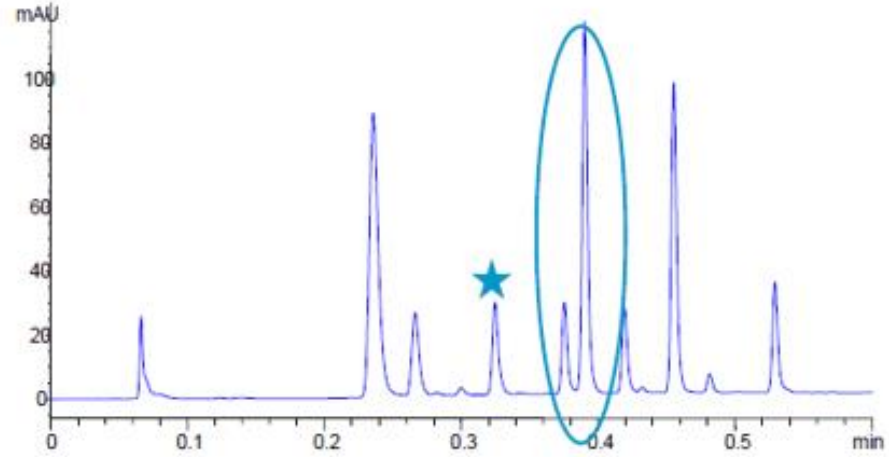




System Data Collection Rate: optimize for peak Rs

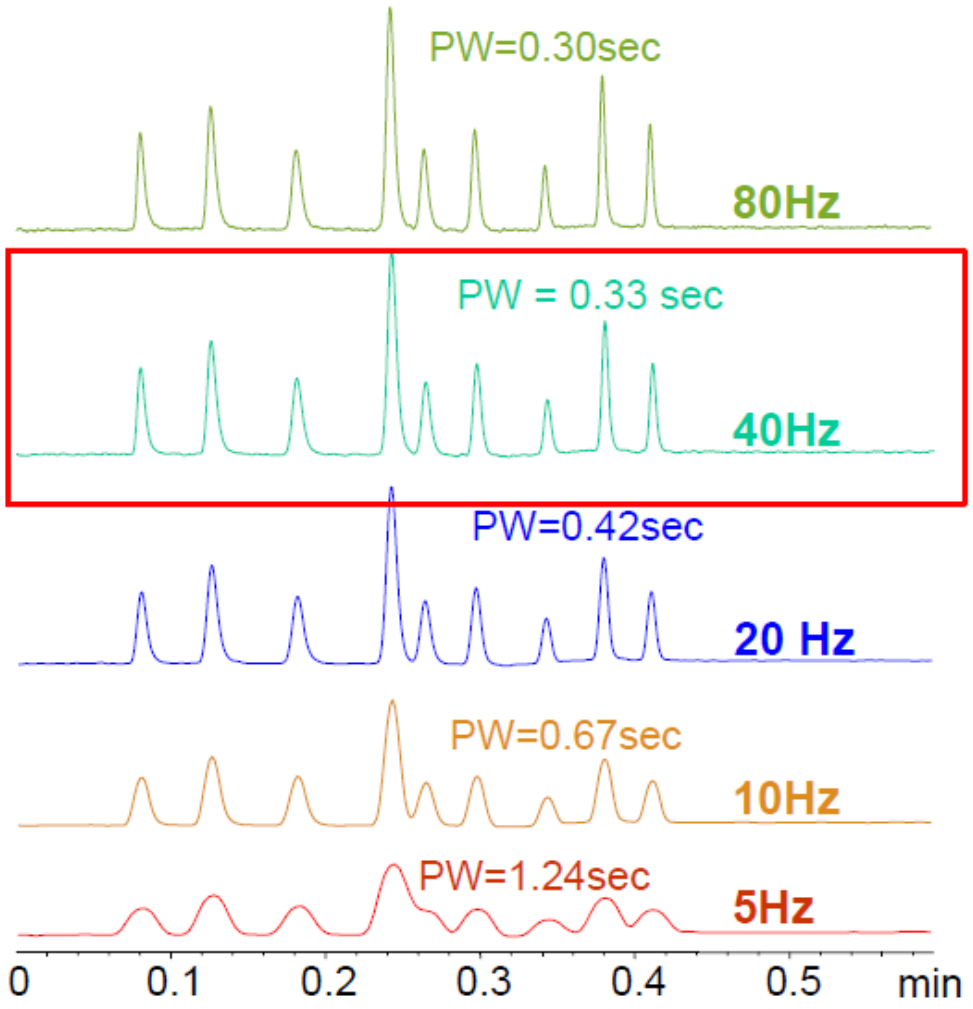


★ *Peak width = 0.021min at 10Hz*



★ *Peak width = 0.017min at 80Hz*

Effect of Data Acquisition Rate



- Increased Data Rate
- More Accurate " Picture"
- Make Sure Rate is Adequate
- Faster Rates Generate More Noise and Take up More Memory

High Defination UHPLC requires high definition chromatograms

My chromatogram isn't like it is Supposed to be!

My column is bad - I need a new one

Why has my peak shape changed?

The pressure is too high?

My peak retention time has shifted!

The baseline is noisy!

Argghhhh!!!!!

Summary

Baseline issues System pressure/Column pressure	Problem could be related to either column OR to the system. Test with and without the column. Further troubleshoot and determine the cause. Take corrective action to reduce or eliminate
Undesirable peak shape/ peak shape change	Whether Tailing, Splitting, Broadening, one needs to consider the many causes for why. Consider column 'health', correct conditions for analysis, & optimizing lc system can help to reduce occurrences
Changes in Retention or Selectivity	Be Consistent. Know what you are working with as it relates to the components for your analysis. Small changes in mobile phase prep, pH or other analysis parameters can have an adverse effect.
NOT always the column	Problems with your chromatography are not always a problem with the column. Considerations of experimental conditions & Instrumentation must be also investigated
Preventive Measures/Maintenance	Use proper precautions to prevent problems. Taking time with your sample & mobile phase prep, & doing routine instrumentation maintenance all help to keep analyses running smoothly

THANK YOU FOR ATTENDING

ANY QUESTIONS??

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 8am – 5pm EST – PST in US and Canada

- gc-column-support@agilent.com
- lc-column-support@agilent.com
- spp-support@agilent.com
- spectro-supplies-support@agilent.com