New To HPLC
Avoiding Beginner Pitfalls

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April 24, 2013

Outline

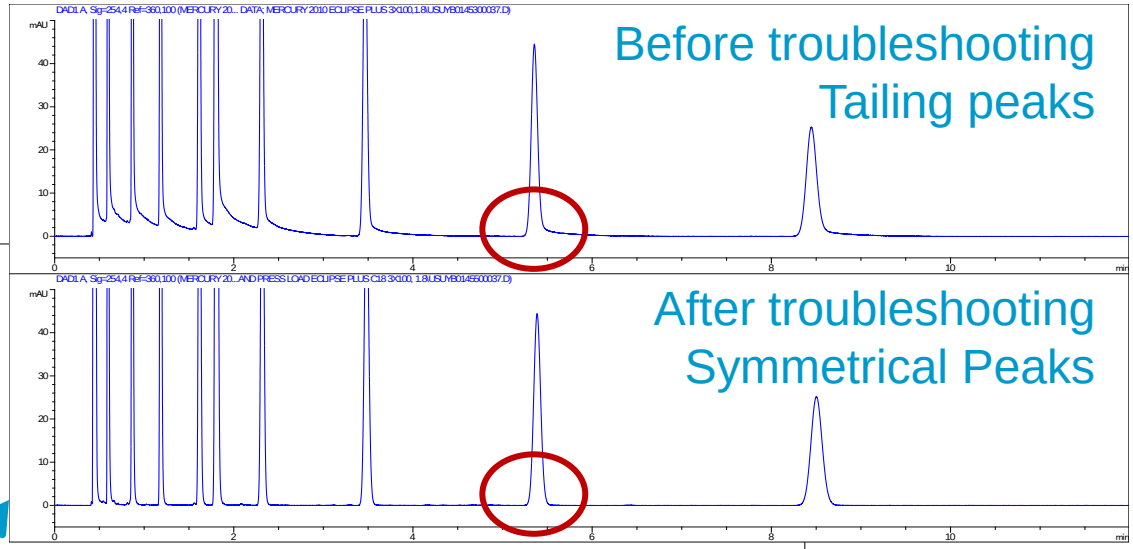
- Instrument
  - Connections
  - Performance
  - Detector
- Column
  - Characteristics
  - Lifetime
- Method Conditions
  - Mobile phase
  - pH
  - Temperature
- Sample
  - Cleanup
  - Injection

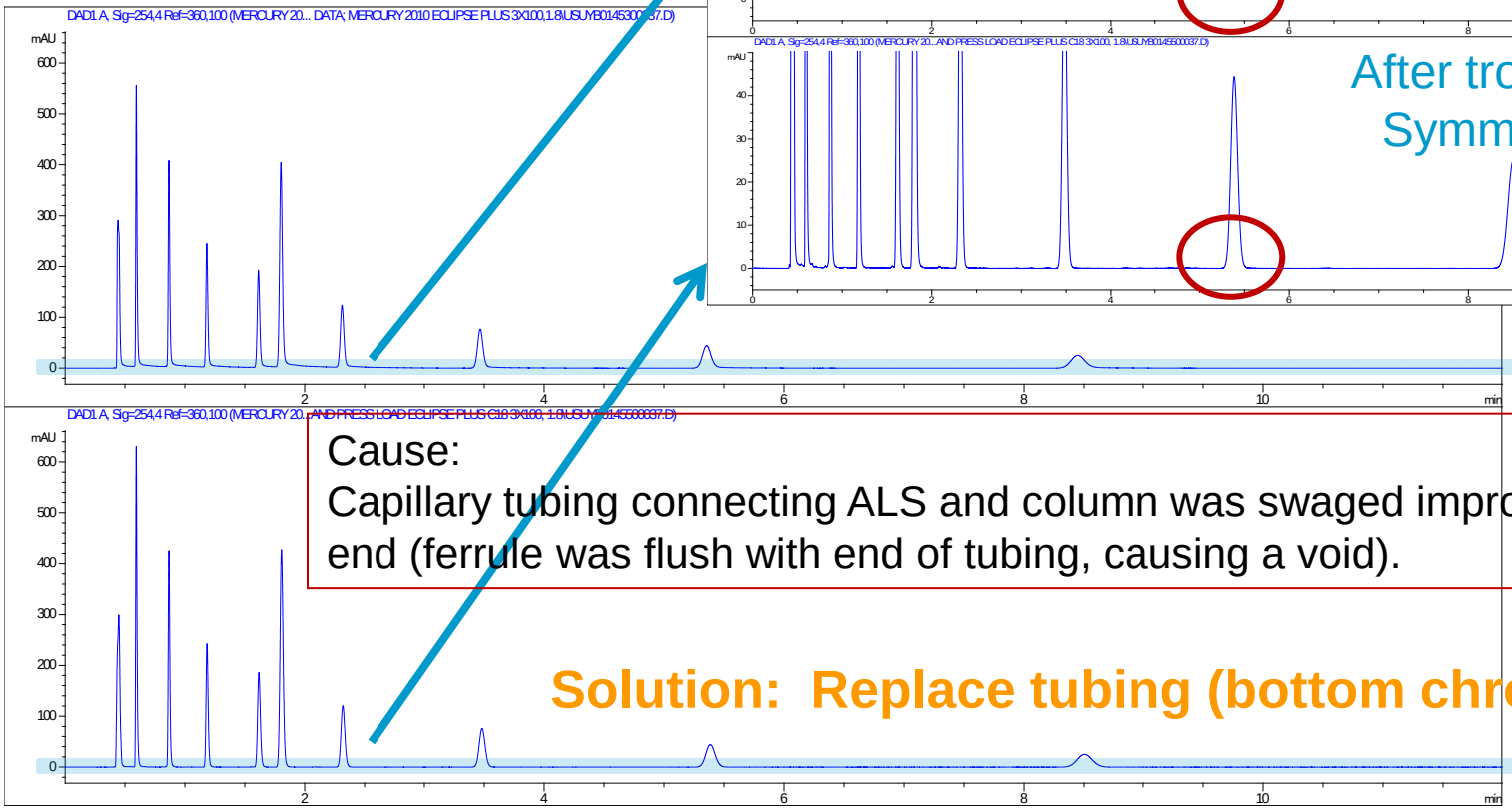
Instrument

- Fittings
  - Connections
    - Improper – Results in areas where the flow does not move smoothly
- Tubing
  - ID, Length
  - NO RANDOM PIECES
- Instrument Performance
  - Worn seals
  - Proportioning valve
- Detector
  - Which one
  - Flow cell
  - Proper settings

Poor Fitting
Peak Tailing – Extra Column Effects

Problem:
All peaks tail
(top chromatogram).



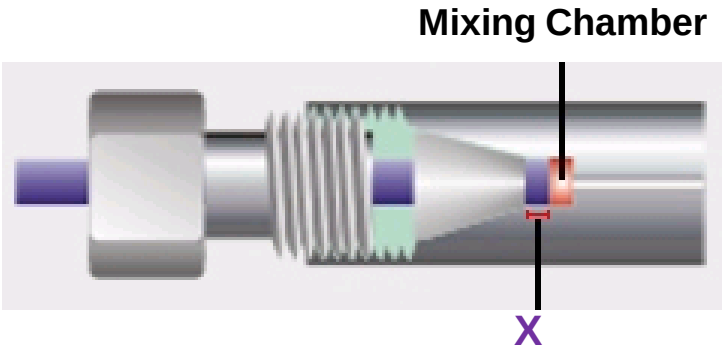


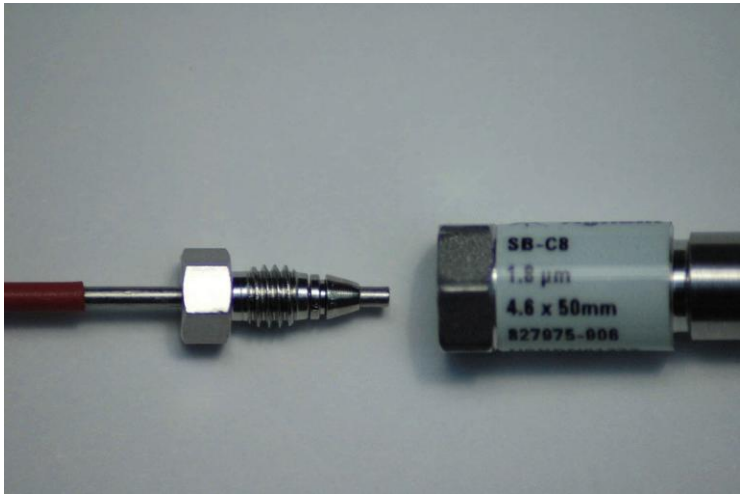
Cause:
Capillary tubing connecting ALS and column was swaged improperly on the ALS end (ferrule was flush with end of tubing, causing a void).

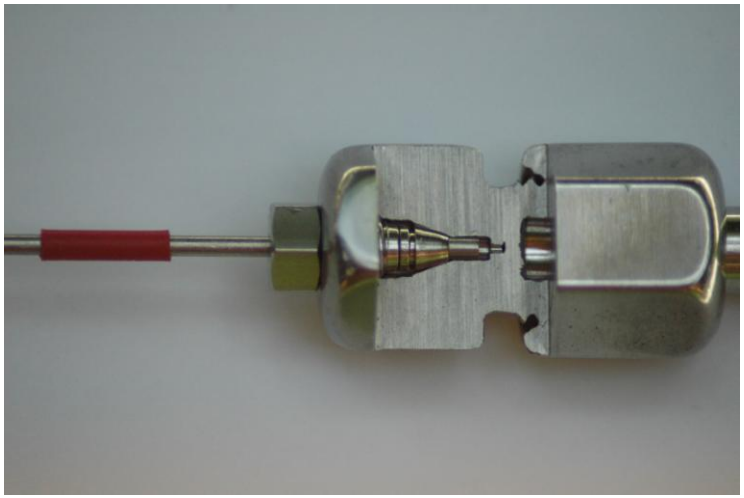
Solution: Replace tubing (bottom chromatogram)

Poorly Made Connections = Peak tailing/fronting

Wrong … too short







If Dimension X is too short, a dead-volume, or mixing chamber, will occur.

This can broaden or split peaks and/or cause tailing.

It will typically affect all peaks, but especially early eluting peaks.

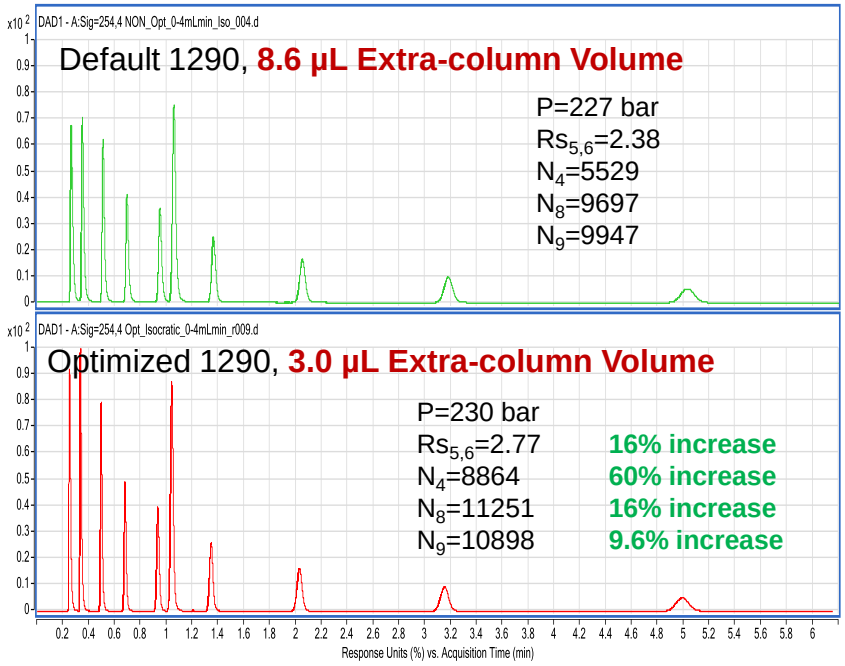
For information on making proper connections check out The LC Handbook, Pub. No.5990-7595EN

Extra Column Volume

Effect of ECV on Alkylphenones Analysis

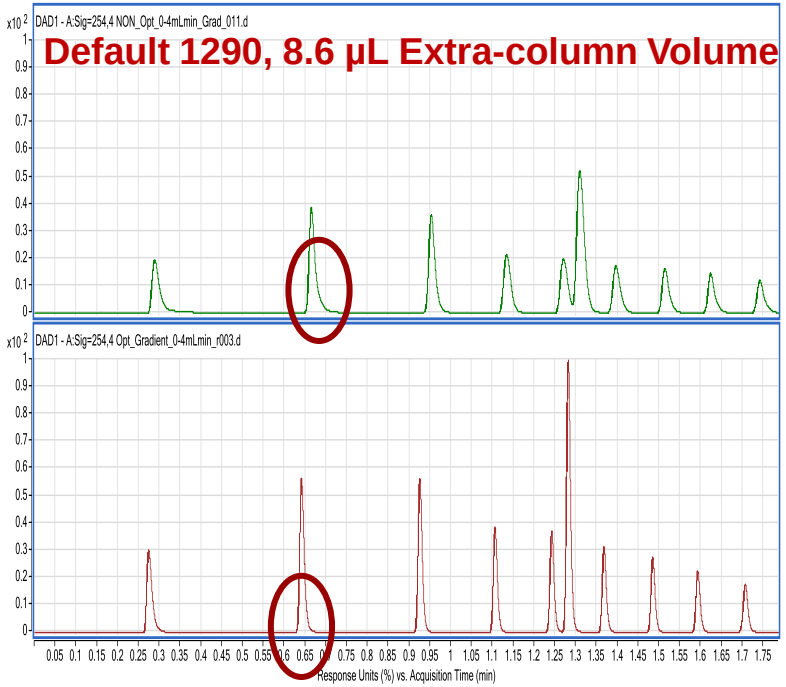
Isocratic

Efficiency



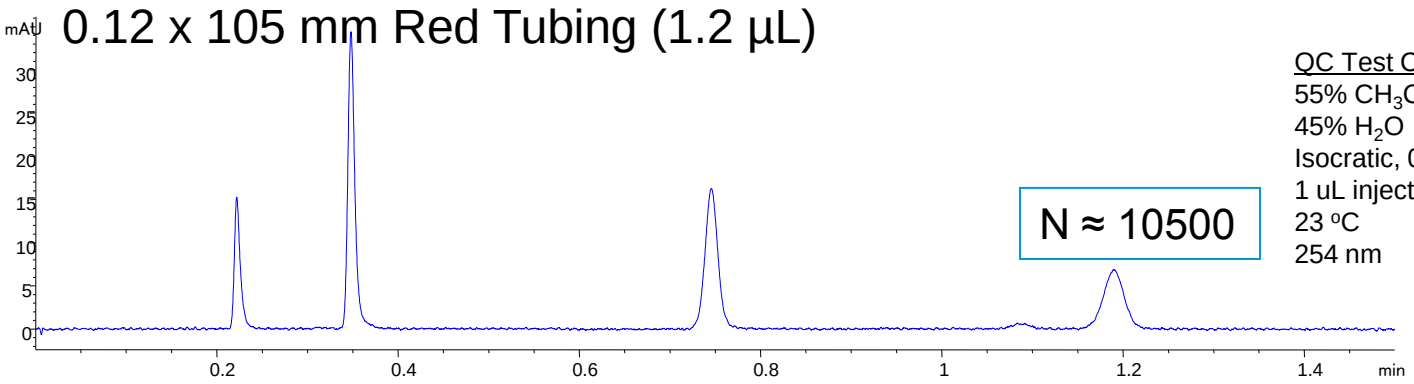
Gradient

Efficiency and Tailing

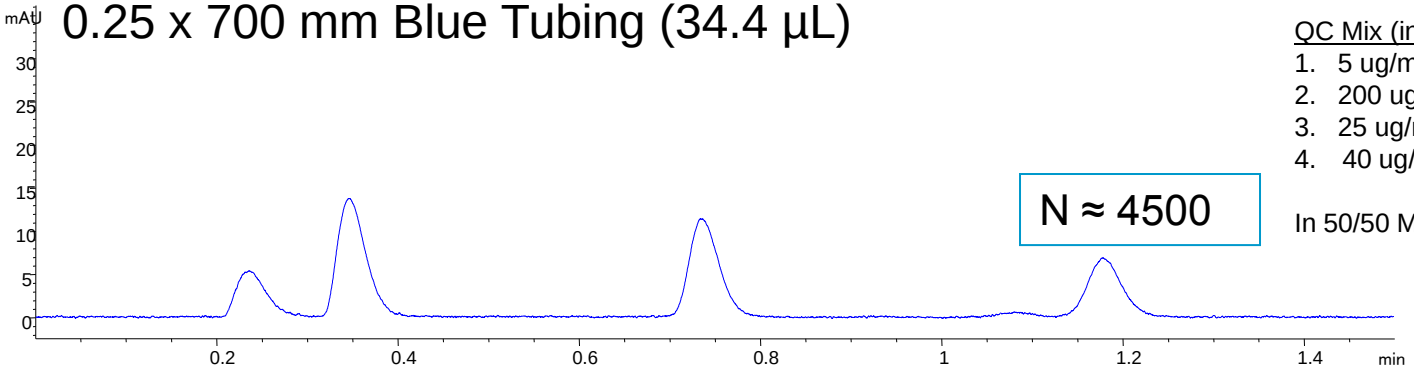


Column used: RRHD Eclipse Plus C18, 2.1 x 50mm, 1.8um, PN 959757-902

Do NOT Use Random Pieces of Tubing



<u>QC Test Conditions</u>:
55% CH_3CN
45% H_2O
Isocratic, 0.6 mL/min
1 uL injection of QC Mix
23 °C
254 nm



<u>QC Mix (in elution order)</u>:
1. 5 ug/mL uracil
2. 200 ug/mL phenol
3. 25 ug/mL 4-chloro-nitrobenzene
4. 40 ug/mL naphthalene

In 50/50 MeCN/Water

➢ QC test of a 2.1 x 50 mm, 1.8-µm Eclipse Plus C18 showing peak broadening when larger volume tubing is installed between the autosampler and column. 43% of the efficiency lost with too much ECV

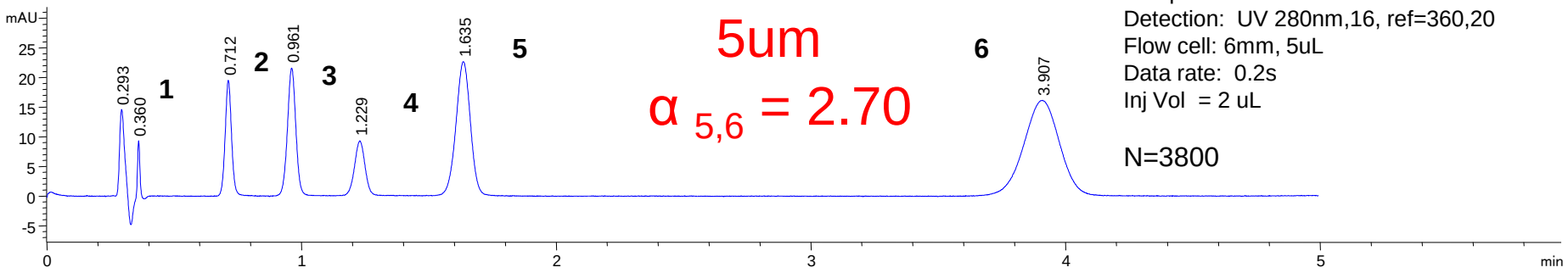
➢ To avoid this pitfall - Minimize extra column volume contribution from tubing by using the shortest length you can with the appropriate diameter and make proper connections

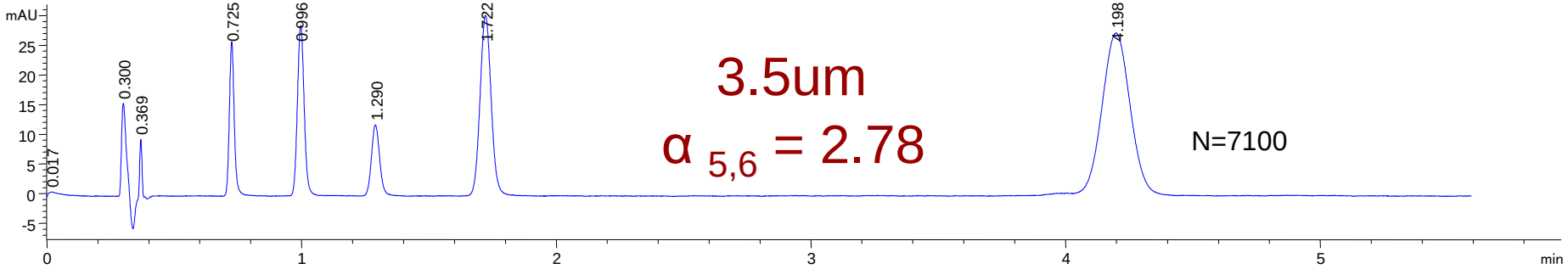
Inconsistent Selectivity between Particle Sizes

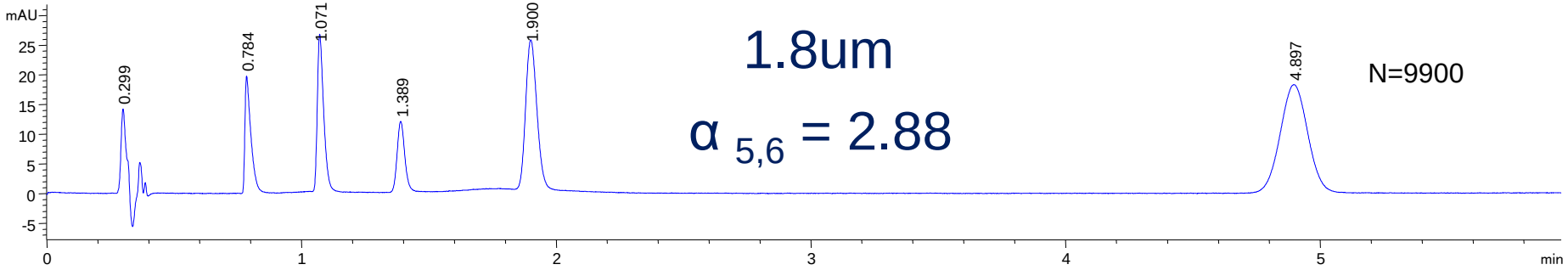
What Could Cause This

Column: Eclipse Plus C18, 4.6 x 50mm
MP: H2O:ACN; 60:40
Flow : 1.5 mL/min
Temp: 25 C
Detection: UV 280nm,16, ref=360,20
Flow cell: 6mm, 5uL
Data rate: 0.2s
Inj Vol = 2 uL

N=3800



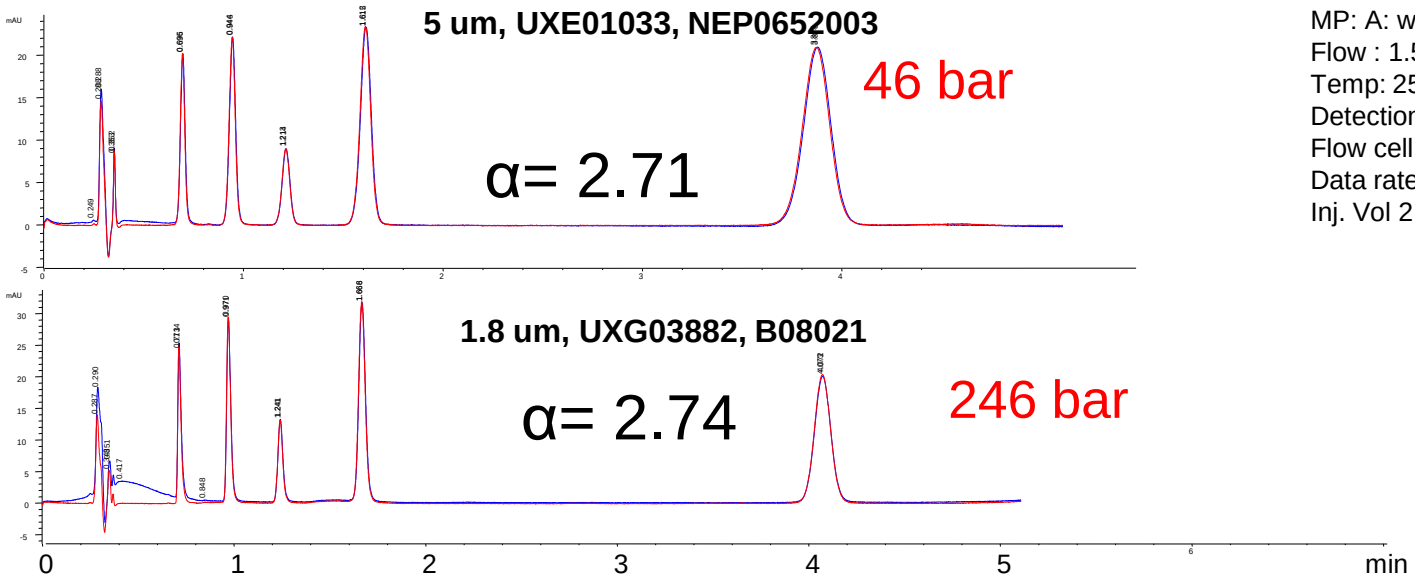




Inconsistent Selectivity between Particle Sizes, cont'd

One channel **premixed** mobile phase shows similar α

Problem with Proportioning Valve



MP: A: water, acetonitrile (60:40 v/v)
Flow : 1.5 mL/min
Temp: 25 C
Detection: UV 280nm,16, ref=360,20
Flow cell: 6mm, 5uL
Data rate: 0.2s
Inj. Vol 2 uL

Column Type	Alpha of peaks 5,6 proportioned	Alpha of peaks 5,6 premixed
5um	2.70	2.71
3.5um	2.75	2.74
1.8um	2.88	2.74

- Chromatography problems can come from more than just the column
- Good instrument maintenance can avoid instrument related problems with your chromatography

Detector – Which One & Why

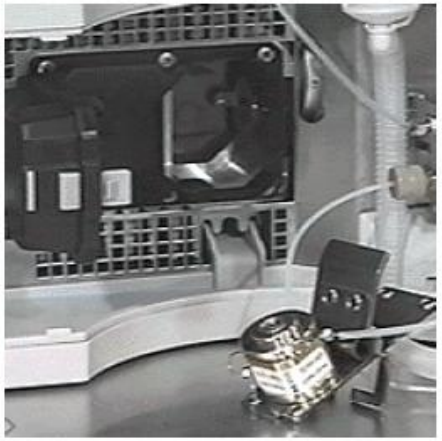
- UV/DAD
  - Popular, simple to use, reliable, sensitive
  - Sample must have UV absorbance
- MS
  - Sensitive
  - Sample must be ionizable
- RI
  - Refractive Index; difference between analyte and mobile phase
  - Need strict temperature control
- ELSD
  - Independent of a compound's absorbance, fluorescence, or electro-activity
  - Enables detection of semi-volatile and thermally sensitive compounds
- FLD
  - More selective and can be more sensitive
  - Compounds must fluoresce; Compounds often derivatized
- ECD
  - Very sensitive
  - Can produce severe noise

UV Detector

- Sample
- Appropriate wavelength
  - Sample
  - Reference
    - Selecting Reference Wavelength in DAD Spectroscopy, http://www.chem.agilent.com/Library/Support/Documents/faq184.pdf
    - How To Select Reference Wavelength on DAD or MWD, http://www.chem.agilent.com/Library/Support/Documents/faq182.pdf
  - Mobile phase components
    - UV Cutoff
- Flow Cell
- Data rate
  - Column size
  - Response

Flow Cells

Match flow cell volume to chromatographic peak widths



Flow Cell Volume/Pathlength	Uv Signal /Noise	Chrom. Resolution*
13 µl / 10 mm	+++	+
5 µl / 6 mm	++	++
1.7 µl / 6 mm	+	+++

* Depends on analytical conditions and column dimension

13 µl Standard Flow Cell:

For highest sensitivity and linearity
4.6-3 mm ID, 2.7, 3.5, 5 µm columns

1.7 µl Micro Flow Cell:

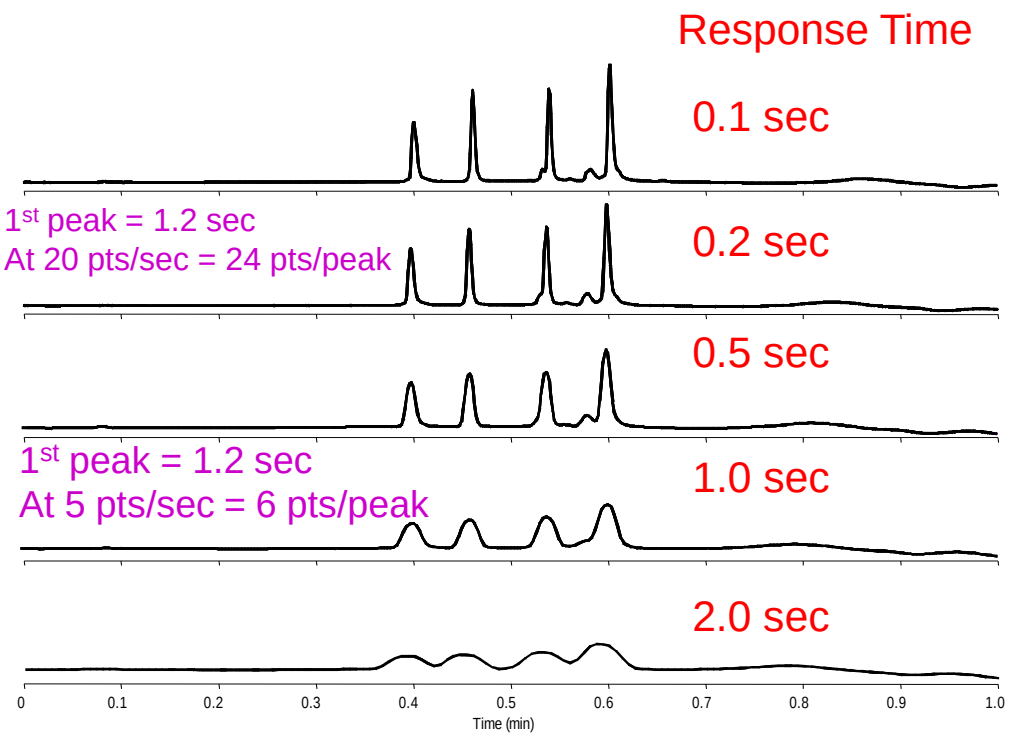
For highest resolution
UHPLC, 1.8, 2.7 µm
2.1-1 mm ID columns

5 µl Semi-micro Flow Cell:

Best compromise of sensitivity & selectivity
HPLC/UHPLC, 1.8 to 5 µm
4.6 – 1 mm ID columns

Other flow cells include
Max-Light Cartridge cells for Infinity DAD
500 nL for capillary LC
80 nL for nano LC
0.6 mm for Prep LC

Effect of Detector Response Time on Fast Gradient Analyses



Agilent 1100 DAD
Agilent 1100 WPS with ADVR

Column: Poroshell 300SB-C18
2.1 x 75 mm, 5 mm

Mobile Phase:
A: 95% H_2O, 5% ACN with 0.1% TFA
B: 5% H_2O, 5% ACN with 0.1% TFA

Flow Rate: 2 mL/min

Temperature: 70°C

Detector: UV 215 nm

Piston stroke: 20

Sample:
1. Neurotensin 3. Lysozyme
2. RNaseA 4. Myoglobin

- You may have to adjust the response rate of your detector for rapid peak detection
- To avoid missing the peak, make sure detector is set properly
  - Need ~25 data points to accurately "describe" a chromatographic peak.

http://www.chem.agilent.com//Library/Support/Documents/FAQ_Approved_PDF_Template_enough_datapoints.pdf

Column

- Performance Report
  - Mfg. tests – tailing, efficiency, selectivity
    - Optimized instrument
- Not all C18 (L1) columns are the same
  - Silica, surface area, bonding chemistry, end-capping, etc.
- Equilibration
  - See Appendix
- What kills a column
  - Method conditions
  - Crud
- Pressure
- Guards
- Lot-to-lot

Performance Report

SERIAL NUMBER: USDAZ01333

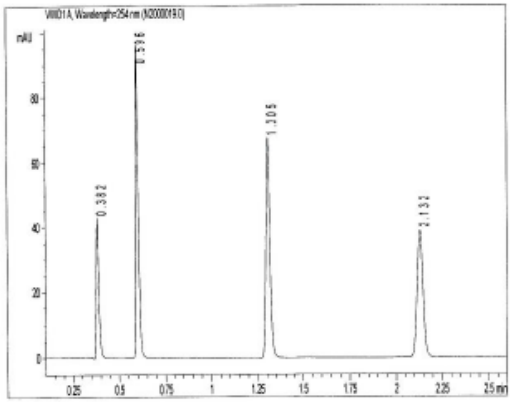
PART NUMBER: 959758-902
COLUMN TYPE: ZORBAX RRHD Eclipse Plus C18 2.1 x 100 mm, 1.8 µm
PACKING LOT #: B09089

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	



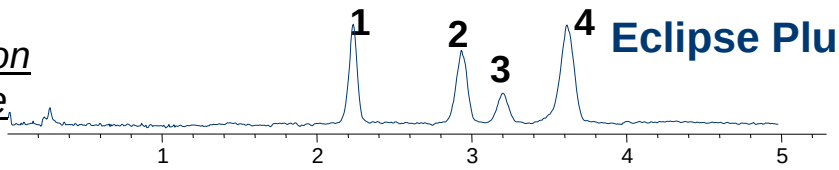
Sample components with concentrations diluted in mobile phase in the following elution order.

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

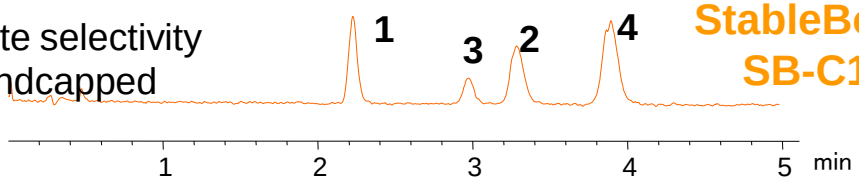
Good News/Bad News
Not All C18s Are The Same

1st choice
Best Resolution & Peak Shape



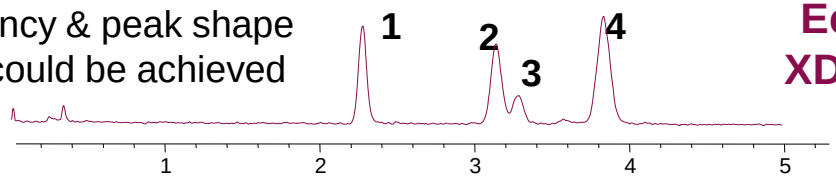
Eclipse Plus C18

2nd choice
Good alternate selectivity due to non-endcapped



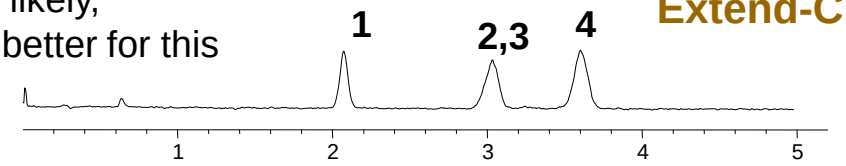
StableBond SB-C18

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
- *See appendix for available method development kits*

- Don't assume every C18 will behave the same

Method Development Kits
See Appendix

Column Lifetime

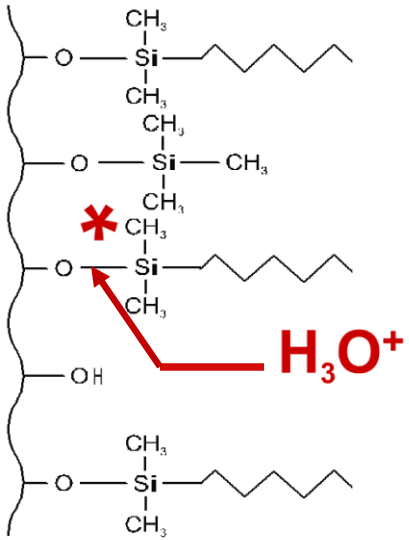
- Follow manufacturer's guidelines
- Method conditions that can affect column lifetime
  - pH
    - Low pH
      - pH<3 – acid hydrolysis of bonded phase
      - Retention time changes, resolution changes, increased peak tailing
    - High pH
      - Silica-based packing has some solubility in pH>6
  - Temperature
    - Improper temp can accelerate the dissolution of the silica above pH 6.
  - Crud
    - Column contamination
    - Plugged frit
  - Buffer Choice
    - Avoid phosphate & carbonate buffers at high pH
- Storage conditions
  - Columns should not be maintained at neutral or elevated pH or elevated temperature when not in use

Operational Conditions

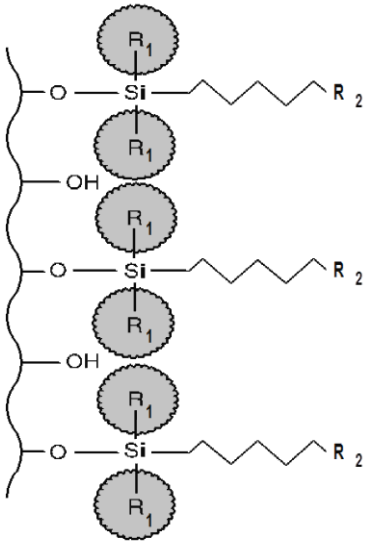
Mobile Phase Effects on Column Life

Low pH (1-3) - Bonded Phase Loss by Acid Catalyzed Hydrolysis

Conventional



StableBond

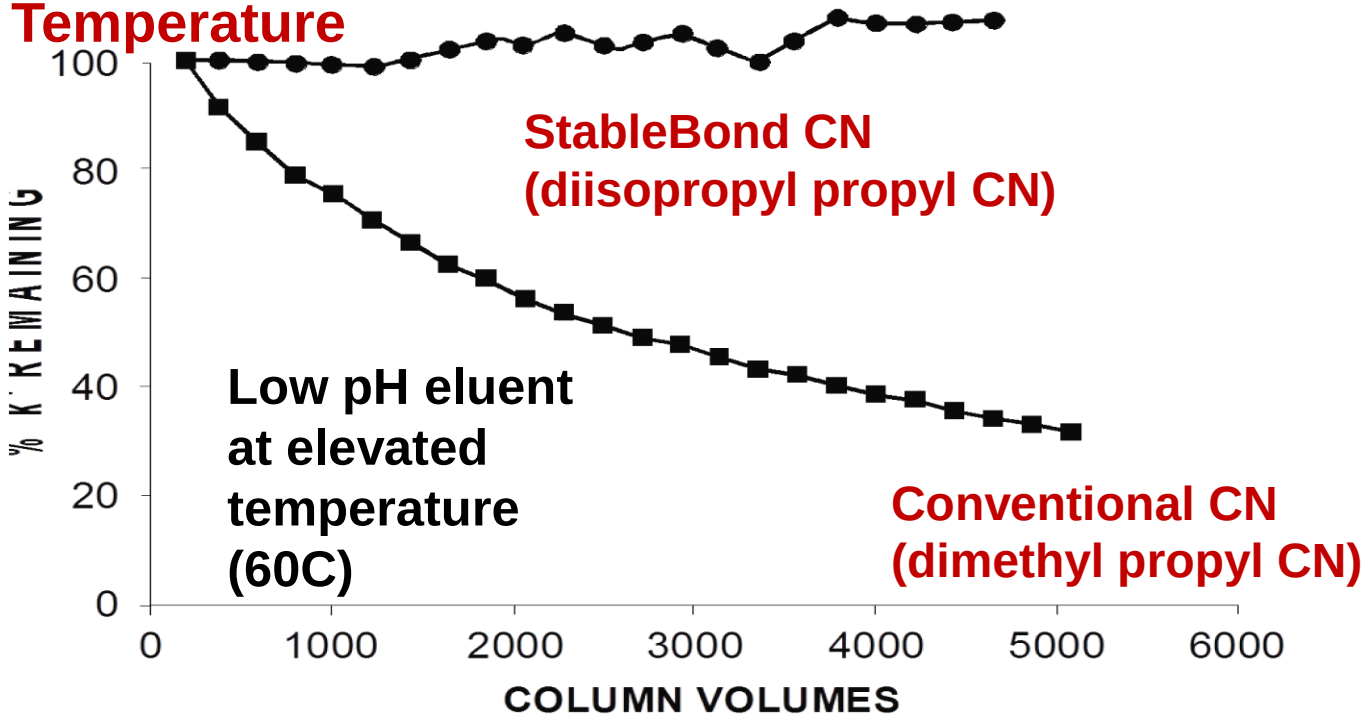


* Hydrolytically Sensitive Siloxane Bond

Operational Conditions

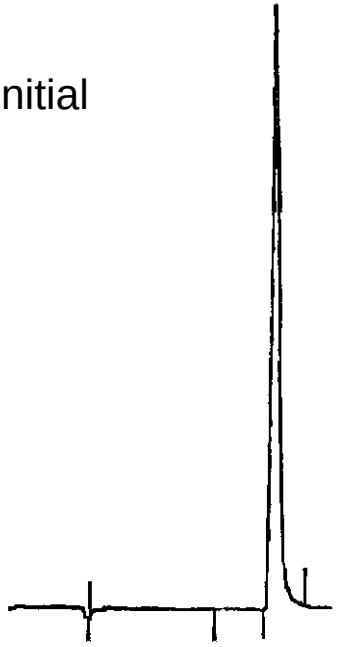
Mobile Phase Effects on Column Life

Bonded-Phase Hydrolysis Rate Increases With Temperature

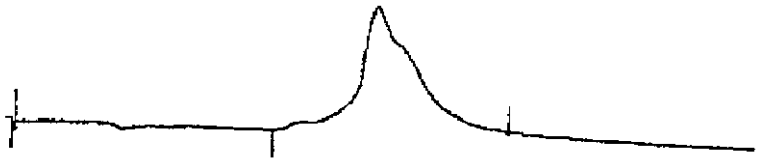


Peak Broadening, Splitting
Column Void

Initial



After 30 injections



Mobile Phase: 50%ACN: 50% Water : 0.2% TEA (~ pH 11)

- Multiple peak shape changes can be caused by the same problem.
- In this case a void resulted when the silica dissolved at high pH.

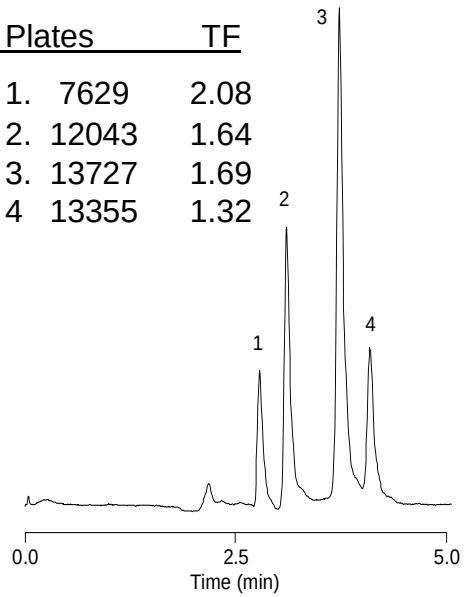
- To help get good column lifetime, follow manufacturer's guidelines for pH

CRUD

Peak Tailing from Column Contamination

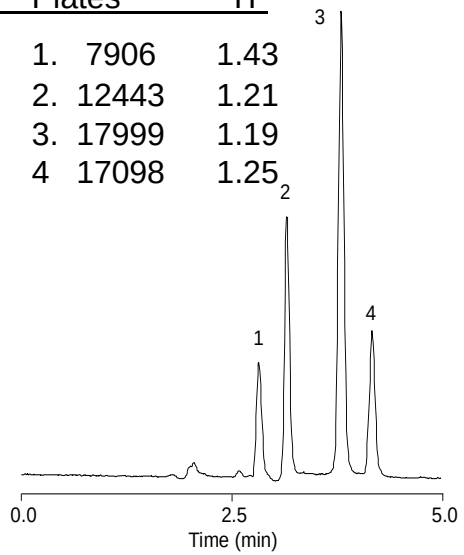
Column: StableBond SB-C8, 4.6 x 250 mm, 5µm Mobile Phase: 20% H_2O : 80% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

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



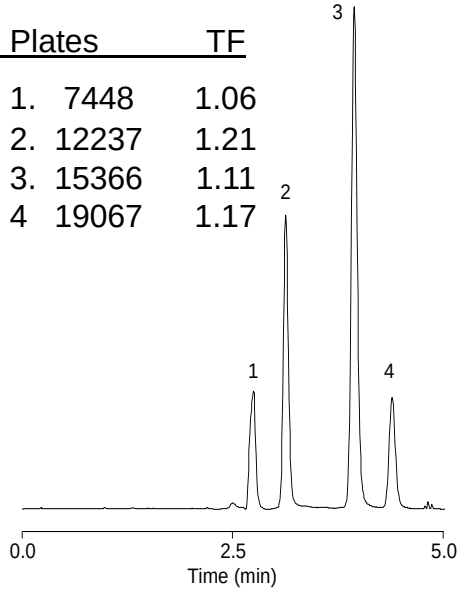
QC test forward direction

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



QC test reverse direction

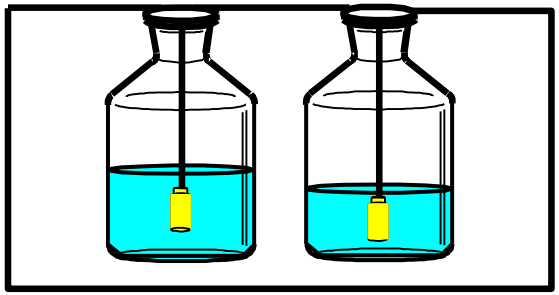
	Plates	TF
1.	7448	1.06
2.	12237	1.21
3.	15366	1.11
4	19067	1.17



QC test after cleaning
100% IPA, 35°C

- Good column hygiene can extend the life of your column
  - Column cleaning procedure (see appendix)

Preventing Column Back Pressure Problems



1. Filter mobile phase:
   - filter non-HPLC grade solvents
   - filter buffer solutions
   - Install an in-line filter between auto-sampler and column (removes pump seal debris, ALS rotor debris, and sample particulates). Use 2 um frit for 3.5 um/5um columns, use 0.5 um frit for 1.8 um/2.7 columns.
2. Filter all samples and standards
3. Perform sample clean-up (i.e. SPE, LLE) on dirty samples.
4. Appropriate column flushing – flush buffers from entire system with water/organic mobile phase.
5. Replace buffers every 24-48 hours, never add to the bottle, always use a new one.

Guard Columns

What They Do

- ✓ Increase total "column length"
  - Increase retention
    - $(tR_T = (L_{col} + L_{gcol})/(L_{col}) \times tR_{Col})$
  - Also - t_0, w, P, N and R_s
- ✓ Should increase efficiency
- ✓ Provide protection to the analytical column

What They Don't Do

- ✓ Replace good sample clean-up
- ✓ Replace column hygiene

Not a *"magic device"*

➢Any device in the flow path (guard column, filter, switching valve, detector, etc.) can adversely affect your chromatography

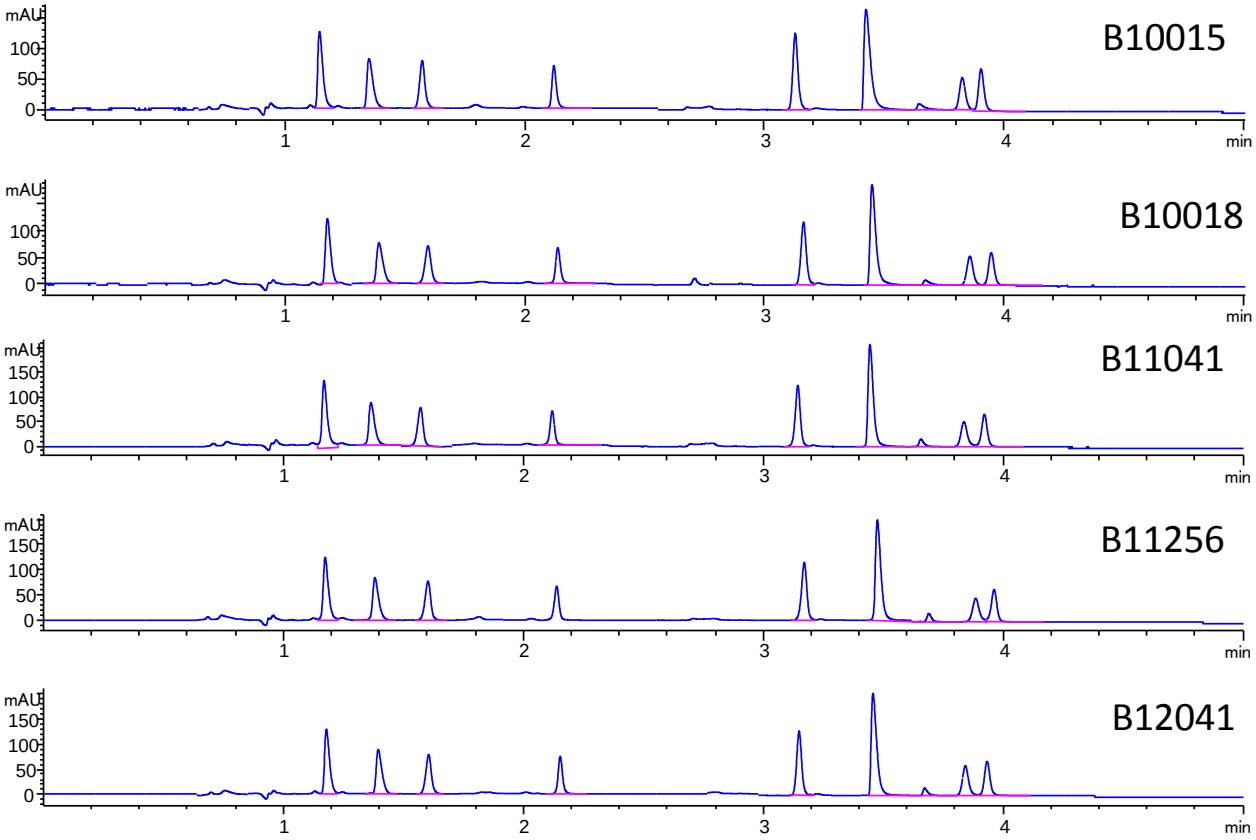
➢To avoid a potential pitfall with your guard column

- Test clean sample or standard with and without guard column in flow path
- Chromatography should be equivalent

"First, do no harm"

Lot-to-Lot Comparison of 5 Lots
Poroshell 120 EC-C18

2010



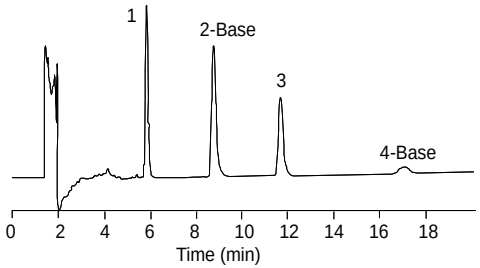
2012

A 20 mM Ammonium Acetate pH 4.80 adjusted with 20 mM Acetic Acid B: Acetonitrile, 30 C, Sig= 230 nm,4: ref 360,100
0.638 mL/ min 10 % to 40 % B over 4 min, 3 x100 mm Poroshell 120 EC-C18
2ul Ascorbic acid, acesulfane K, saccharin, caffeine, aspartame ,sorbic acid , quinine, dehydroacetic acid

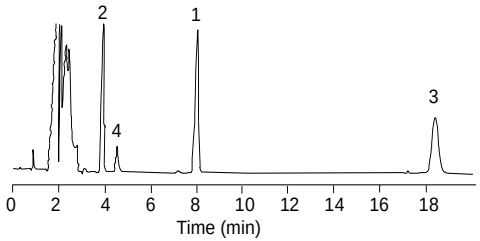
Agilent Poroshell 120 Columns for HPLC and UHPLC", March 15, 2013, Pub. No. 5990-5951EN; Brochure

Lot-to-Lot Selectivity Change Related to pH Choice

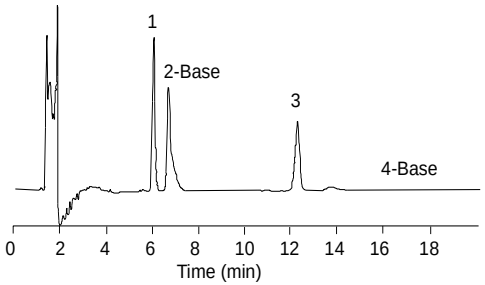
pH 4.5 - Lot 1



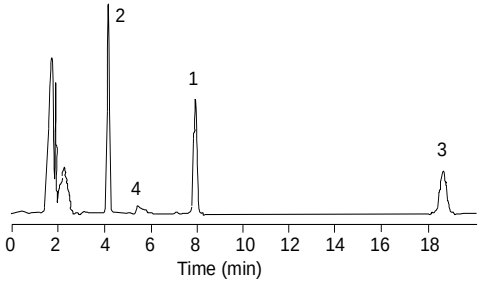
pH 3.0 - Lot 1



pH 4.5 - Lot 2



pH 3.0 - Lot 2



- pH 4.5 - selectivity change from lot-to-lot for basic compounds
- pH 3.0 - no selectivity change from lot-to-lot

- For Method Ruggedness
  - Test 3 different column lots
  - Compare R_s for the 3 lots
    - If ΔR_s is too large, modify method

New Method Validation Kits
Available May 2013
See appendix

Method Conditions

- Mobile phase
  - What's in your mobile phase and why
  - Don't believe everything you read; Focus of paper may not be chromatography
  - Microbial growth
  - Baseline
    - Drift
      - Additives, e.g., TFA
      - Detector, e.g., RI
    - Problems, http://www.chem.agilent.com/Library/Support/Documents/Baseline_problems.pdf
  - MP prep
  - Ghost Peaks
    - Sample
    - Mobile phase components; H_2O, solvent, additives
- pH
  - High
  - Low
- Temperature
  - Effect on Column
  - Effect on Sample
- Pressure
  - Sample/Sample matrix
  - Solvent viscosity, e.g., MeOH v. ACN

Microbial Growth

➢ Potential problems

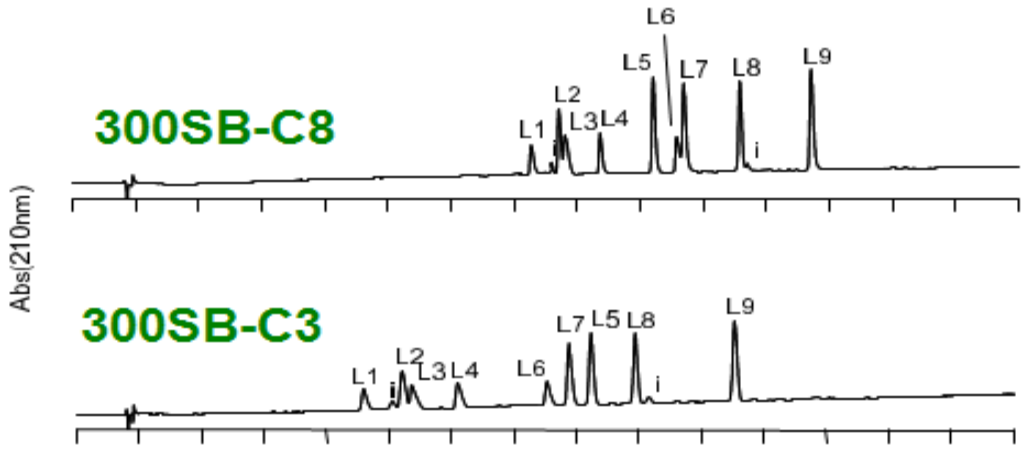
- Increased system pressure or pressure fluctuations
- Increased column pressure, premature column failure
- Can mimic application problems
- Gradient inaccuracies
- Ghost peaks
- Difficult to remove if gets in degasser and rest of system

➢ 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

Baseline Drift



Conditions:

Columns: ZORBAX 300SB, 4.6 x 150 mm, 5 µm
Mobile Phase: Gradient, 0 - 26% B in 30min.
A = 0.1% TFA in Water
B = 0.1% TFA in Acetonitrile
Temperature: 40°C
Sample: 2 µg of each peptide
Flow Rate: 1.0 mL / min.
Detection: UV-210nm

➢ Know the UV Cutoff of your mobile phase components

Mobile Phase Preparation

- HPLC grade or better
- Buffer preparation procedure
  - Be consistent
    - Document process
  - See appendix
- 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

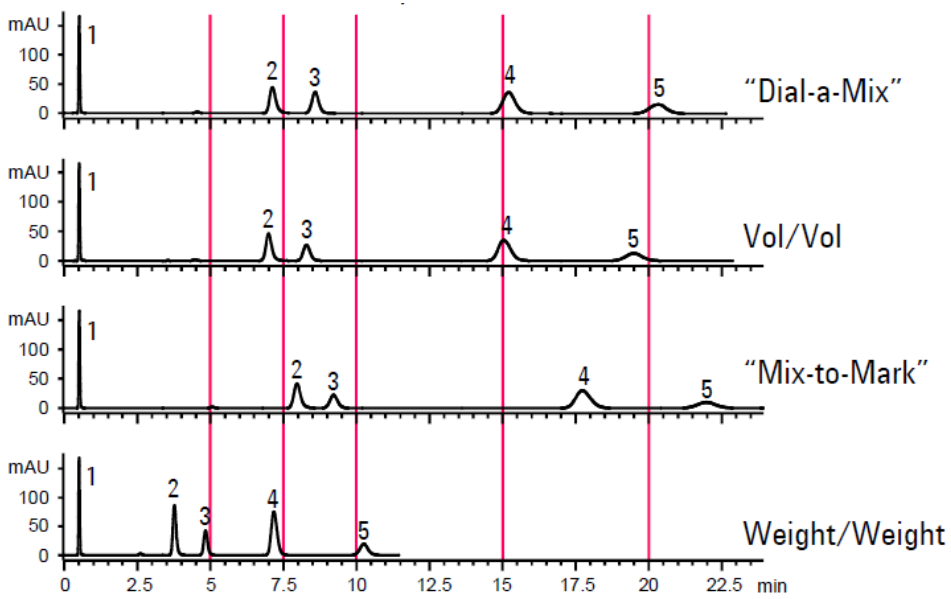
≠

500 ml H_2O added to 500 ml ACN

- ✓ Degree of contraction is affected by the relative quantities of each
- ✓ Temperature

- Small changes in mobile phase strength can have a large effective on retention
- To avoid this pitfall, be consistent and document your procedure

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

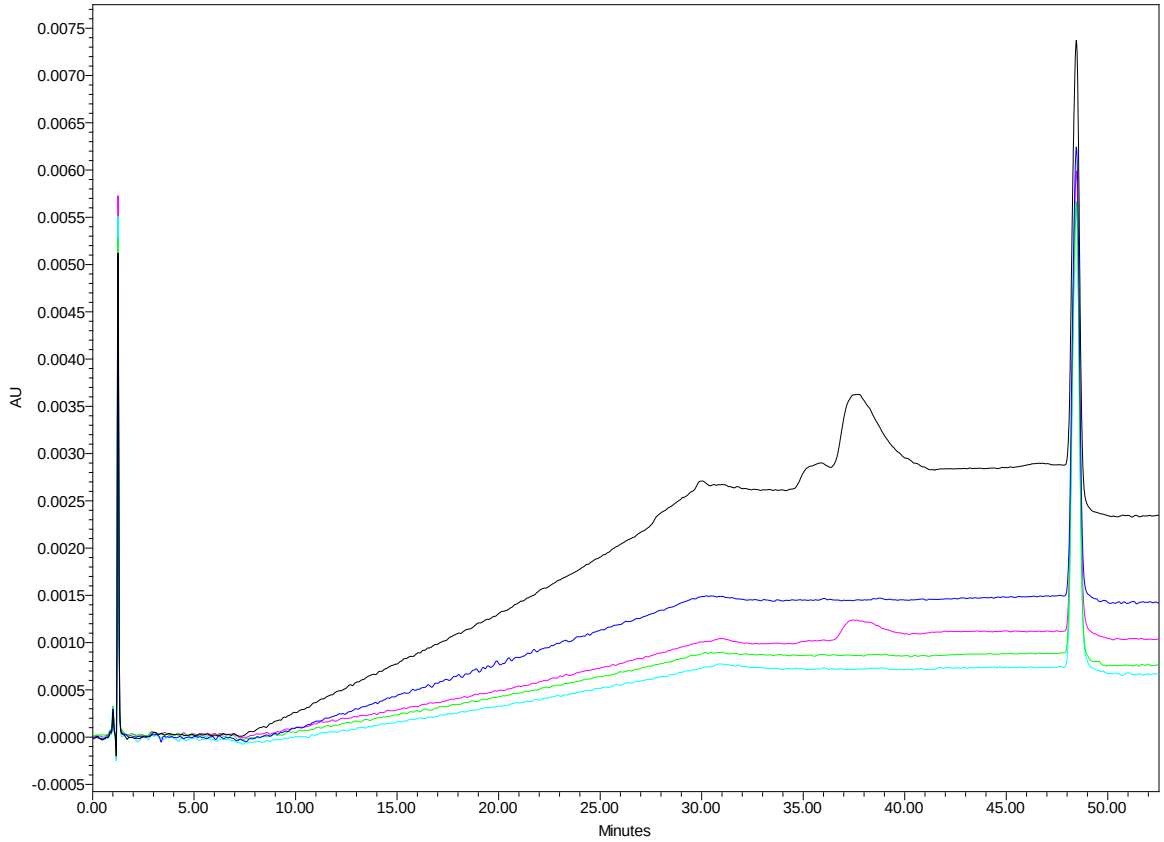
- Method used to prepare MP can significantly affect the elution pattern
- To avoid this potential pitfall
  - Be consistent
  - w/w is more accurate than v/v

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

Ghost Peaks
Where Do They Come From

- Organic
- Additives
  - TFA
  - Salts
- H2O
- Sample
- Other

Acetonitrile Comparison

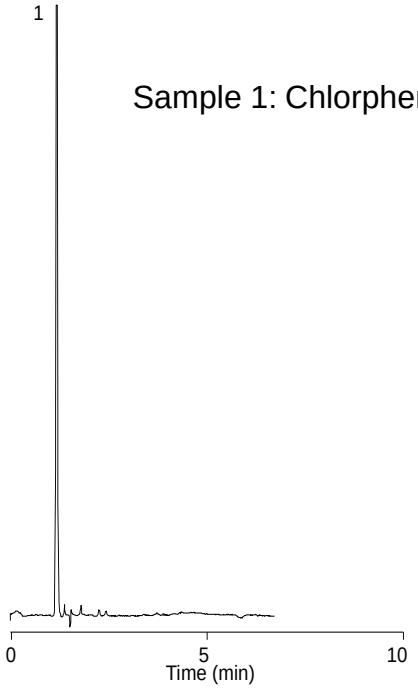


Multiple suppliers and lots of ACN tested

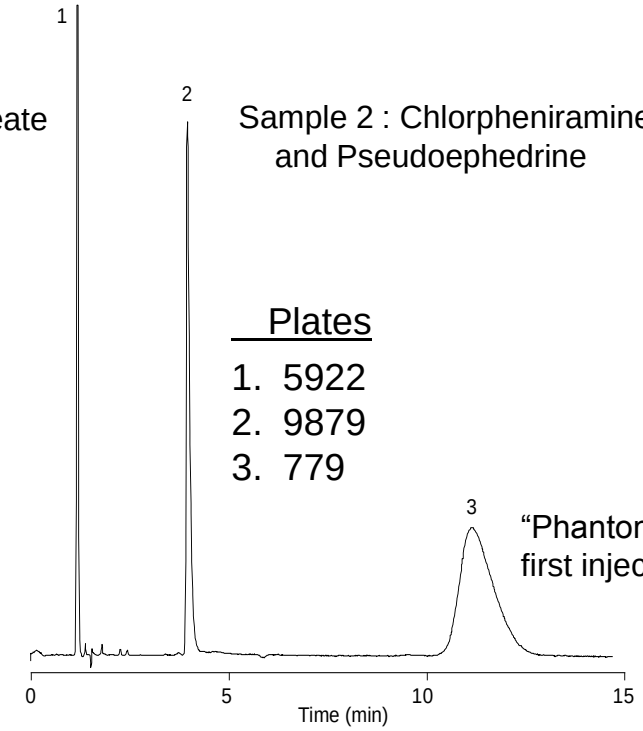
- Solvent - quality and consistency

Broad Peaks
Unknown “Phantom” Peaks

Column: Extend-C18, 4.6 x 150 mm, 5 μm Mobile Phase: 40% 10 mM TEA, pH 11 : 60% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 Sample: 1. Maleate 2. Pseudoephedrine 3. Chlorpheniramine



Sample 1: Chlorpheniramine maleate



Sample 2 : Chlorpheniramine maleate and Pseudoephedrine

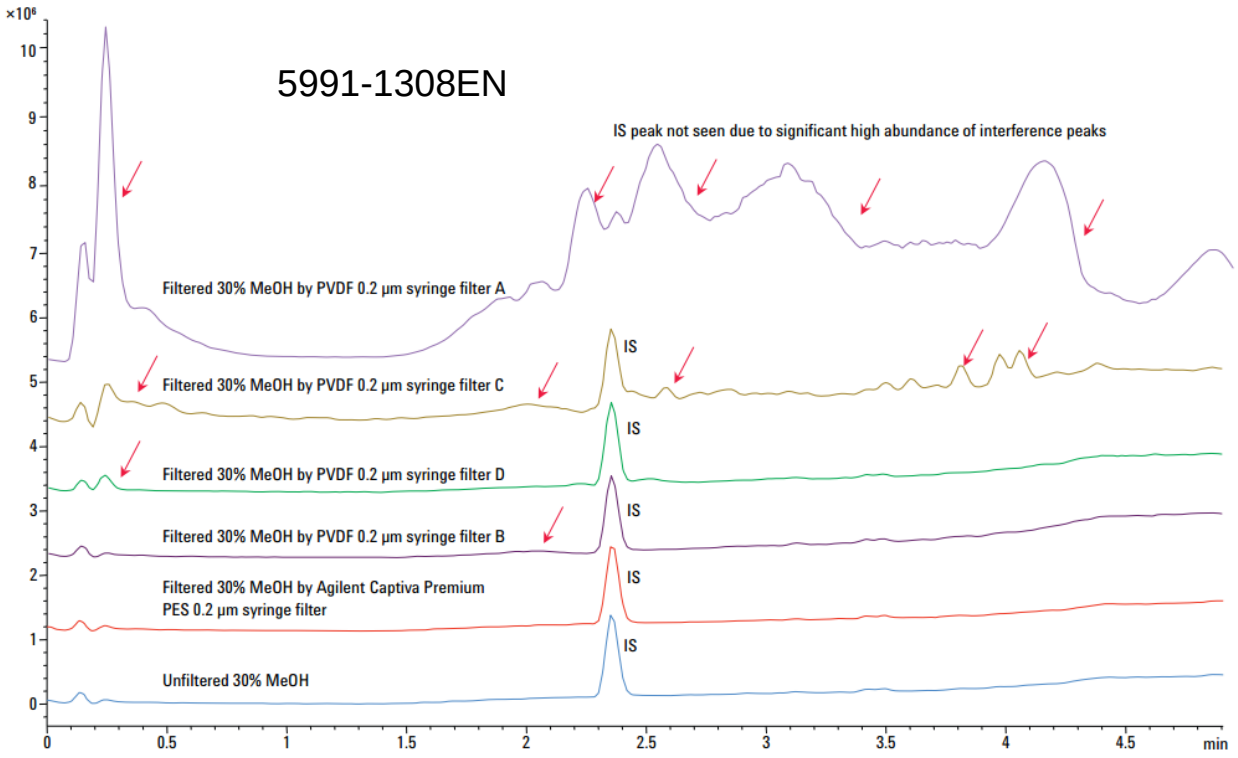
Plates

1. 5922
2. 9879
3. 779

“Phantom” peak from first injection

- Extremely low plates are an indication of an extremely late eluting peak from the preceding run
- Disproportionally broad peak can be an indicator too.
- Make sure everything has come off the column

Choosing the Best Syringe Filters



Filter cleanliness comparison of the Agilent Captiva Premium PES syringe filter with non-Agilent PVDF syringe filters using LC/MS under positive mode.

UHPLC:	Agilent 1290 Infinity LC System
Column:	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (p/n 959757-902)
Mobile phases:	A: H_2O + 0.01% formic acid (FA) B: Acetonitrile + 0.01% FA
Flow rate:	0.5 mL/min, gradient
Total run time:	5 min plus 1 min post run
Gradient:	Hold at 30% B for 1 min, then ramped to 90% B in 3 min, and hold at 90% B for 1 min
Injection volume:	8 µL
Internal standard:	50 µg/mL Naproxin
MS:	Agilent 6150 Single Quadrupole LC/MS System
Source:	ESI with Agilent Jet Stream Technology (AJS-ES)
Capillary voltage:	4,000 V
Nozzle voltage:	2,000 V
Drying gas flow:	12 L/min
Drying gas temp:	250 °C
Nebulizer pressure:	35 psig
Sheath gas flow:	3.0 L/min
Sheath gas temp:	150 °C
Mass range:	100 – 1350 *m/z*
Fragmentor:	150 V (pos), 80 V (neg)

- When selecting a syringe filter, make sure it is appropriate for the sample
- *And* make sure it does not add unwanted interferences

http://filtrationselectiontool.chem.agilent.com

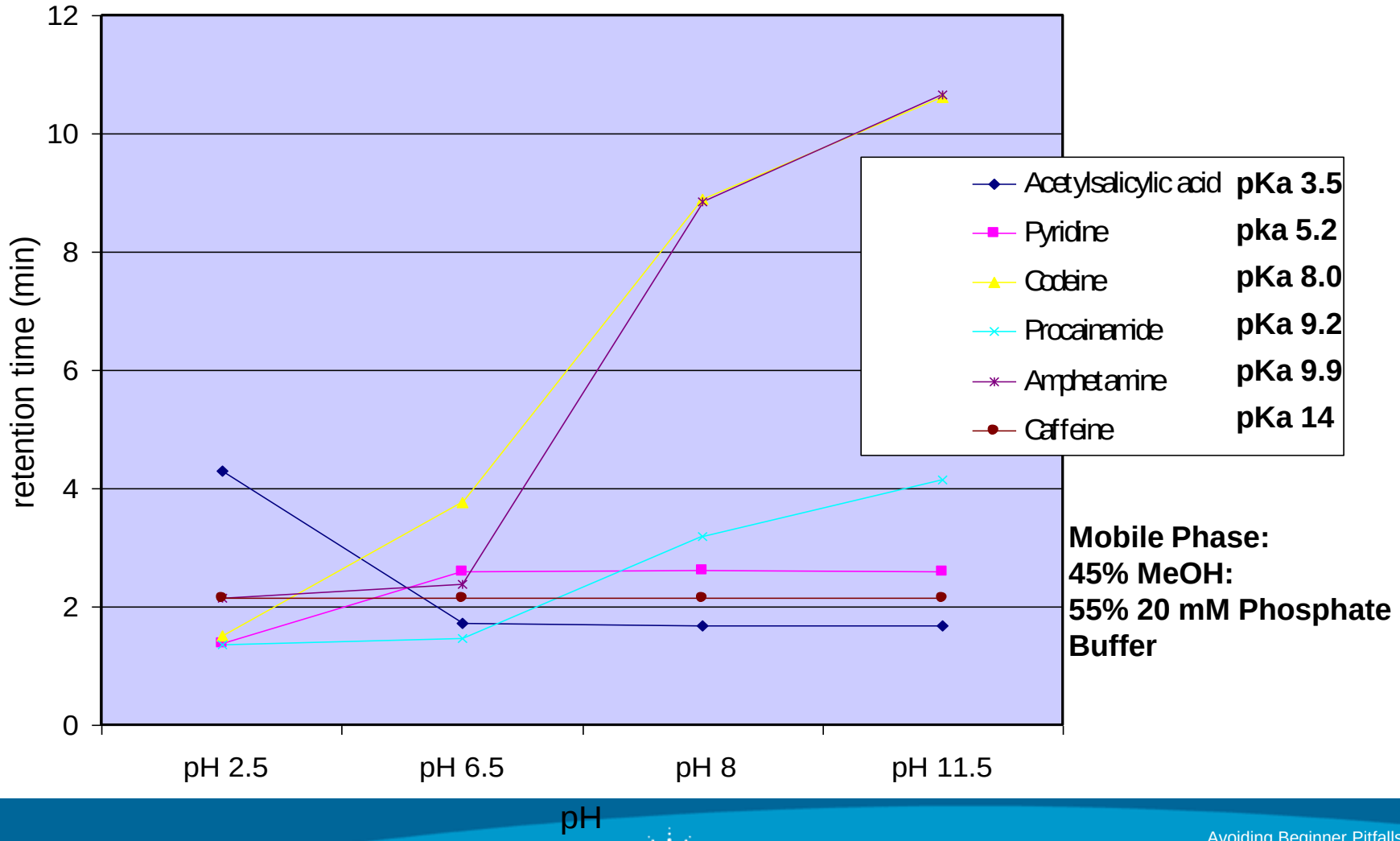
Captiva Filtration Selection Guide: 5991-1230EN

Method Conditions

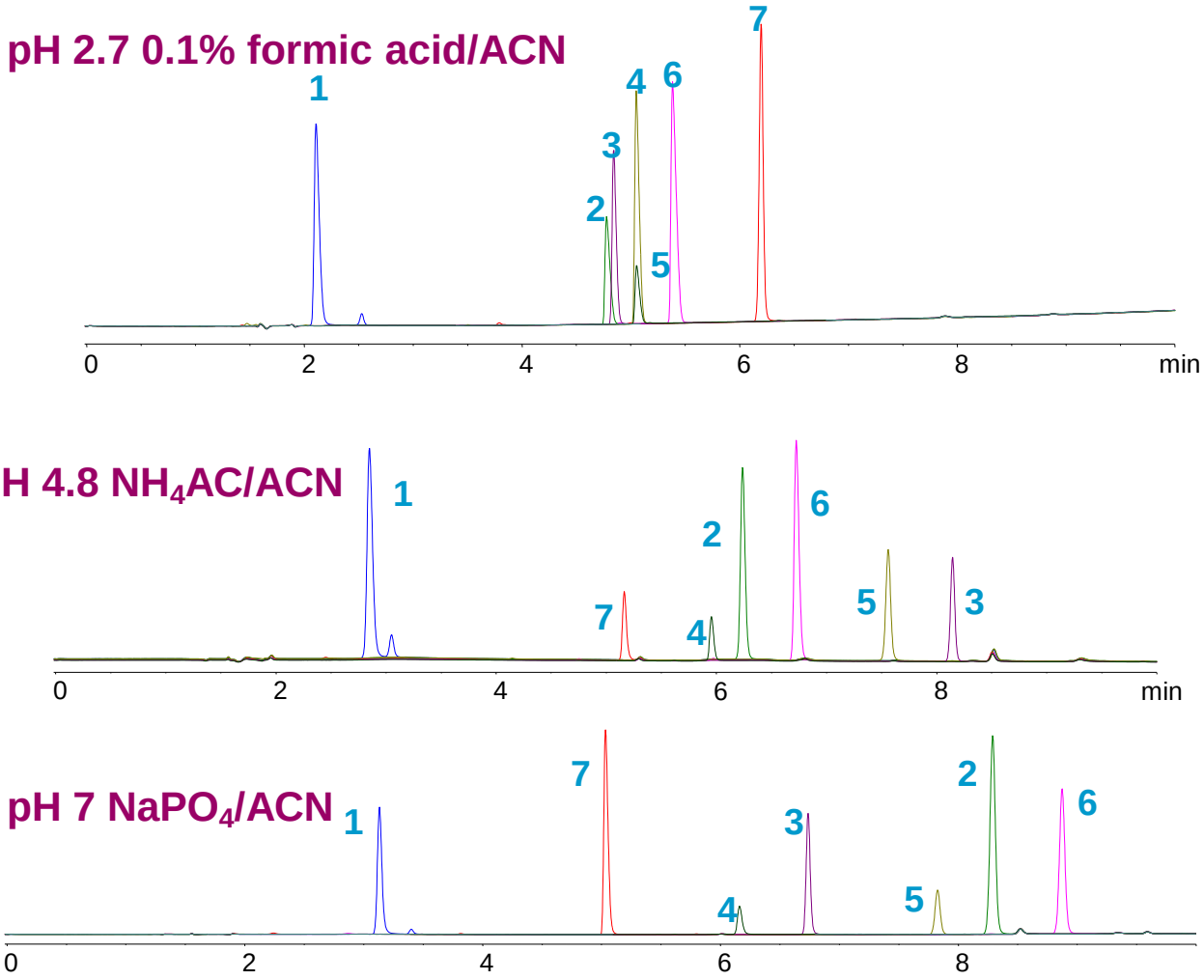
- Mobile phase
  - What's in your mobile phase and why
  - Don't believe everything you read; Focus of paper may not be chromatography
  - Microbial growth
  - Baseline
    - Drift
      - Additives, e.g., TFA
      - Detector, e.g., RI
    - Problems, http://www.chem.agilent.com/Library/Support/Documents/Baseline_problems.pdf
  - MP prep
  - Ghost Peaks
    - Sample
    - Mobile phase components; H_2O, solvent, additives
- pH
  - High
  - Low
- Temperature
  - Effect on Column
  - Effect on Sample
- Pressure
  - Sample/Sample matrix
  - Solvent viscosity, e.g., MeOH v. ACN

Change in Retention with pH for Ionizable Compounds is Compound Dependent

Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)



pH Can Affect Your Separation

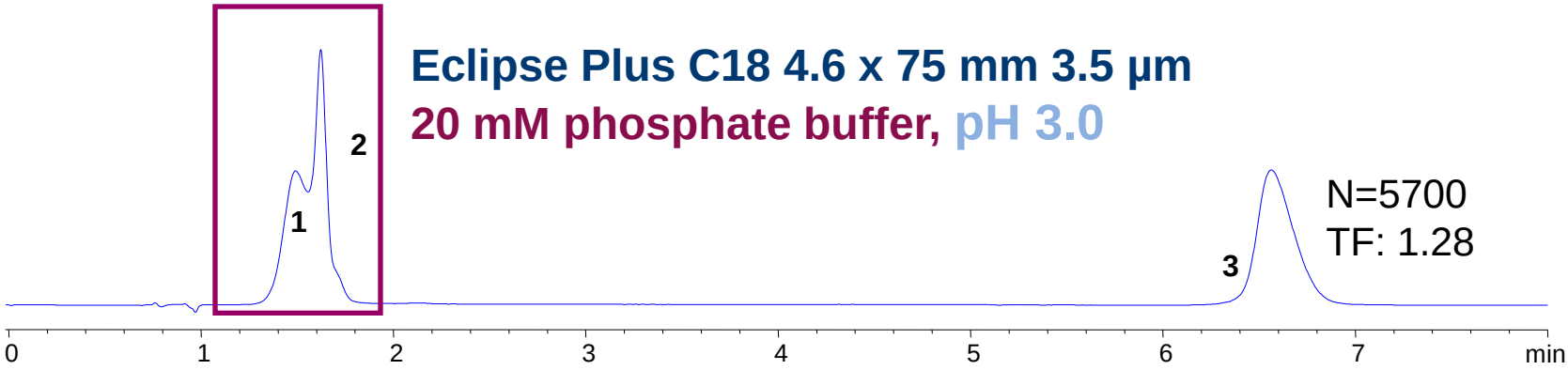


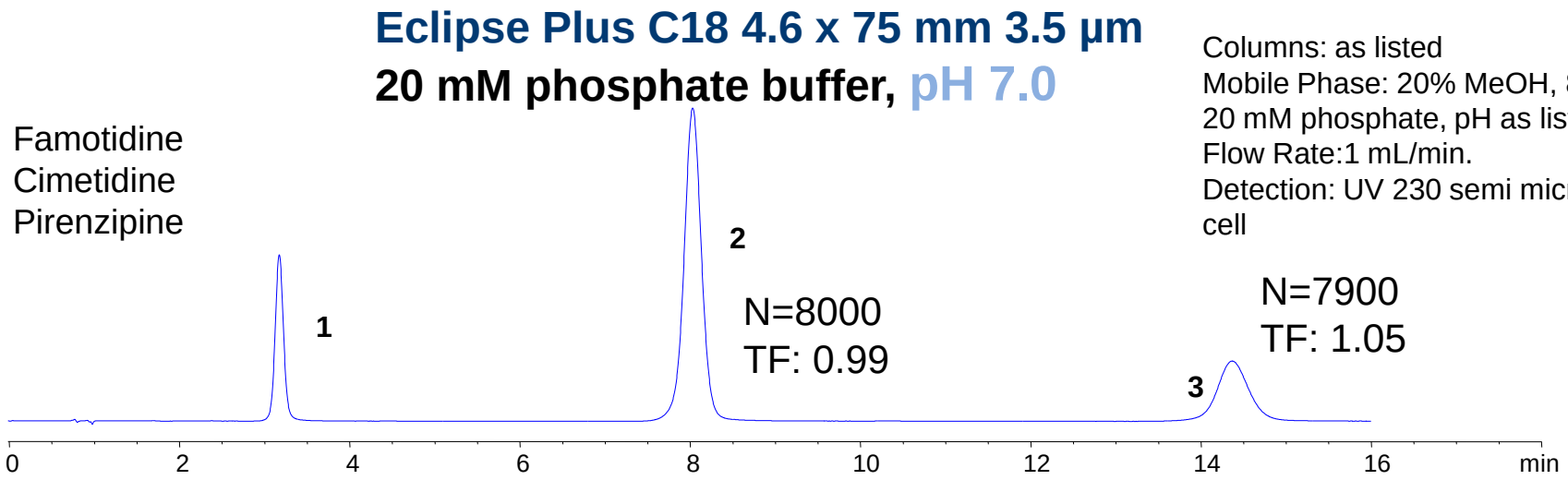
1. procainamide
2. buspirone
3. pioglitazone
4. eletriptan
5. dipyridamole
6. diltiazem,
7. furosemide

- **Selectivity and resolution can change with pH**
- **Eclipse Plus can be used with many mobile phases and pH's**

Conditions: Column: Eclipse Plus C18 4.6 x 100mm, 5um Gradient: 10 – 90% in 10 minutes Detection: UV 254 nm

Selectivity Differences at Mid pH Key to Resolution



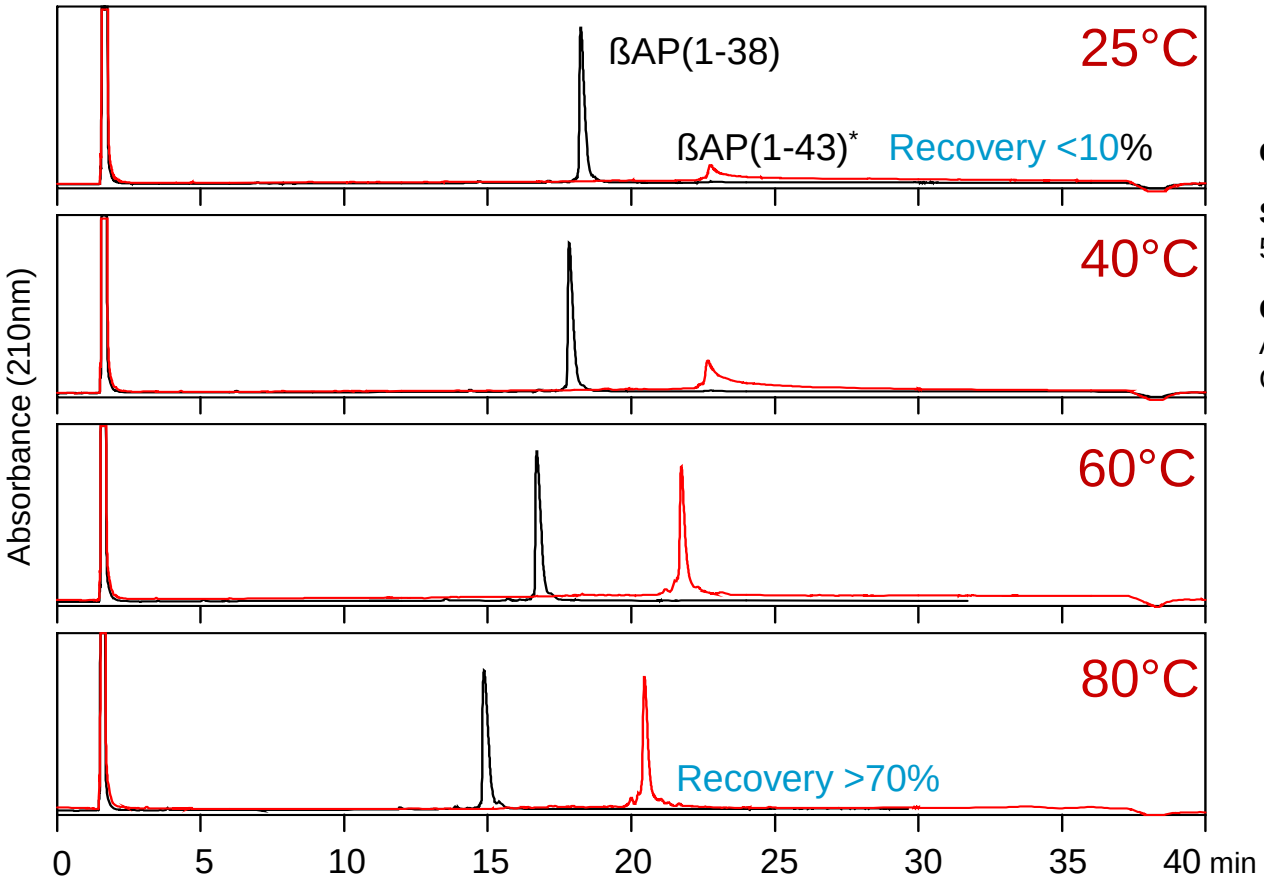


1. Famotidine
2. Cimetidine
3. Pirenzipine

Columns: as listed
Mobile Phase: 20% MeOH, 80% 20 mM phosphate, pH as listed
Flow Rate:1 mL/min.
Detection: UV 230 semi micro flow cell

- **For this sample resolution is not possible at pH 3, but is at pH 7**
- **Column may be fine but method conditions may not be optimal**

Temperature



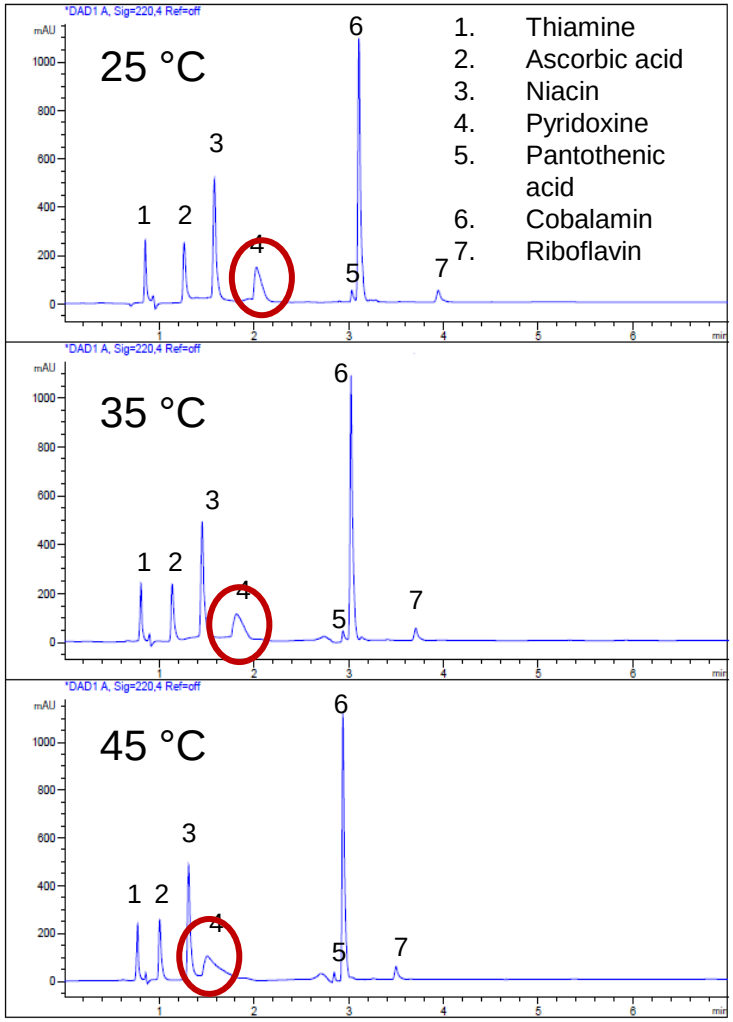
Column: ZORBAX 300 SB-C18, 4.6 x 150 mm

Sample: 10 µl injection,
5 µg peptide in 6M Urea/5% HOAc

Conditions: 1 ml/min
A=0.1%TFA in H2O, B=0.09%TFA in ACN
Gradient: 20-45% B / 35 min

- Changes k* and α*
- Potentially Improves Resolution (R)

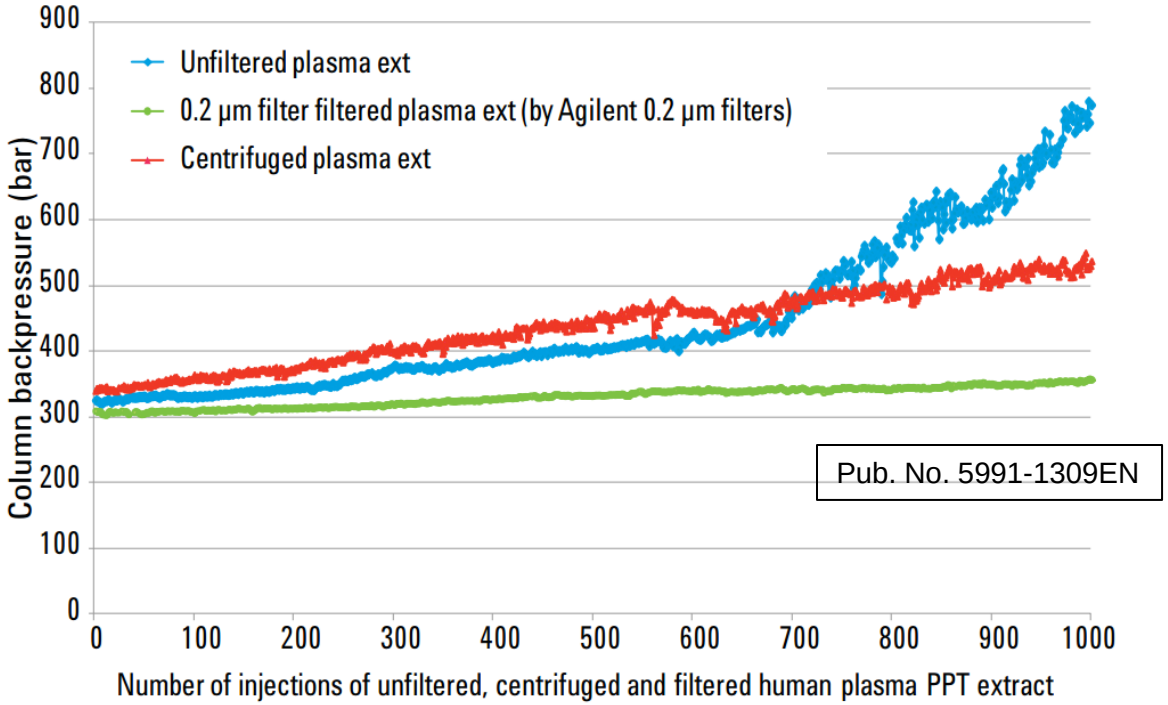
Temperature - Effect on Sample



Columns: Poroshell 120 EC-C18, 2.7 µm, 3.0 x 100 mm
Mobile Phase: A=10 mM ammonium formate, pH 3.8
B = methanol
Gradient: 0 min, 1% B; 0.53 min, 12% B; 0.55 min, 30% B; 3.8 min, 30% B
Flow Rate: 0.8 mL / min.

- Generally, increasing temperature improves peak shape
- But depending on the conditions, the unexpected can occur
- To avoid temperature related pitfalls, it is a good idea to try several different temperatures to see how it affects peak shape and retention
- Remember room temperature is not the same in every room!

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

- To help avoid this pitfall, use an appropriate 0.2 µm filter with all samples prior to injection

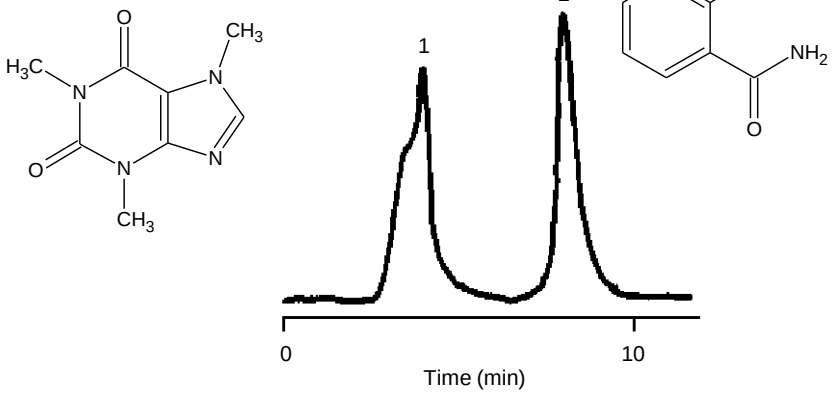
http://filtrationselectiontool.chem.agilent.com

Captiva Filtration Selection Guide: 5991-1230EN

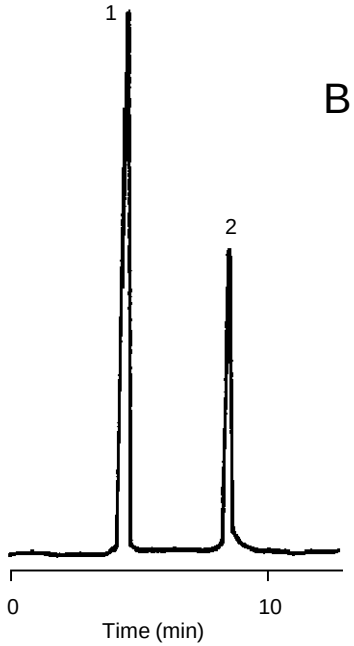
Split Peaks
Injection Solvent Effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 mm Mobile Phase: 82% H_2O : 18% ACN
Injection Volume: 30 mL Sample: 1. Caffeine 2. Salicylamide

A. Injection Solvent
100% Acetonitrile



B. Injection Solvent
Mobile Phase



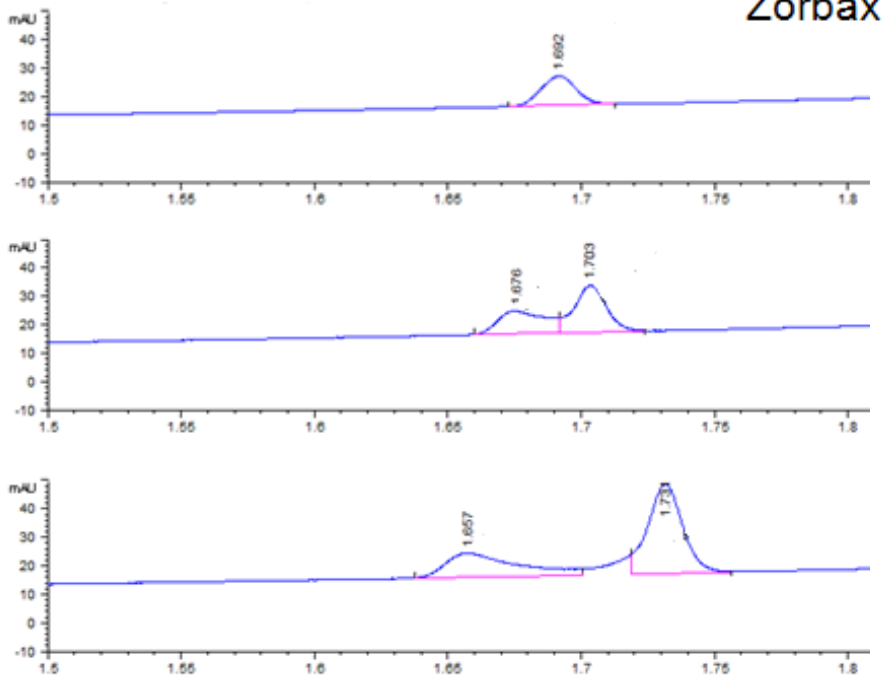
Injection Solvent Effects

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

- To avoid this pitfall
  - 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

Conclusion

Chromatography is a powerful tool but it's important to know, there can be pitfalls.

The more you understand about chromatography, the easier it is to recognize potential pitfalls and avoid them.

These are just a few of the potential pitfalls. Have you encountered other pitfalls or do you have an example of one we talked about today? If you have one you'd like to share for a future pitfalls talk, please send it to lc-column-support@agilent.com or rita_steed@agilent.com.

Thank You

Try a Poroshell 120 Column of Your Choice *for Free*

A special offer for attending today's e-Seminar

If you are interested, go to www.agilent.com/chem/poroshellinvitation

Your Agilent Sales Rep will contact you to assist with your column choice

We ask that you try it within 45 days and tell us about your experience

For more information on Poroshell 120 columns, see Pub. No. 5990-5951EN

For more information on other Agilent columns and consumables request a copy of the 2013 catalog, Pub. No. 5991-1065EN or view the on-line version of the catalog at http://www.chem.agilent.com/en-US/promotions/Pages/catalog.aspx

Appendix

Method Validation Kits - Available May 2013

PN	Description
959990-912K	Eclipse Plus Phe-Hex, 4.6x250mm, 5um, MVK
993967-902K	Eclipse XDB-C18, 4.6 x 150mm, 5um, MVK
990967-902K	Eclipse XDB-C18, 4.6 x 250mm, 5um, MVK
883975-902K	SB-C18, 4.6 x 150mm, 5um, MVK
880975-902K	SB-C18, 4.6 x 250mm, 5um, MVK
963967-902K	Eclipse XDB-C18, 4.6 x 150mm, 3.5um, MVK
959963-912K	Eclipse Plus Ph-Hex,4.6x150mm,3.5um,MVK
863953-902K	SB-C18, 4.6 x 150mm, 3.5um, MVK
695775-902K	Poroshell 120 EC-C18,2.1x100mm,2.7um,MVK
699775-902K	Poroshell 120 EC-C18,2.1x50mm,2.7um,MVK
699975-302K	Poroshell 120 EC-C18,3.0x50mm,2.7um,MVK

PN	Description
695975-902K	Poroshell 120 EC-C18,4.6x100mm,2.7um,MVK
699975-902K	Poroshell 120 EC-C18,4.6x50mm,2.7um,MVK
827975-902K	RRHT SB-C18, 4.6 x 50mm, 1.8um, MVK
959941-902K	RRHT Eclipse Plus C18, 4.6 x 50mm, 1.8um, MVK
927975-902K	RRHT Eclipse XDB-C18, 4.6 x 50mm, 1.8um, MVK
959758-902K	RRHD Eclipse Plus C18,2.1x100,1.8um,MVK
959757-902K	RRHD Eclipse Plus C18,2.1x50mm,1.8um,MVK
981758-902K	RRHD Eclipse XDB-C18,2.1x100mm,1.8um,MVK
981757-902K	RRHD Eclipse XDB-C18,2.1x50mm,1.8um,MVK
858700-902K	RRHD SB-C18, 2.1 x 100mm, 1.8um, MVK
857700-902K	RRHD SB-C18, 2.1 x 50mm, 1.8um, MVK

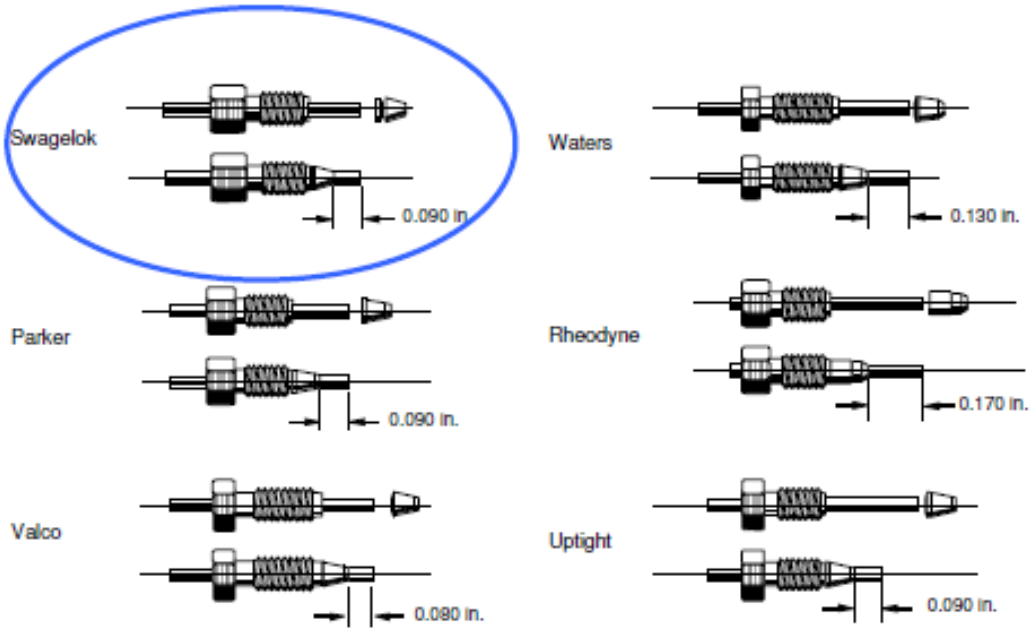
More kits coming Summer 2013

Method Development Kits

PN	MD Kits	Description
5190-6160	**P120, USP Method Dev Kit, 3.0x100mm**	Poroshell 120 EC-C18, EC-C8, EC-CN columns, 3.0 x 100 mm
5190-6159	**P120, USP Method Dev Kit, 4.6x100mm**	Poroshell 120 EC-C18, EC-C8, EC-CN columns, 4.6 x 100 mm
5190-6155	**P120, Selectivity Meth Dev, 2.1x50mm**	Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm
5190-6156	**P120, Selectivity Meth Dev, 4.6x50mm**	Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 4.6 x 50 mm
5190-6157	**P120, Aqueous Meth Dev Kit, 2.1x50mm**	Poroshell 120 Sb-Aq, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm
5190-6158	**P120, Aqueous Meth Dev Kit, 4.6x50mm**	Proshell 120 SB-Aq, Phenyl-Hexyl, and Bonus RP columns, 4.6 x 50 mm
5190-6153	**RRHD Eclipse Plus Meth Dev Kit, 2.1mm ID**	RRHD Eclipse Plus C18, Eclipse Plus C8, Eclipse Plus Phenyl-Hexyl, 2.1 x 50 mm columns
5190-6154	**RRHD Aqueous Method Dev Kit, 2.1mm ID**	RRHD SB-Aq, Bonus RP, and Eclipse Plus Phenyl-Hexyl columnc, 2.1 x 50 mm
5190-6152	**RRHD pH Method Dev Kit, 2.1mm ID**	RRHD StableBond SB-C18, Eclipse Pluse C18, and Extend-C18 column, 2.1 x 50 mm
5190-6160	**P120, USP Method Dev Kit, 3.0x100mm**	Poroshell 120 EC-C18, EC-C8, EC-CN columns, 3.0 x 100 mm
5190-6159	**P120, USP Method Dev Kit, 4.6x100mm**	Poroshell 120 EC-C18, EC-C8, EC-CN columns, 4.6 x 100 mm
5190-6155	**P120, Selectivity Meth Dev, 2.1x50mm**	Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm
5190-6156	**P120, Selectivity Meth Dev, 4.6x50mm**	Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 4.6 x 50 mm
5190-6157	**P120, Aqueous Meth Dev Kit, 2.1x50mm**	Poroshell 120 Sb-Aq, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm

PN	MD Kits	Description
5190-6158	**P120, Aqueous Meth Dev Kit, 4.6x50mm**	Proshell 120 SB-Aq, Phenyl-Hexyl, and Bonus RP columns, 4.6 x 50 mm
5190-6153	**RRHD Eclipse Plus Meth Dev Kit, 2.1mm ID**	RRHD Eclipse Plus C18, Eclipse Plus C8, Eclipse Plus Phenyl-Hexyl, 2.1 x 50 mm columns
5190-6154	**RRHD Aqueous Method Dev Kit, 2.1mm ID**	RRHD SB-Aq, Bonus RP, and Eclipse Plus Phenyl-Hexyl columnc, 2.1 x 50 mm
5190-6152	**RRHD pH Method Dev Kit, 2.1mm ID**	RRHD StableBond SB-C18, Eclipse Pluse C18, and Extend-C18 column, 2.1 x 50 mm
5190-6160	**P120, USP Method Dev Kit, 3.0x100mm**	Poroshell 120 EC-C18, EC-C8, EC-CN columns, 3.0 x 100 mm
5190-6159	**P120, USP Method Dev Kit, 4.6x100mm**	Poroshell 120 EC-C18, EC-C8, EC-CN columns, 4.6 x 100 mm
5190-6155	**P120, Selectivity Meth Dev, 2.1x50mm**	Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm
5190-6156	**P120, Selectivity Meth Dev, 4.6x50mm**	Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 4.6 x 50 mm
5190-6157	**P120, Aqueous Meth Dev Kit, 2.1x50mm**	Poroshell 120 Sb-Aq, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm
5190-6158	**P120, Aqueous Meth Dev Kit, 4.6x50mm**	Proshell 120 SB-Aq, Phenyl-Hexyl, and Bonus RP columns, 4.6 x 50 mm
5190-6153	**RRHD Eclipse Plus Meth Dev Kit, 2.1mm ID**	RRHD Eclipse Plus C18, Eclipse Plus C8, Eclipse Plus Phenyl-Hexyl, 2.1 x 50 mm columns
5190-6154	**RRHD Aqueous Method Dev Kit, 2.1mm ID**	RRHD SB-Aq, Bonus RP, and Eclipse Plus Phenyl-Hexyl columnc, 2.1 x 50 mm
5190-6152	**RRHD pH Method Dev Kit, 2.1mm ID**	RRHD StableBond SB-C18, Eclipse Pluse C18, and Extend-C18 column, 2.1 x 50 mm
5190-6155	**P120, Selectivity Meth Dev, 2.1x50mm**	Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm
5190-6157	**P120, Aqueous Meth Dev Kit, 2.1x50mm**	Poroshell 120 Sb-Aq, Phenyl-Hexyl, Bonus RP columns, 2.1 x 50 mm
5190-6153	**RRHD Eclipse Plus Meth Dev Kit, 2.1mm ID**	RRHD Eclipse Plus C18, Eclipse Plus C8, Eclipse Plus Phenyl-Hexyl, 2.1 x 50 mm columns
5190-6154	**RRHD Aqueous Method Dev Kit, 2.1mm ID**	RRHD SB-Aq, Bonus RP, and Eclipse Plus Phenyl-Hexyl columnc, 2.1 x 50 mm
5190-6152	**RRHD pH Method Dev Kit, 2.1mm ID**	RRHD StableBond SB-C18, Eclipse Pluse C18, and Extend-C18 column, 2.1 x 50 mm

HPLC Fittings



Troubleshooting LC Fittings Part II,
J.W. Dolan and P. Upchurch, LC/GC Magazine 6:788 (1988)

Stainless Steel and Polymer Fittings

Stainless steel fittings – can be used anywhere and are especially popular for highest pressure connections

Agilent uses Swagelok type fittings with front and back ferrules, which give best sealing performance throughout our LC system (use this on the instrument connections, i.e. valves, heaters etc)

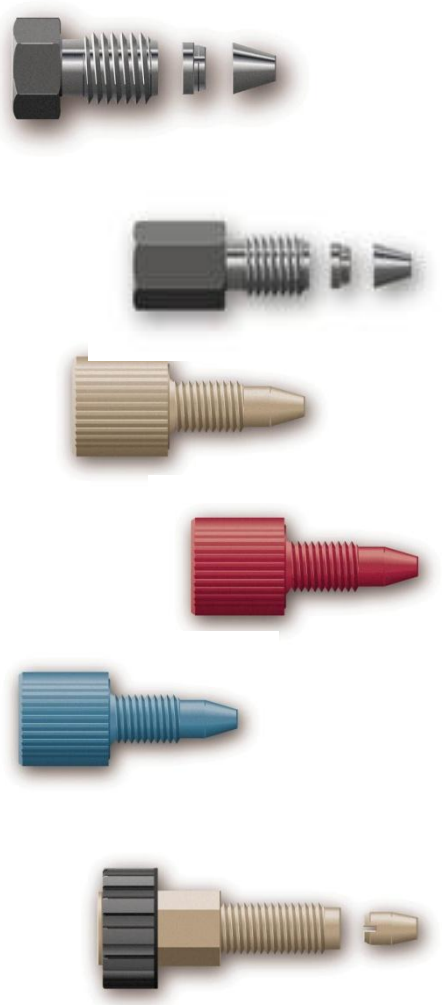
PEEK fittings (< 400 bar) are most popular when:

- Connections are changed frequently, i.e. connecting columns
- Bio-compatibility is needed
- Pressure is less critical

Polyketone fittings can be used up to 600 bar

- Use this fitting on column connections with Poroshell 120 (PN 5042-8957)

New 1200 bar removable fittings are available for UHPLC type systems



Some typical column connectors shown here

New 1200 Bar Removable Fittings

Part Number	Description	Picture
5067-4733	1200 Bar Removable Fitting	
5067-4738	1200 Bar Removable Long Fitting	
5067-4739	1200 Bar Removable Extra Long Fitting	

Fitting Description: Stainless steel screw, an internal stainless steel ferrule, and a front ferrule in PEEK

Where to Use It: **Anywhere in the flow path**
Ideal for the connection between the heat exchanger and the column because it can be re-used without losing tightness.
On competitive instruments

What Does it Replace: The standard stainless steel Swagelock fitting

Why: Because the heat exchanger had to be replaced when changing the column if the non-removable Swagelok fitting was used

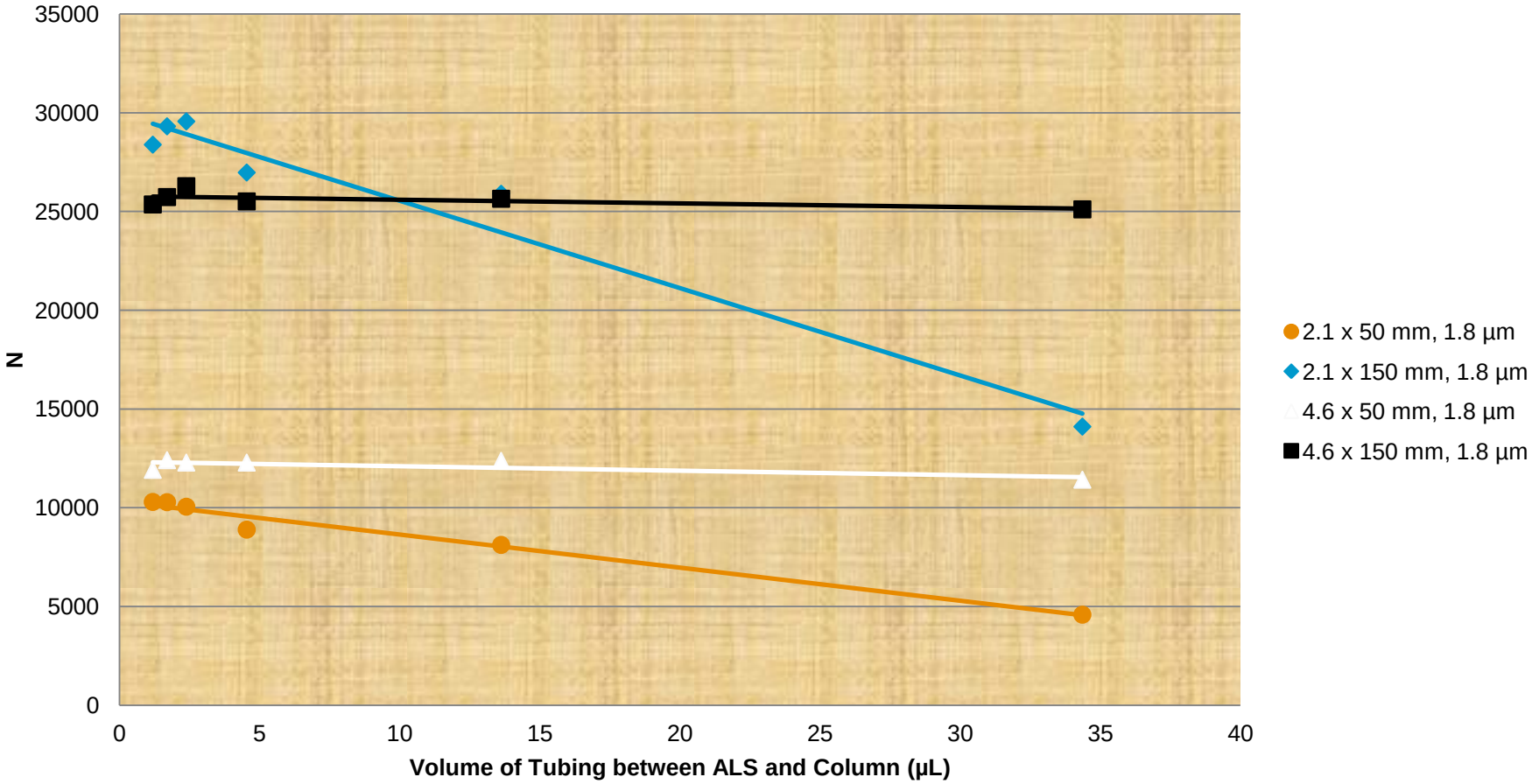
Use of 0.12 mm Tubing instead of 0.17 mm Tubing Reduces extra column volume by half.

Inside Diameter (mm)	Length (mm)	Material	Color	Connections	Part Number	Volume (ul)
0.12	180	SS	Red	1 end pre-swaged	G1313-87304	2.0
0.12	280	SS	Red	1 end pre-swaged	01090-87610	3.2
0.12	105	SS	Red	1 end pre-swaged	01090-87611	1.2
0.12	150	SS	Red	pre-swaged	G1315-87312	1.7
0.12	105	SS	Red	Without fittings	5021-1820	1.2
0.12	150	SS	Red	Without fittings	5021-1821	1.7
0.12	280	SS	Red	Without fittings	5021-1822	3.2
0.12	400	SS	Red	Without fittings	5021-1823	4.5
0.17	180	SS	Green	1 end pre-swaged	G1313-87305	4.1
0.17	280	SS	Green	1 end pre-swaged	01090-87304	6.4
0.17	130	SS	Green	1 end pre-swaged	01090-87305	2.9
0.17	90	SS	Green	1 end pre-swaged	G1316-87300	2.0
0.17	105	SS	Green	Without fittings	5021-1816	2.4
0.17	150	SS	Green	Without fittings	5021-1817	3.4
0.17	280	SS	Green	Without fittings	5021-1818	6.4
0.17	400	SS	Green	Without fittings	5021-1819	9.1

Use Lower Volume RED Tubing When Possible

GREEN Tubing Has 2x Volume Of RED TUBING of Same Length

2.1 mm columns show a significant efficiency dependence on extra-column volume



The above scatter plots compare the effects of column length and internal diameter on extra column volume; length has only a slight effect, while internal diameter is greatly affected by extra-column volume.

Comparison of alpha values from proportioned and premixed mobile phase

Column Type	Alpha of peaks 5,6 proportioned	Alpha of peaks 5,6 premixed
5um	2.70	2.71
3.5um	2.75	2.74
1.8um	2.88	2.74

Selectivity changes from column to column and lot-to-lot are the hardest to resolve.
Problems can be more than just the column.
Increasing pressure can make problems harder to troubleshoot.
Proportion of mobile phase changes with pressure.

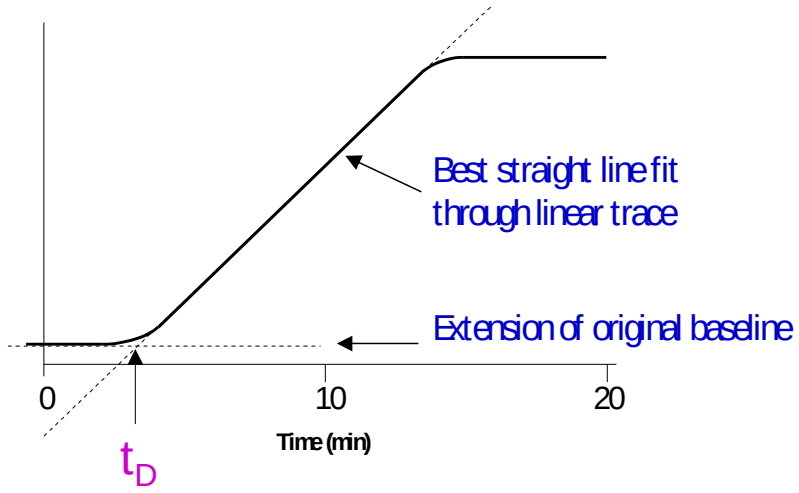
Determining the Dwell Volume (System Equilibration Volume) For Your HPLC System

- Replace column with Zero Dead Volume (ZDV) union
- Prepare mobile phase components

  A = methanol
  B = methanol with 0.2% v/v acetone

- Monitor at 254 nm
- Run gradient from 0 to 100%B in 10 minutes
- Use flow rate appropriate with column and HPLC system
  - Standard 1100 binary or quaternary system
    - 4.6 mm (e.g., 1 mL/min),
  - Capillary HPLC system plumbed for
    - 0.5 mm capillary column (10 - 20 µL/min)
    - 0.3 mm capillary column (3 – 5 µL/min)

$V_D = t_D \times F$



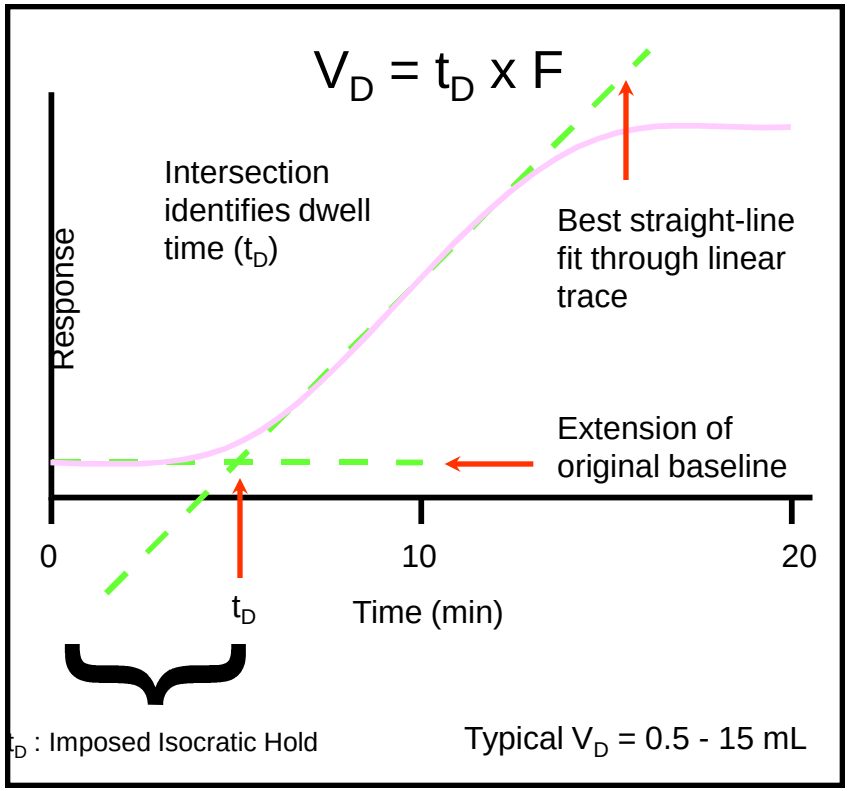
- Intersection of the two lines identifies dwell time (t_D)
- Dwell volume is equal to product of the flow rate and the dwell time.

Measuring Dwell Volume

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

Dwell Volume Impact

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



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

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

Column Cleaning

Flush with stronger solvents than your mobile phase.

Use at least 25 mL of each solvent for analytical (4.6 mm) columns

Reversed-Phase Column Flush Solvent Choices
(in order of increasing strength)

1. Mobile phase minus any buffer salt(s)
2. 100% Methanol
3. 100% Acetonitrile
4. 75% Acetonitrile:25% Isopropanol
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.**

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.

Injection Volumes for Different ID's

Column ID	Column Volume	Peak Volume, k=1	Typical Injection Volume	Typical Inj Vol Range
4.6 mm	1500 µL	148 µL	20 µL	5 – 50 µL
3.0 mm	640 µL	44 µL	10 µL	3 – 30 µL
2.1 mm	320 µL	22 µL	2 µL	0.5 – 15 µL
1.0 mm	70 µL	4 µL	0.5 µL	0.1 – 3 µL
0.5 mm	15 µL	1 µL	150 nL	40 – 500 nL
0.3 mm	6 µL	0.3 µL	50 nL	15 – 250 nL
0.1 mm	700 nL	32 nL	10 nL	1 – 10 nL
0.075 mm	400 nL	18 nL	2 nL	0.5 – 5 nL

Column length = 150 mm, N =13,000

~Column Volume = 3.14 x (Column ID/2)^2 x Column Length x 0.60

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