HPLC Column and Separation Troubleshooting

What Every HPLC User Should Know

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Columns & Consumables
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Trouble Shooting Steps

- Recognize the Problem
- When Did the System Last Function Properly?
- Has Anything Been Changed?
- Is The Procedure Being Followed Properly?
- Review Method for Compliance

HPLC Components

- Pump
- Injector/Autosampler
- **Column**
- Detector
- Data System/Integrator

Problems Can Be Related to All Components

Categories of Column Problems

A. Pressure

B. Peak shape

C. Retention

Pressure Issues

Column Observations	Potential Problems
High pressure	- Plugged frit
	- Column contamination
	- Plugged packing
Low Pressure	- Leak
	- Flow Incorrect

Determining the Cause and Correcting High Back Pressure

- Check pressure with/without column - many pressure problems are due to blockages in the system or at the guard

<u>If Column pressure is high:</u>

- Back flush column – Clear plugged frit
- Wash column – Eliminate column contamination and plugged packing
  - high molecular weight/adsorbed compounds
  - precipitate from sample or buffer
- Change frit – Clear plugged frit

Column Cleaning

Flush with stronger solvents than your mobile phase.

Reversed-Phase Solvent Choices
in Order of Increasing Strength

Use at least 25 mL of each solvent for analytical columns

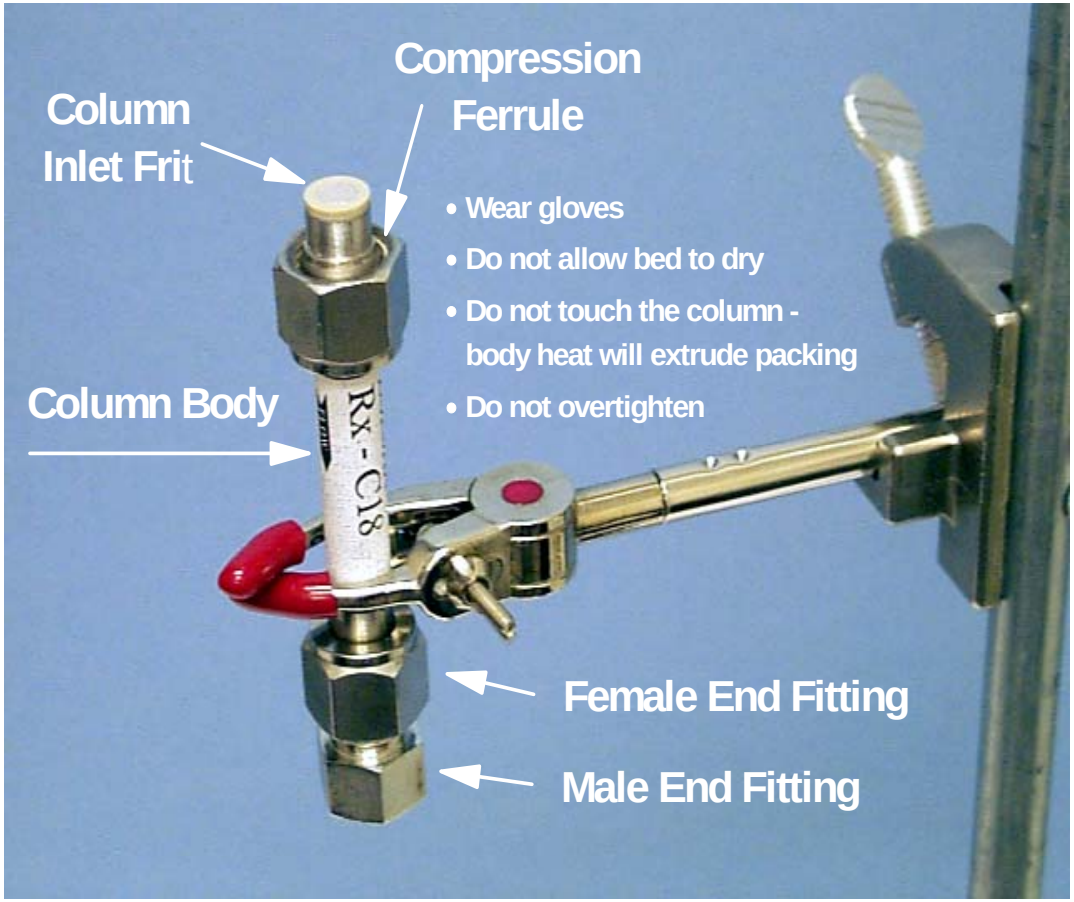
- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile
- 75% Acetonitrile:25% Isopropanol
- 100% Isopropanol
- 100% Methylene Chloride*
- 100% Hexane*

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

How to Change a Frit

May not be possible with new generation columns

May damage very high performance columns

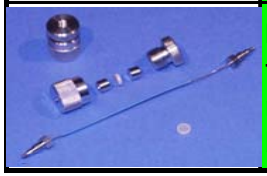


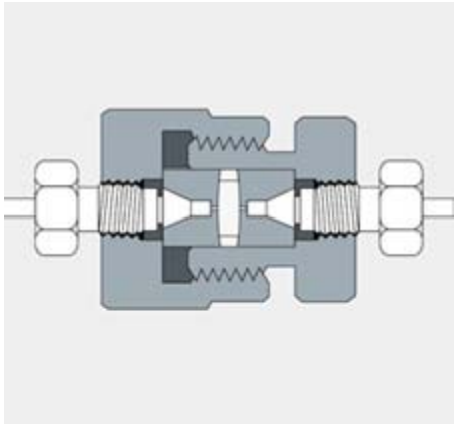
Preventing Back Pressure Problems Is A Better Choice!

- Use column protection
  - In-line filters
    - Guard columns
- Filter samples
- Sample clean-up (i.e. SPE)
- Appropriate column flushing
- Filter buffered mobile phases

In-Line Filters Provide Good Insurance Against System OverPressure

NEW RRLC In-line filter and fitting – max 600 bar

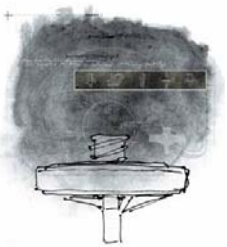
	Description	Part number	Porosity	Frit diameter	Flow rate	Part number Replacement Frits
	**RRLC In-line filter, 2.1 mm, max 600 bar**	**5067-1551**	0.2 µm	2.1 mm	<1 mL/min	**5067-1555 (10/pk)**
	**RRLC In-line filter, 3.0 & 4.6 mm, max 600 bar**	**5067-1553**	0.2 µm	4.6 mm	1 - 5 mL/min	**5067-1562 (10/pk)**

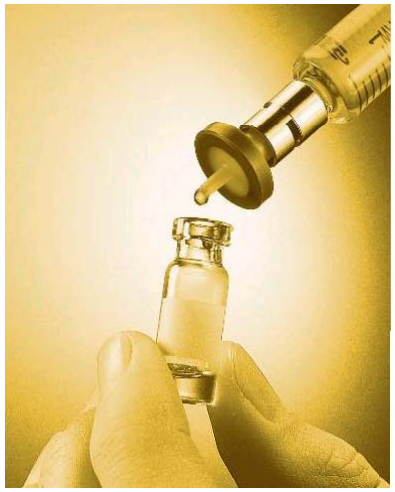


Protect RRHT columns with efficient in-line filter with 0.2 µm pore size frits

Why Filter the Sample?

High Performance Requires Better Sample “Hygiene”





Prevents blocking of capillaries, frits, and the column inlet

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

Results in less downtime of the instrument for repairs

Produces improved analytical results by removing potentially interfering contamination

Choice of Membranes

Regenerated Cellulose (RC)

- Universal hydrophilic membrane, compatible with most solvents - aqueous and organic
- High purity, extremely low extractables and lowest protein binding

PTFE

- Good chemical compatibility with aqueous and organic solvents as well as for acids and bases
- Hydrophobic

Nylon

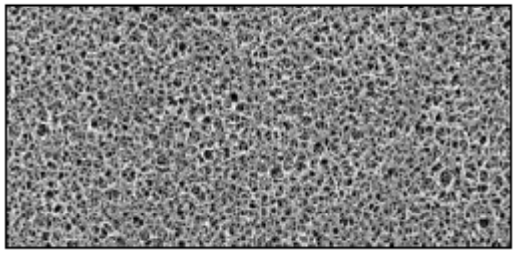
- Universal filter for aqueous and organic solutions
- Hydrophilic

Cellulose Nitrate

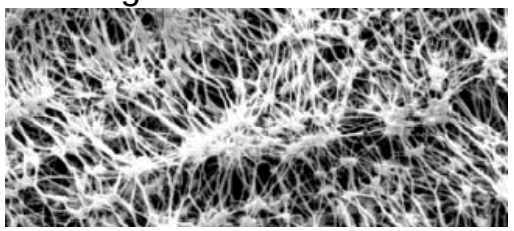
- Compatible with many, but not all, aqueous or non-aqueous solvents
- Most commonly used as a pre-filter

Cellulose Acetate (CA)

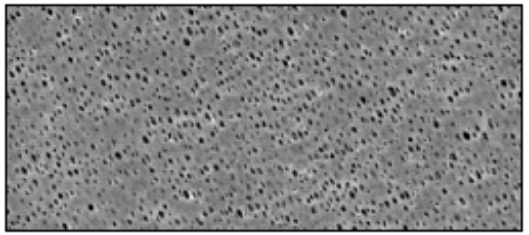
- Only compatible with aqueous solvents
- Used for proteins and protein-related samples



Regenerated Cellulose



PTFE



Nylon

Magnified views showing structural differences of some membrane types

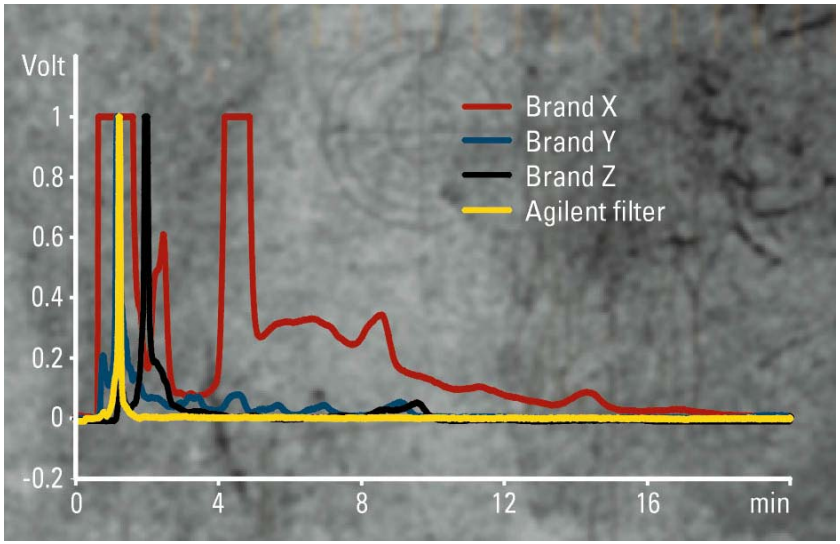
Choose the Right Size for Your Sample Volume

Diameter [mm]	Sample volume [ml]	Hold up volume [µl]	Effective filter area [cm²]
30	1–50	< 50	5.1
25	1–30	< 30	3.5
13	1–10	< 10	0.75

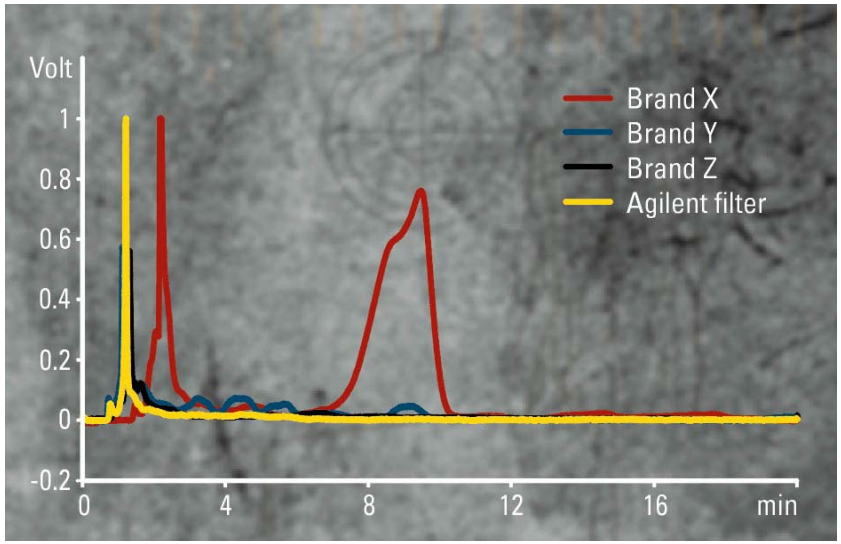
- Hold up volume is the volume needed to wet the membrane and which is lost during the filtration.
- 30 mm filters are recommended for large sample sizes – while for typical LC and GC use 13 mm is the optimum size.
- 25 mm is most often used and very common when sufficient sample is available and the larger hold up volume is not a problem.

Safe Filtration - Low Extractables

- Agilent filters are made of ultra clean membranes and housings, which:
  - Add no extractables to the filtrate to contaminate the sample
  - Produces higher sample recoveries
  - Results in more accurate analyses



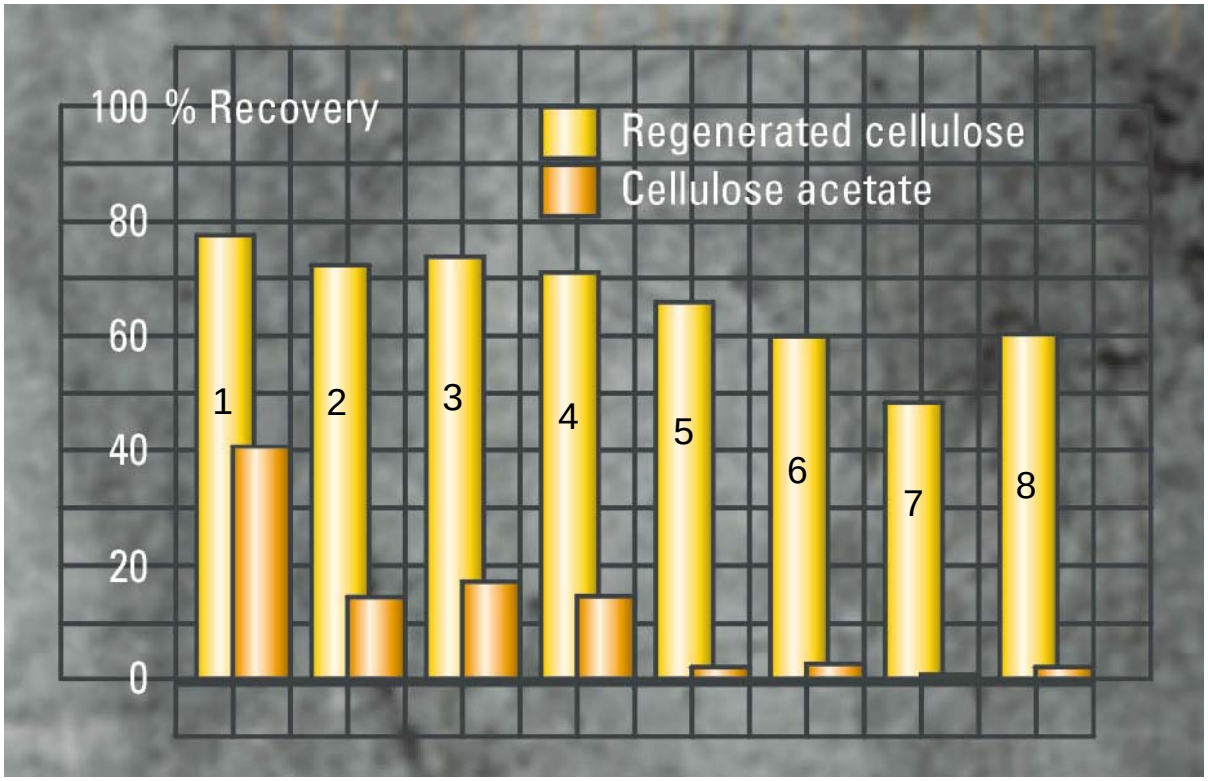
Methanol extract measured with HPLC at 254 nm



Acetonitrile extract measured with HPLC at 254 nm

Regenerated Cellulose
- High Recovery of the Sample

- An Environmental example … recovery of:
  1. PAH,
  2. Naphthalene,
  3. Acenaphthylene,
  4. Acenaphthene,
  5. Fluorene,
  6. Phenanthrene,
  7. Anthracene,
  8. Pyrene



Key Point: Regenerated Cellulose gives much better sample recovery after filtration than cellulose acetate

Key Thing to Remember

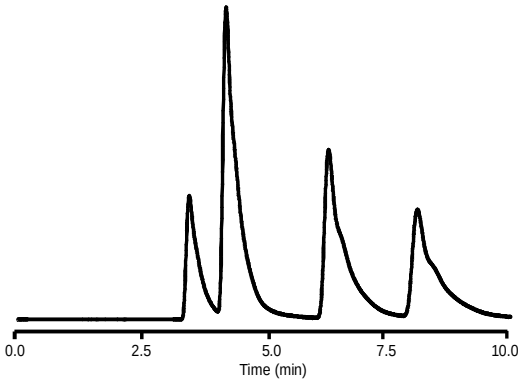
- As Column Particle Size Shrinks, Column Frit Porosity is Reduced (5µm-2µm frit, 3.5µm-0.5µm frit, 1.8µm-0.2µm frit
- Mobile Phase Filtering Reduces Wear on Instrument Parts (Check Valves, Piston Seals, Autosampler)
- Sample Filtering Reduces Wear on Instrument and Prevents Column Plugging Due to Particulates
- A Little Prevention Reduces Downtime and Maintenance Costs

Peak Shape Issues

Split Peaks

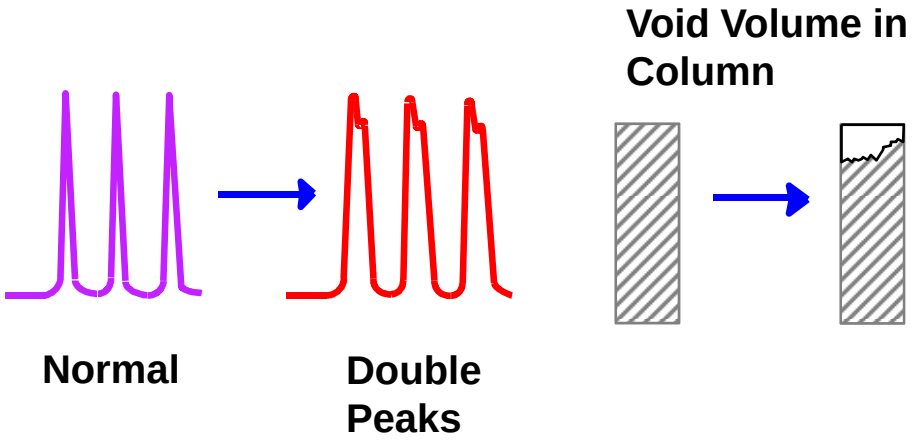
Can be caused by:

- Column contamination
- Partially plugged frit
- Column void
- Injection solvent effects



Peak Shape - Void

Double Peaks



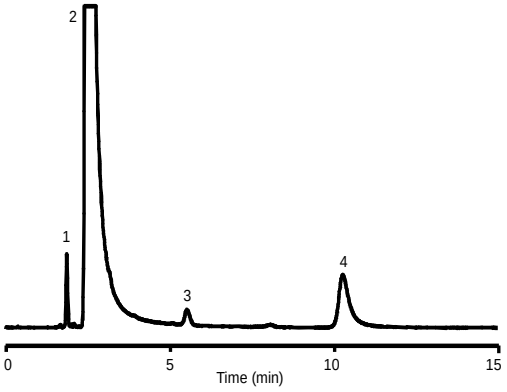
What Might Cause a Column to Void?

Split Peaks

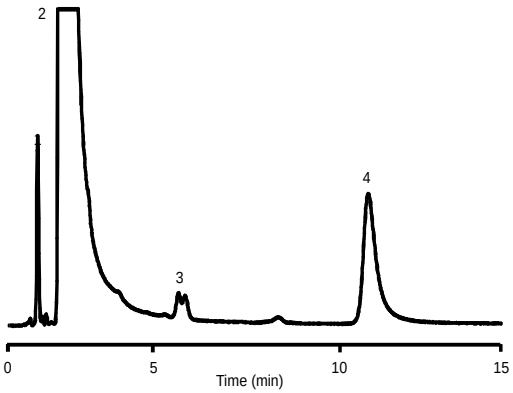
Column Contamination

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

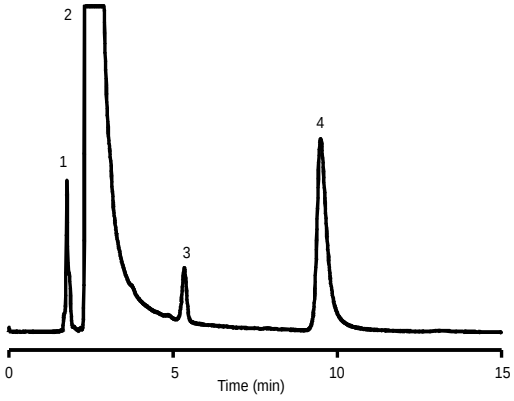
Injection 1



Injection 30



Injection 1 After Column Wash with 100% ACN



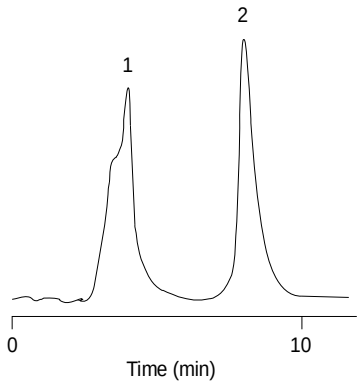
Column washing eliminates the peak splitting, which resulted from a contaminant on the column.

Split Peaks

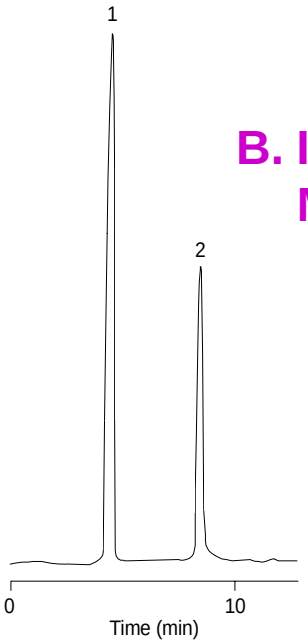
Injection Solvent Effects

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

A. Injection Solvent 100% Acetonitrile



B. Injection Solvent Mobile Phase



Injecting in a solvent stronger than the mobile phase can cause peak shape problems, such as peak splitting or broadening.

Summary of Causes of Split Peaks

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

2. Injection solvent stronger than mobile phase - likely split and broad peaks, dependent on sample volume

3. Mobile phase pH > 7 - likely column void due to silica dissolution (unless specialty column used)

Peak Tailing, Broadening and Loss of Efficiency

May be caused by:

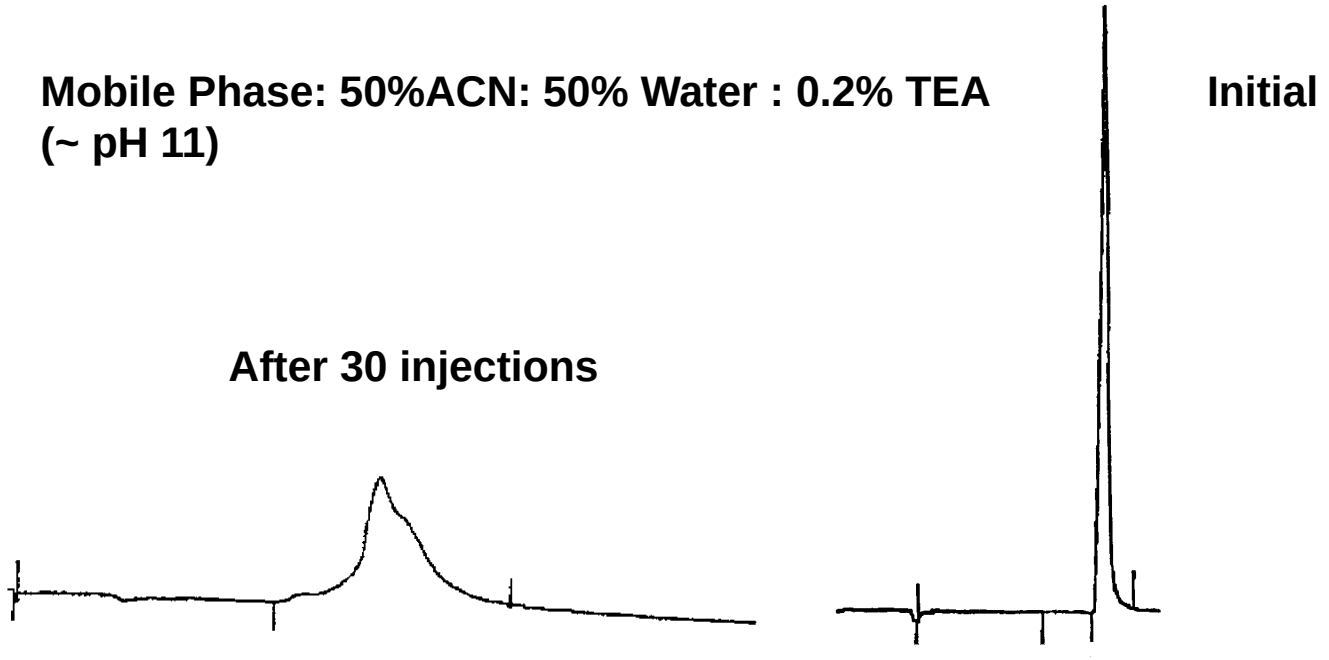
- Column void
- Column “secondary interactions”
- Column contamination
- Column aging
- Column loading
- Extra-column effects

Column Void
Packing Dissolved at High pH

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

Initial

After 30 injections



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

Peak Tailing

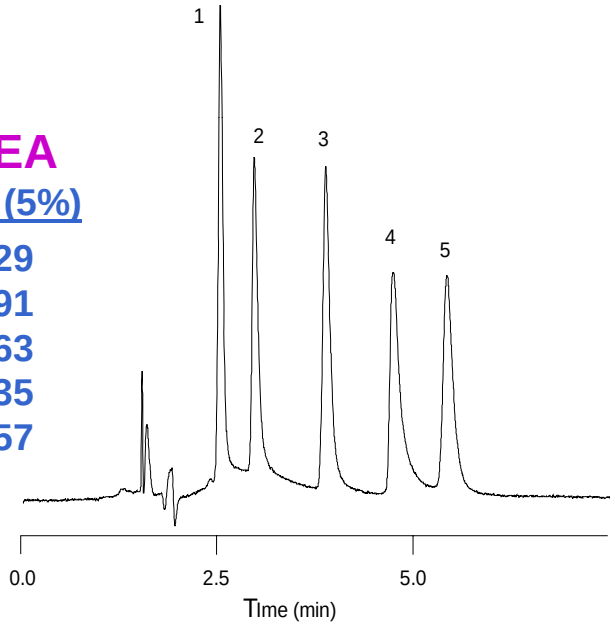
Column “Secondary Interactions”

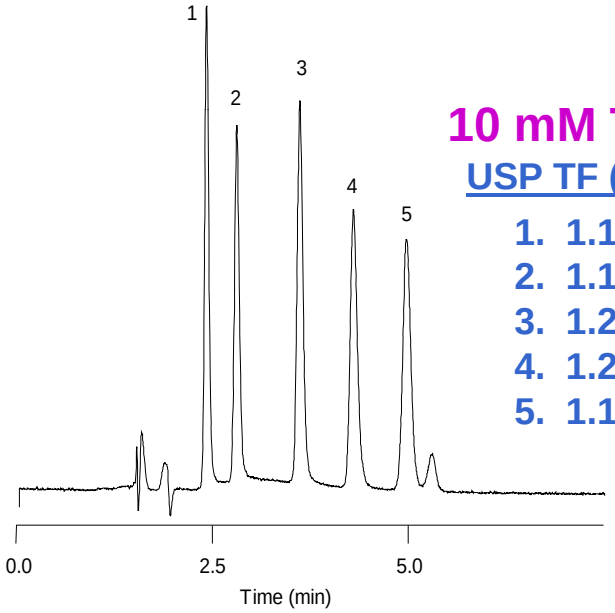
Column: Alkyl-C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 pH 7.0 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

No TEA

USP TF (5%)

1. 1.29
2. 1.91
3. 1.63
4. 2.35
5. 1.57





10 mM TEA

USP TF (5%)

1. 1.19
2. 1.18
3. 1.20
4. 1.26
5. 1.14

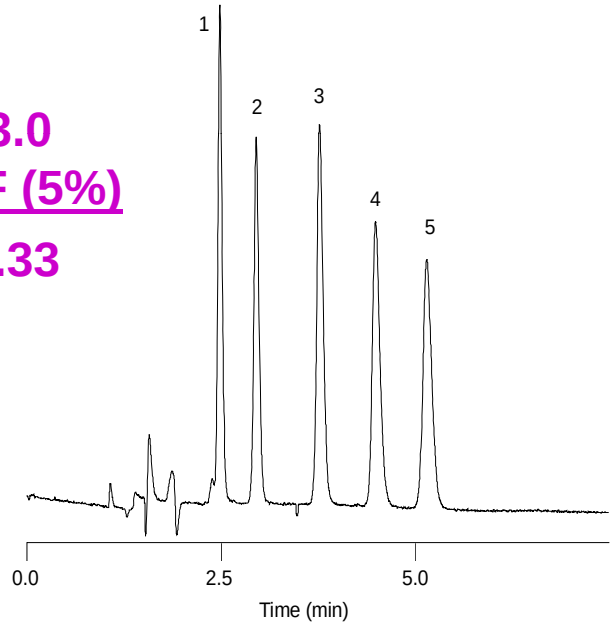
Peak tailing eliminated with mobile phase modifier (TEA) at pH 7

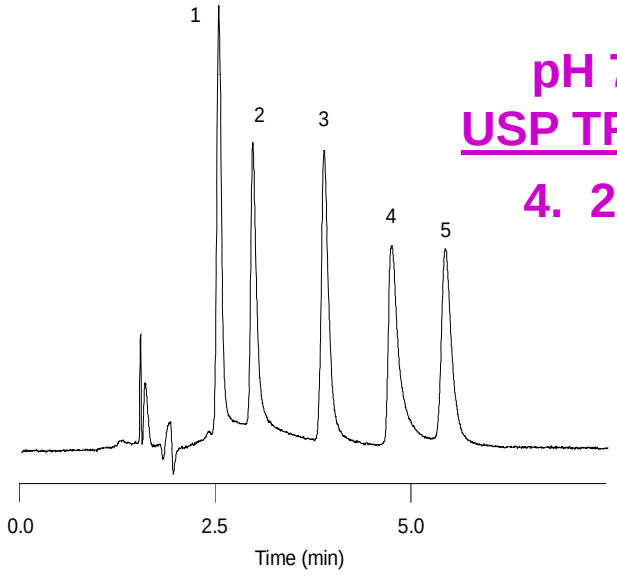
Peak Tailing

Column “Secondary Interactions”

Column: Alkyl-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 cause peak tailing.

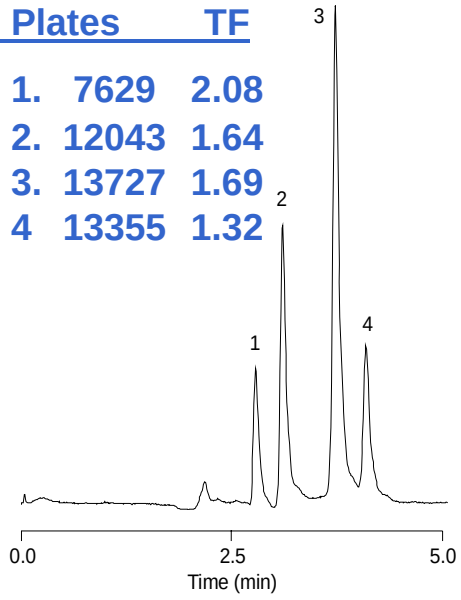
Peak Tailing

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

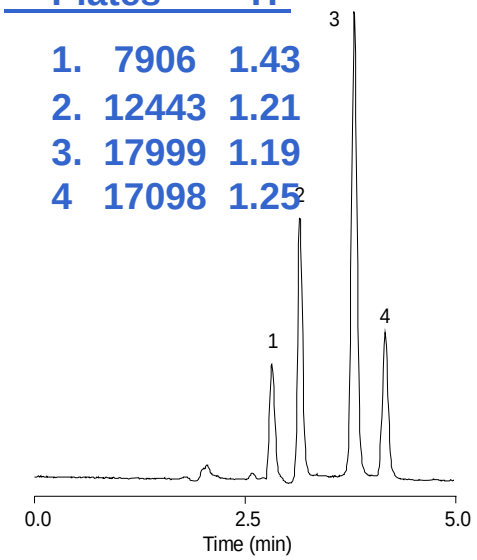
QC test forward direction

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



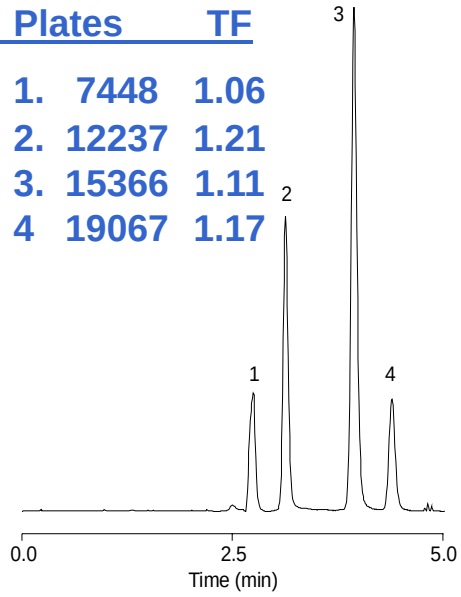
QC test reverse direction

	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



Peak Tailing/Broadening

Sample Load Effects

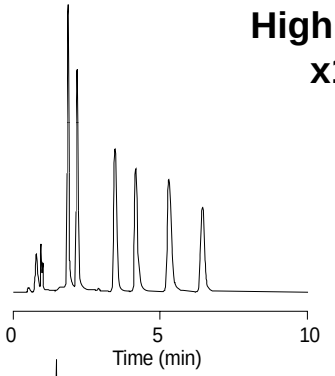
Columns: 4.6 x 150 mm, 5µm Mobile Phase: 40% 25 mM Na_2HPO_4 pH 7.0 : 60% ACN Flow Rate: 1.5 mL/min
Temperature: 40°C Sample: 1. Desipramine 2. Nortriptyline 3. Doxepin 4. Imipramine 5. Amitriptyline 6. Trimipramine

Tailing

Eclipse XDB-C8
USP TF (5%)

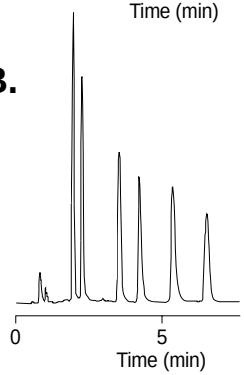
	A	B
1.	1.60	1.70
2.	2.00	1.90
3.	1.56	1.56
4.	2.13	1.70
5.	2.15	1.86
6.	1.25	1.25

A.



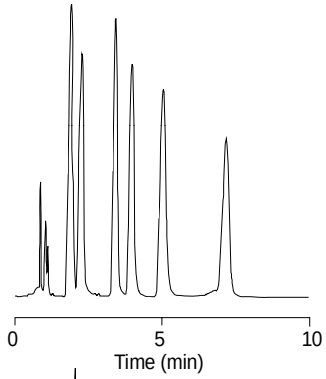
High Load x10

B.

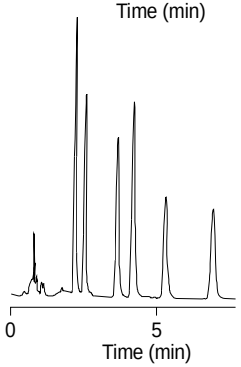


Low Load

C.



D.



Broadening

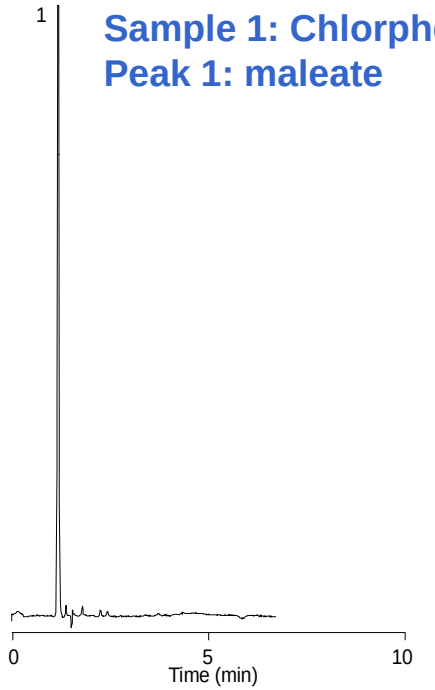
Competitive C8
Plates

	C	D
1.	850	5941
2.	815	7842
3.	2776	6231
4.	2539	8359
5.	2735	10022
6.	5189	10725

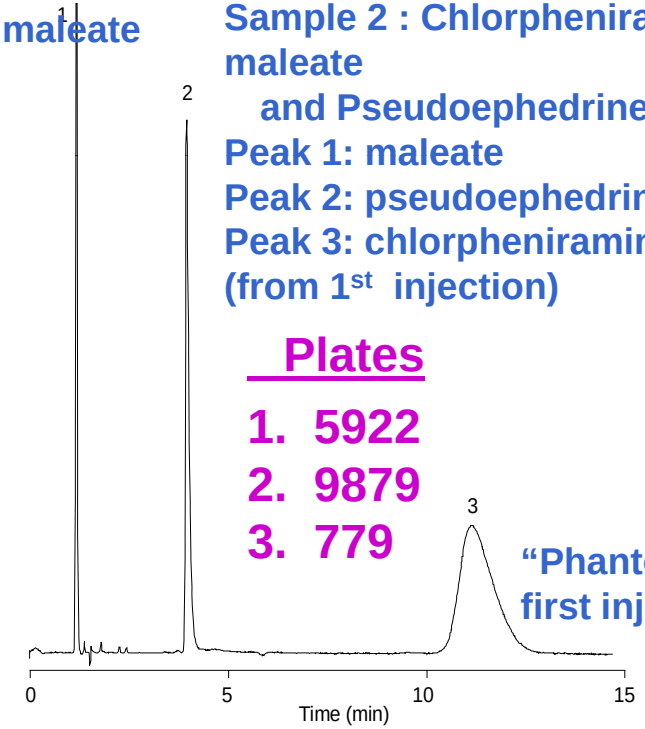
Broad Peaks

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
Peak 1: maleate



Sample 2 : Chlorpheniramine maleate and Pseudoephedrine
Peak 1: maleate
Peak 2: pseudoephedrine
Peak 3: chlorpheniramine (from 1st injection)

Plates

1. **5922**
2. **9879**
3. **779**

“Phantom” peak from first injection

The extremely low plates are an indication of a very late eluting peak from the preceding run.

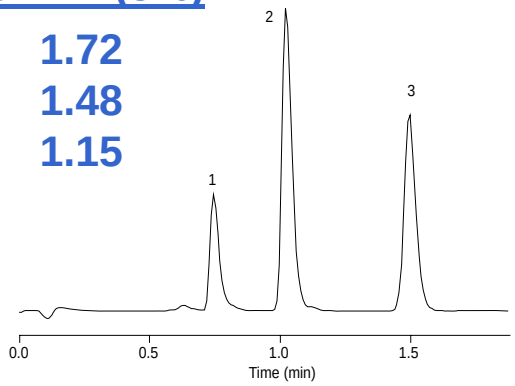
Peak Tailing

Injector Seal Failure

Column: Bonus-RP, 4.6 x 75 mm, 3.5 µm Mobile Phase: 30% H_2O : 70% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. N,N-Dimethylaniline

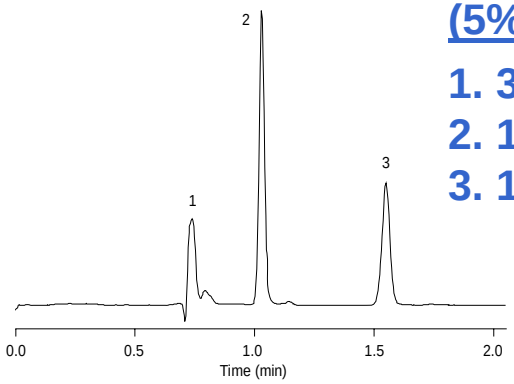
Before

	Plates	USP TF (5%)
1.	2235	1.72
2.	3491	1.48
3.	5432	1.15



After replacing rotor seal and isolation seal

	Plates	USP TF (5%)
1.	3670	1.45
2.	10457	1.09
3.	10085	1.00



Overdue instrument maintenance can cause peak shape problems.

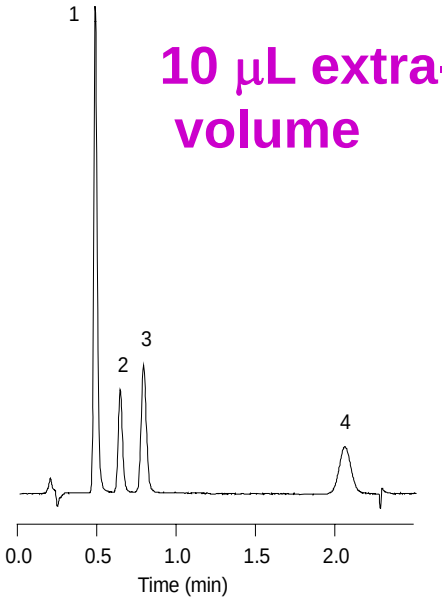
Summary of Correcting Causes of Peak Tailing

- Evaluate mobile phase effects - alter mobile phase pH and additives to eliminate secondary interactions
- Evaluate column choice - try column with high purity silica or different bonding technology
- Reduce sample load
- Eliminate extra-column effects
- Flush column and check for aging/void

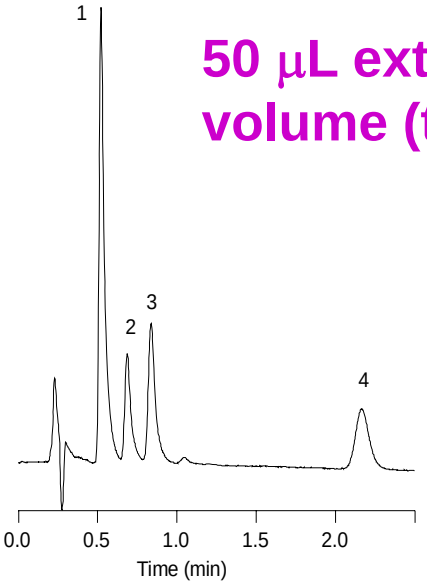
Peak Broadening

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



10 µL extra-column volume



50 µL extra-column volume (tubing)

Agilent Technologies

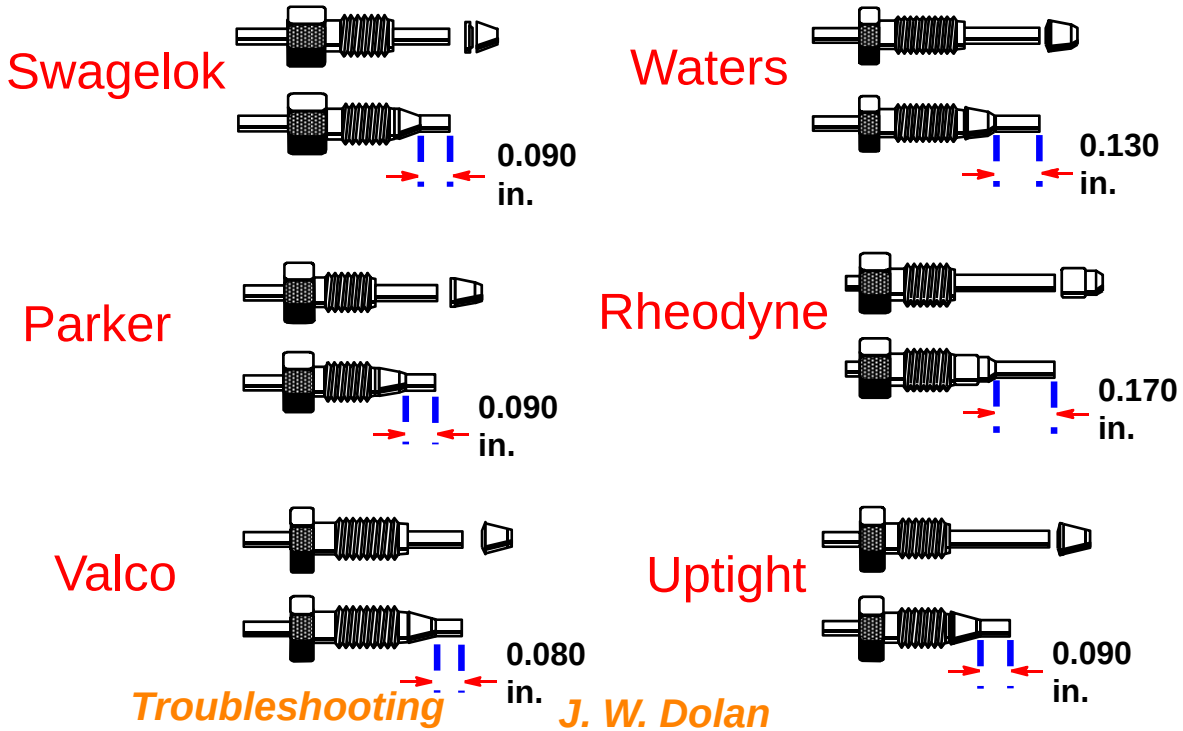
Poor Peak Shape Often Due to Improper Connections

- All Tubing Lengths Are Minimum
- Smallest Diameter Tubing Used
- Proper Flow Cell Volume
- Symptom Still Looks As If There Is Too Much Extra-Column Volume

What Is Wrong?

Have You Made the Connections Properly?

Column Connectors Used in HPLC

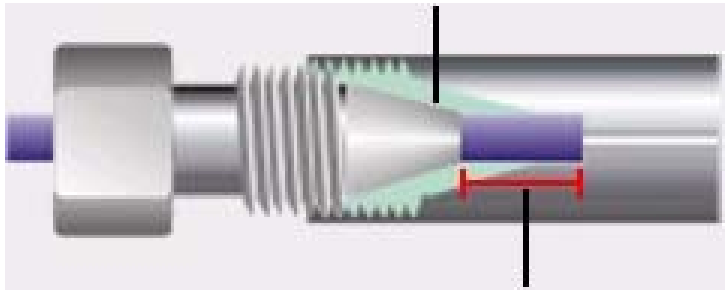


Troubleshooting LC Fittings, Part II. *J. W. Dolan and P. Upchurch.* *LC/GC Magazine 6:788 (1988)*

What Happens If the Connections Poorly Made ?

Wrong … too long

Ferrule cannot seat properly

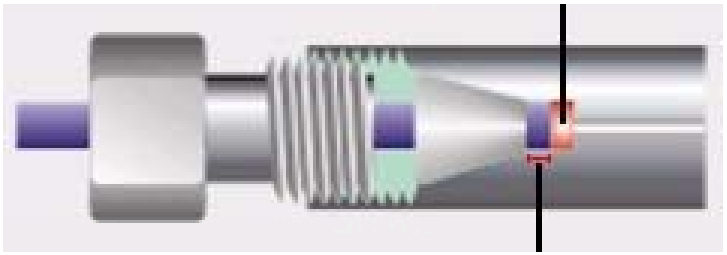


X

If Dimension X is too long, leaks will occur

Wrong … too short

Mixing Chamber



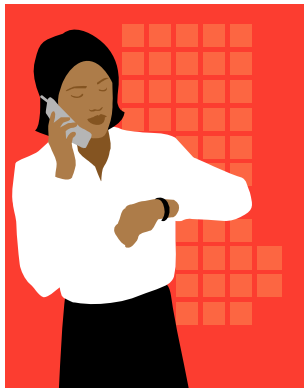
X

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

New Convenience for Column Installation in RRLC Instruments

Easy, hand tighten column connection with **Polyketone** fittings up to 600 bar PN: 5042-8957 (10/pk)

Let Us Take A Break!



Retention Issues

- Retention time changes (t_r)
- Capacity factor (retention) changes (k’)
- Selectivity changes (α)

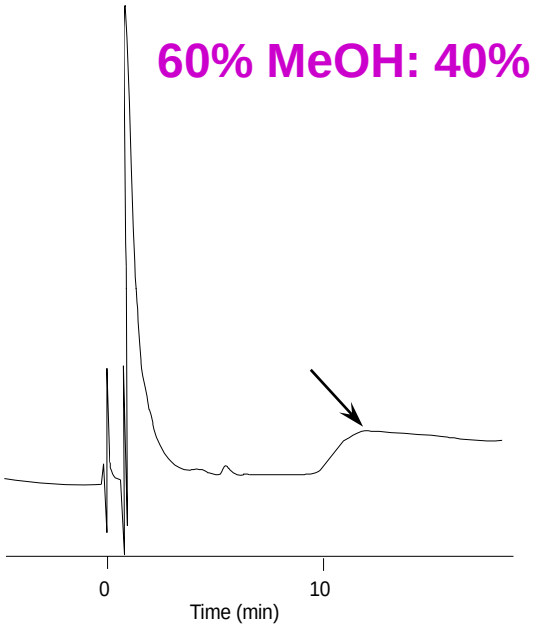
Changes in Retention
Same Column, Over Time

May be caused by:

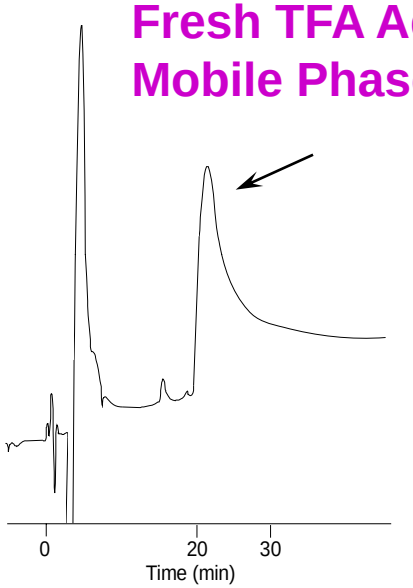
- Column aging
- Column contamination
- Insufficient equilibration
- Poor column/mobile phase combination
- Change in mobile phase
- Change in flow rate
- Other instrument issues

Mobile Phase Change Causes Change in Retention

60% MeOH: 40% 0.1%TFA



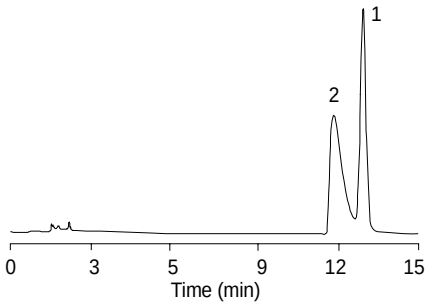
Fresh TFA Added to Mobile Phase



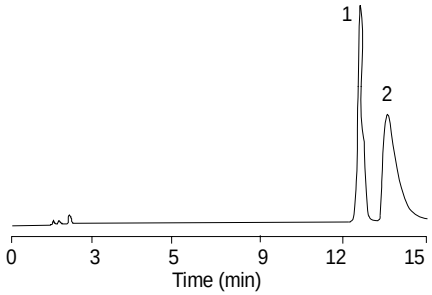
- Volatile TFA evaporated/degassed from mobile phase. Replacing it solved problem.
- Chromatography is from a protein binding study and peak shape as expected.

Column Aging/Equilibration Causes Retention/Selectivity Changes

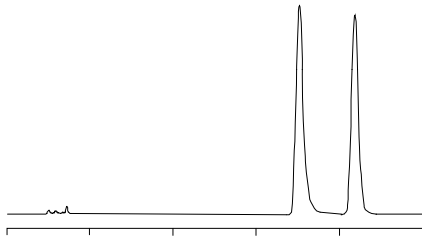
Column 1 - Initial



Column 1 - Next Day



Column 1 - After Cleaning with 1% H_3PO_4



- The primary analyte was sensitive to mobile phase aging of the column.
- The peak shape was a secondary issue (metal chelating compound) resolved by flushing the column.
- Retention and peak shape were as expected after cleaning.

Summary of Determining Cause of Retention Changes

Same Column

1. Determine k', α, and t_r for suspect peaks
2. Wash column
3. Test new column - note lot number
4. Review column equilibration procedures
5. Make up fresh mobile phase and test
6. Check instrument performance

Change in Retention/Selectivity
Column-to-Column

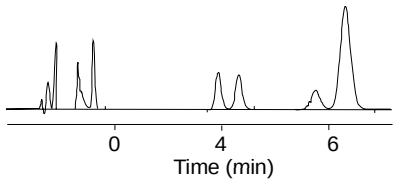
- **Different column histories (aging)**
- **Insufficient/inconsistent equilibration**
- Poor column/mobile phase combination
- Change in mobile phase
- Change in flow rate
- Other instrument issues
- Slight changes in column bed volume (t_r only)

Example Change in Retention/Selectivity

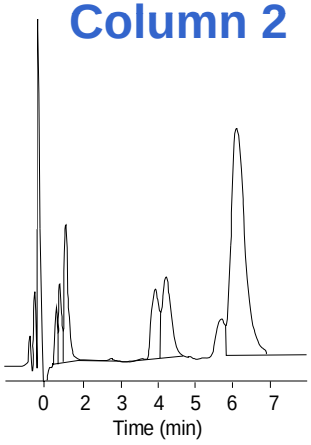
Column-to-Column

Mobile Phase Variation

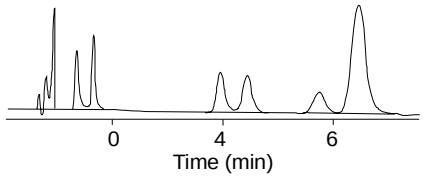
Column 1



Column 2



Column 2 - Fresh mobile phase



"I have experimented with our mobile phase, opening new bottles of all mobile phase components. When I use all fresh ingredients, the problem ceases to exist, and I have narrowed the problem to either a bad bottle of TEA or phosphoric acid. Our problem has been solved."

Summary of Determining the Cause of Retention Changes

Column-to-Column

1. Determine k', α, and t_r for suspect peaks
2. Test new column - note lot number
3. Determine column history of all columns
4. Review column equilibration procedures
5. Make up fresh mobile phase and test
6. Check instrument performance

Minimize Change in Retention/Selectivity
Lot-to-Lot

Evaluate:

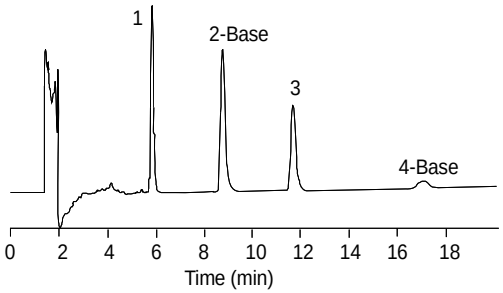
- All causes of column-to-column change*
- Method ruggedness (buffers/ionic strength)
- pH sensitivity (sample/column interactions)

*All causes of column-to-column change should be considered first, especially when only one column from a lot has been tested.

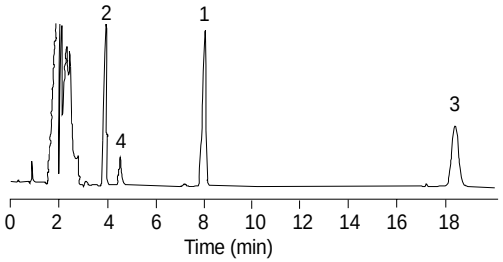
Lot-to-Lot Selectivity Change

pH

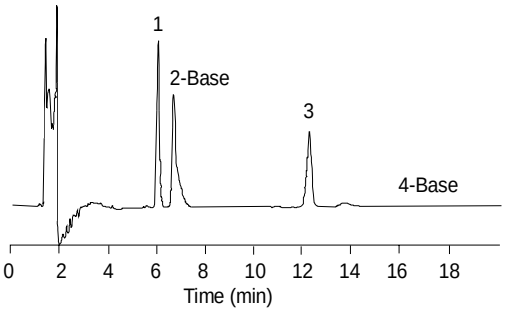
pH 4.5 - Lot 1



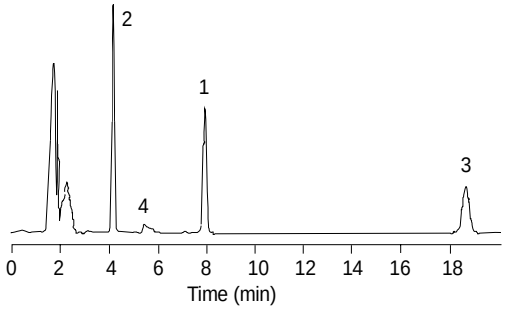
pH 3.0 - Lot 1



pH 4.5 - Lot 2



pH 3.0 - Lot 2



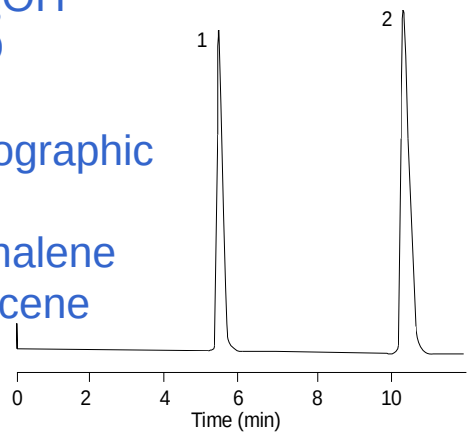
- pH 4.5 shows selectivity change from lot-to-lot for basic compounds
- pH 3.0 shows no selectivity change from lot-to-lot, indicating silanol sensitivity at pH 4.5

Experimental Conditions for Classifying Column Selectivity Changes

Bonded-Phase Test

Mobile Phase:
85% CH_3OH
15% H_2O

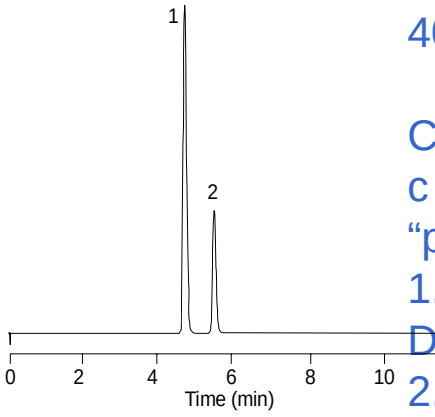
Chromatographic "probes"
1. Naphthalene
2. Anthracene



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

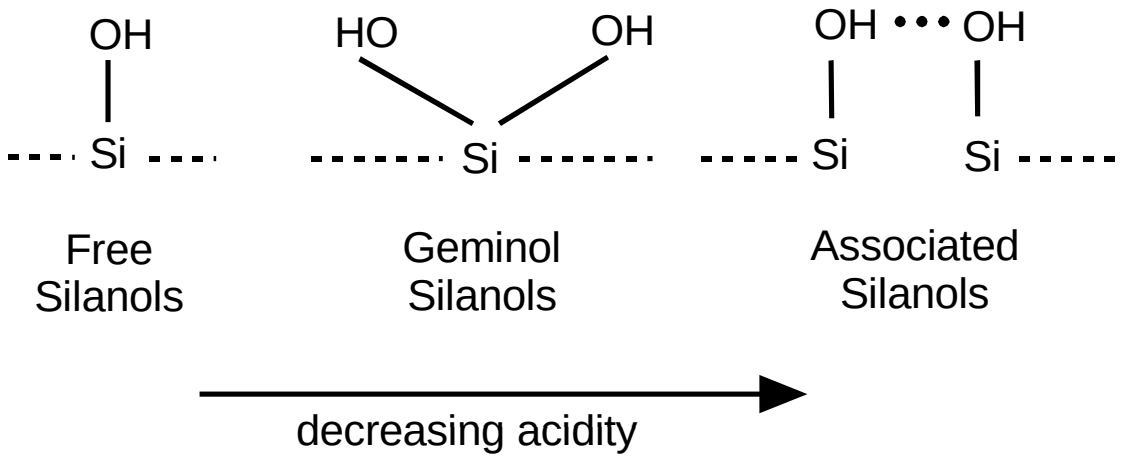
Evaluate Retention Changes
Lot-to-Lot

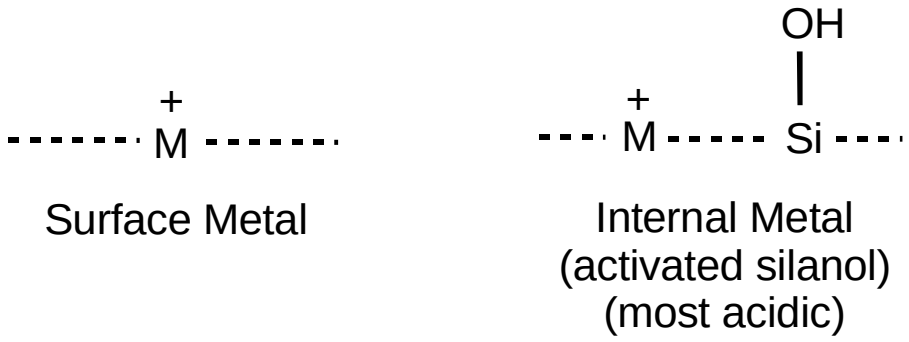
- Eliminate causes of column-to-column selectivity change
- Re-evaluate method ruggedness - modify method
- Determine pH sensitivity - modify method
- Classify selectivity changes
- Contact manufacturer for assistance

Mobile Phase pH and pH Buffers
Why Are These So Important in HPLC?

- pH Effects Ionization
  - Silica Surface of Column
  - Sample Components of Interest
  - Sample Matrix
- Buffers
  - Resist Changes in pH
  - Improve Peak Shape for Ionizable Compounds
- Detector Effects Choice of Buffers
  - UV Transparency
  - Volatility (ELSD, LC/MS, etc.)
- Effects Column Life

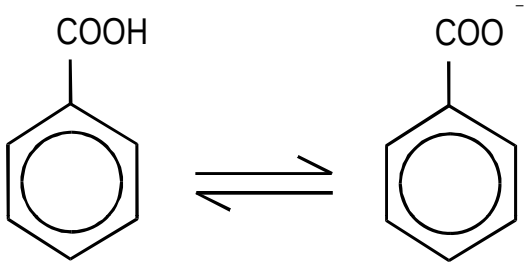
The Surface of Silica Supports for HPLC





Why Worry About pH?
pH, pKa and Weak Acids

$$RCOOH \rightleftharpoons RCOO^- + H^+ \qquad K_a = \frac{[RCOO^-][H^+]}{[RCOOH]}$$



$$+ \quad H^+$$

$$K_a = 6.4 \times 10^{-5}$$
$$pK_a = 4.2$$

At pH 4.2 – the sample exists as benzoic acid and the benzoate ion in a ratio of 1:1. Peak shape can be poor

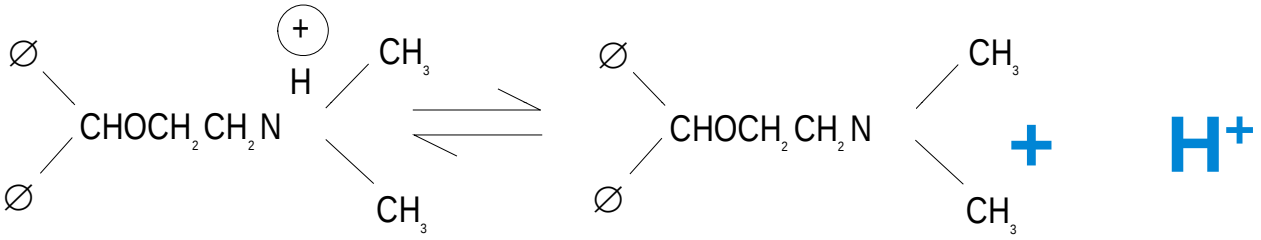
At pH 5.2 – 91% of the sample exists as the benzoate ion. RP retention decreases.

At pH 3.2 – 91% of the sample exists as benzoic acid. RP retention increases.

Why Worry About pH?
pH, pKa and Weak Bases

$$R_3NH^+ \rightleftharpoons R_3N + H^+$$

$$K_a = \frac{[R_3N][H^+]}{[R_3NH^+]}$$



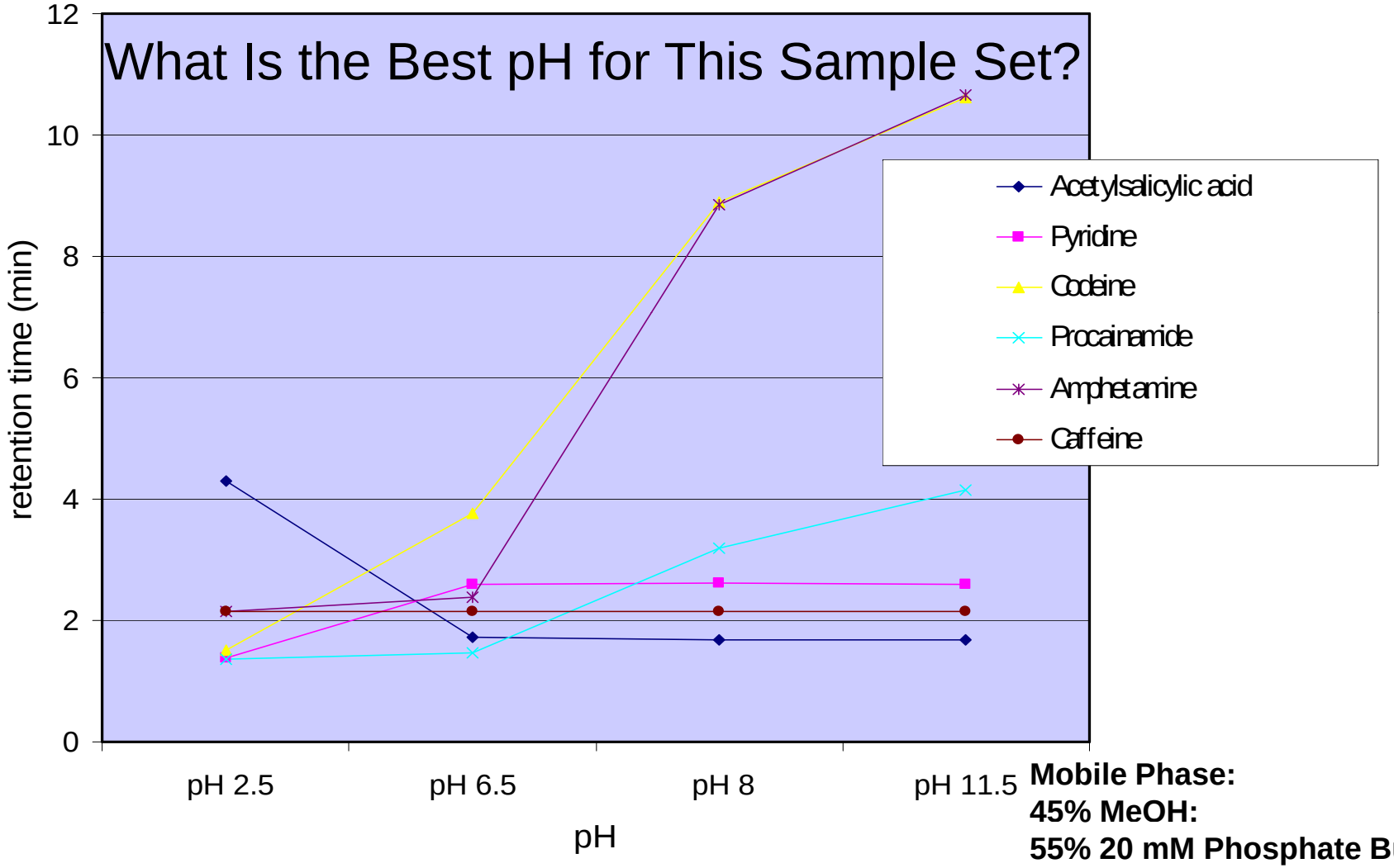
$K_a = 1 \times 10^{-9}$

$pK_a = 9$

At pH 9 – the sample exists as protonated and unprotonated diphenhydramine in a ratio of 1:1. Peak shape can be poor.
At pH 10 – 91% of the sample exists as unprotonated diphenhydramine.
At pH 8 – 91% of the sample exists as protonated diphenhydramine.

Retention vs. pH for Ionizable Compounds

Effects are Compound Dependent



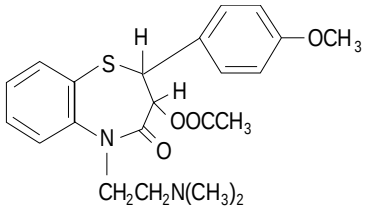
Mobile Phase:
45% MeOH:
55% 20 mM Phosphate Buffer

Importance of Buffers – A Practical Example

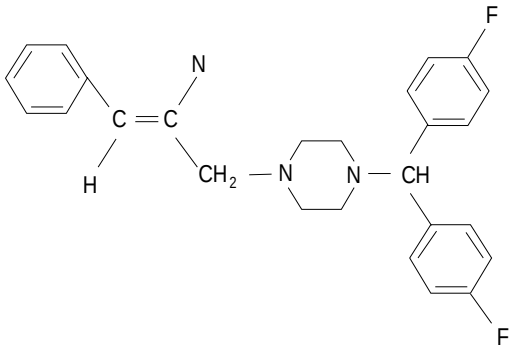
- Why the Sample Dictates Use
- What Happens When Buffer Used Effectively
- What Happens When Buffer Ignored or Used Improperly

Cardiac Drug Separation

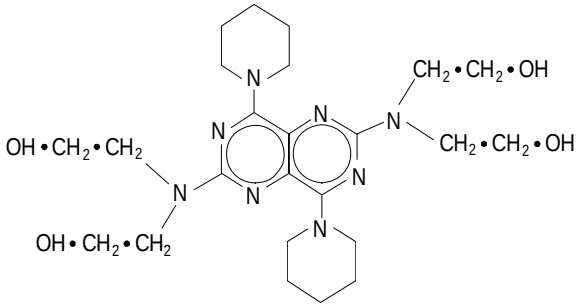
Diltiazem
pKa: unknown
calcium channel blocker



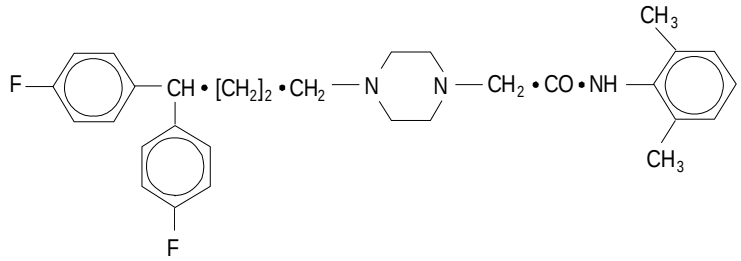
Flunarizine
pKa: unknown
calcium channel blocker



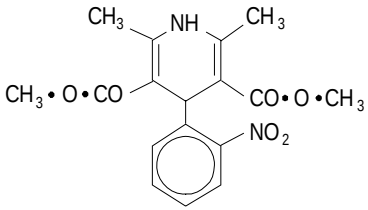
Dipyridamole
pKa: 6.4
anti-thrombotic/anti-anginal



Lidoflazine
pKa: unknown
calcium channel blocker



Nifedipine
pKa: unkbown
anti-anginal vasodilator

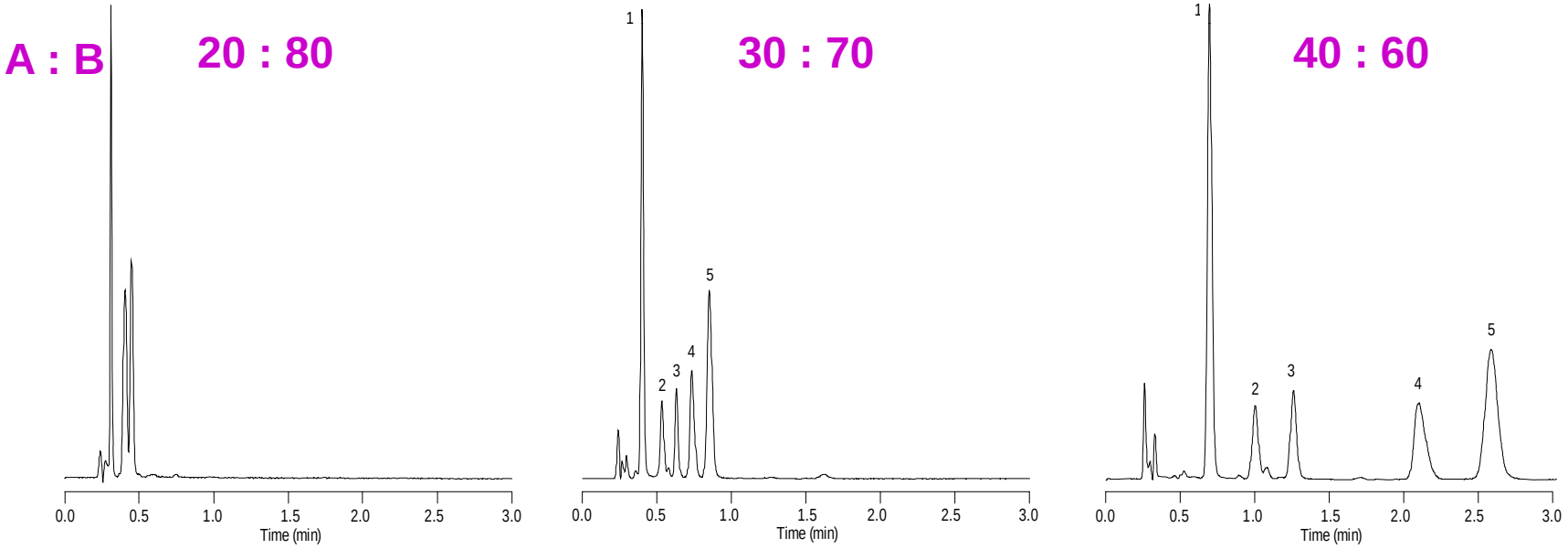


Fast Scouting Isocratic Runs
Cardiac Drugs with Methanol

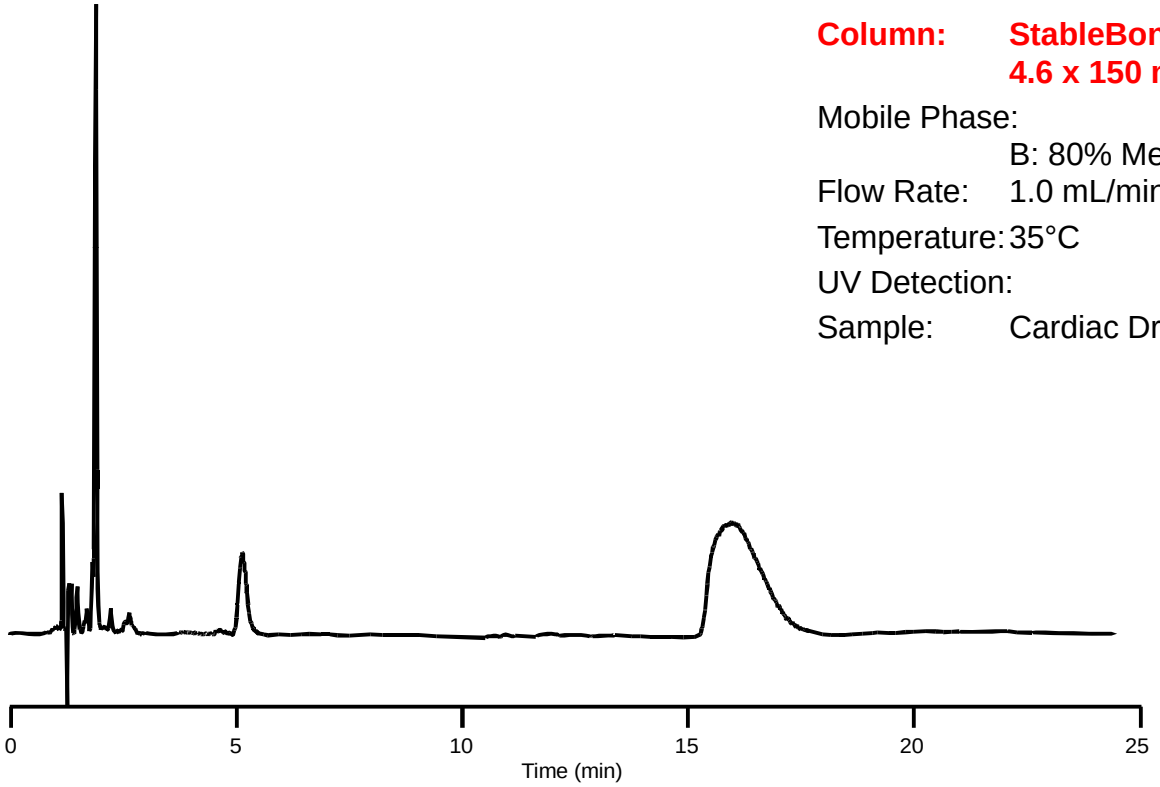
Column: Zorbax Rapid Resolution SB-C18, 4.6 x 75 mm, 3.5 µm Mobile Phase: A: 25 mM NaH_2PO_4 , pH 3.0 B: MeOH

Flow Rate: 2.0 mL/min Temperature: 35°C Detection: UV 254 nm

Sample: Cardiac Drugs 1. Diltiazem 2. Dipyridamole 3. Nifedipine 4. Lidoflazine 5. Flunarizine



I Don’t Have Time to Make Buffers…

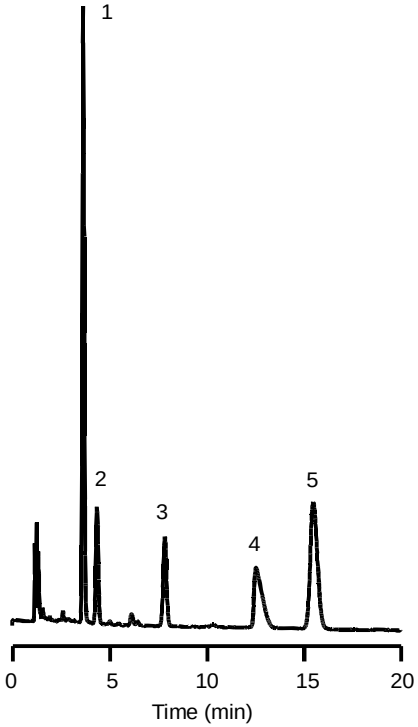


Column: **StableBond SB-C18**
4.6 x 150 mm, 5 µm
Mobile Phase: A: 20% H_2O
B: 80% MeOH
Flow Rate: 1.0 mL/min.
Temperature: 35°C
UV Detection: 254 nm
Sample: Cardiac Drugs

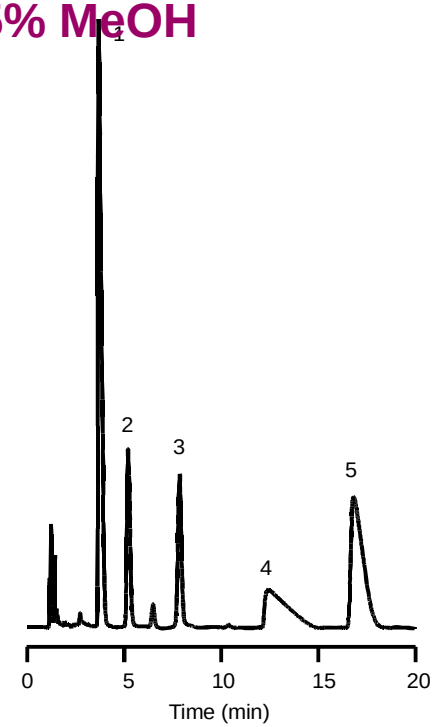
- **Buffers are critical to good retention and peak shape in many separations.**

So I'll Just Put Some Phosphoric Acid in the Water….

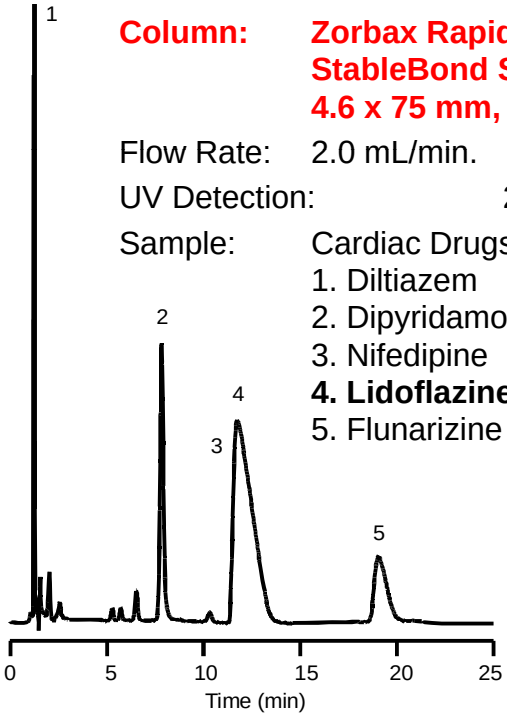
30% H_2O (pH 2.0)
70% MeOH



45% H_2O (pH 3.0)
55% MeOH



45% H_2O (pH 4.0)
55% MeOH



Column: **Zorbax Rapid Resolution StableBond SB-C18 4.6 x 75 mm, 3.5 µm**

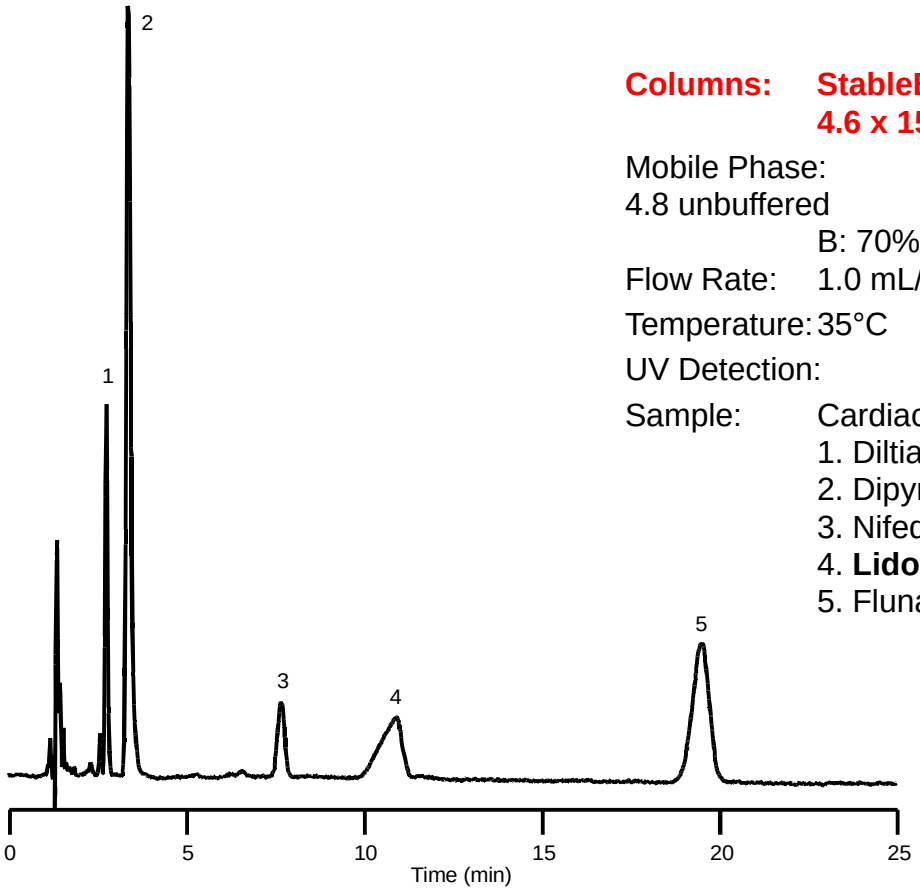
Flow Rate: 2.0 mL/min.

UV Detection: 254 nm

Sample: Cardiac Drugs
1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. **Lidoflazine**
5. Flunarizine

Phosphoric acid provides insufficient buffer strength for optimum peak shape

What If I Work Outside the Buffer Range?



Columns: **StableBond SB-C18**
4.6 x 150 mm, 5 µm

Mobile Phase: A: 30% 25 mM NaH_2PO_4, pH 4.8 unbuffered
B: 70% MeOH

Flow Rate: 1.0 mL/min.

Temperature: 35°C

UV Detection: 254 nm

Sample: Cardiac Drugs
1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. **Lidoflazine**
5. Flunarizine

Common Buffers Used in HPLC
General Purpose Buffers

*Potassium dihydrogen phosphate (monobasic)	1.1 – 3.1
*Dipotassium hydrogen phosphate (dibasic)	6.2 – 8.2
Tripotassium phosphate (tribasic)	11.3 – 13.3
Sodium dihydrogen citrate	2.1 – 4.1
Disodium hydrogen citrate	3.7 – 5.7
Trisodium citrate	4.4 – 6.4
*Sodium acetate	3.8 – 5.8
TRIS [tris(hydroxymethyl)aminomethane]	8.0 – 10.0
Ammonium hydroxide	8.3 – 10.3

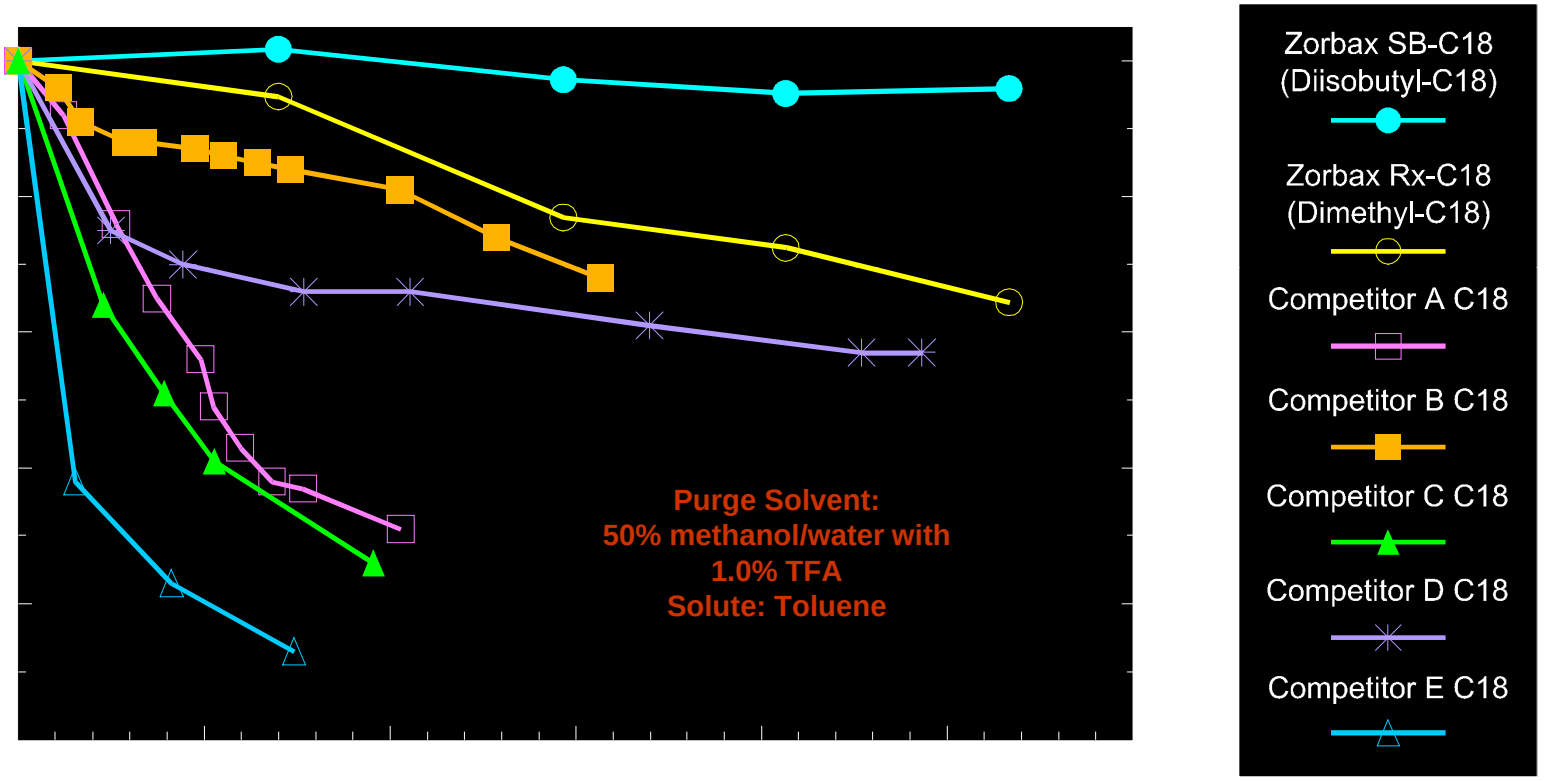
***most common**

Common Buffers Used in HPLC
Volatile Buffers

Ammonium formate	3.0 – 5.0
Pyridinium formate	3.0 – 5.0
Ammonium acetate	3.8 – 5.8
Ammonium carbonate	5.5 – 7.5 and 9.3 – 11.3
Ammonium hydroxide	8.3 – 10.3
Pyrrolidine	10.3 – 12.3
Triethylamine (TEA)	9.7 – 11.7
1-methyl-piperidine	9.3 – 11.3
glycine	8.8 – 10.8
TRIS	7.1 – 9.1

Don’t Forget - Match Column to pH of Mobile Phase for Maximum Column Lifetime

low pH and high temperature (pH 0.8, 90°C)

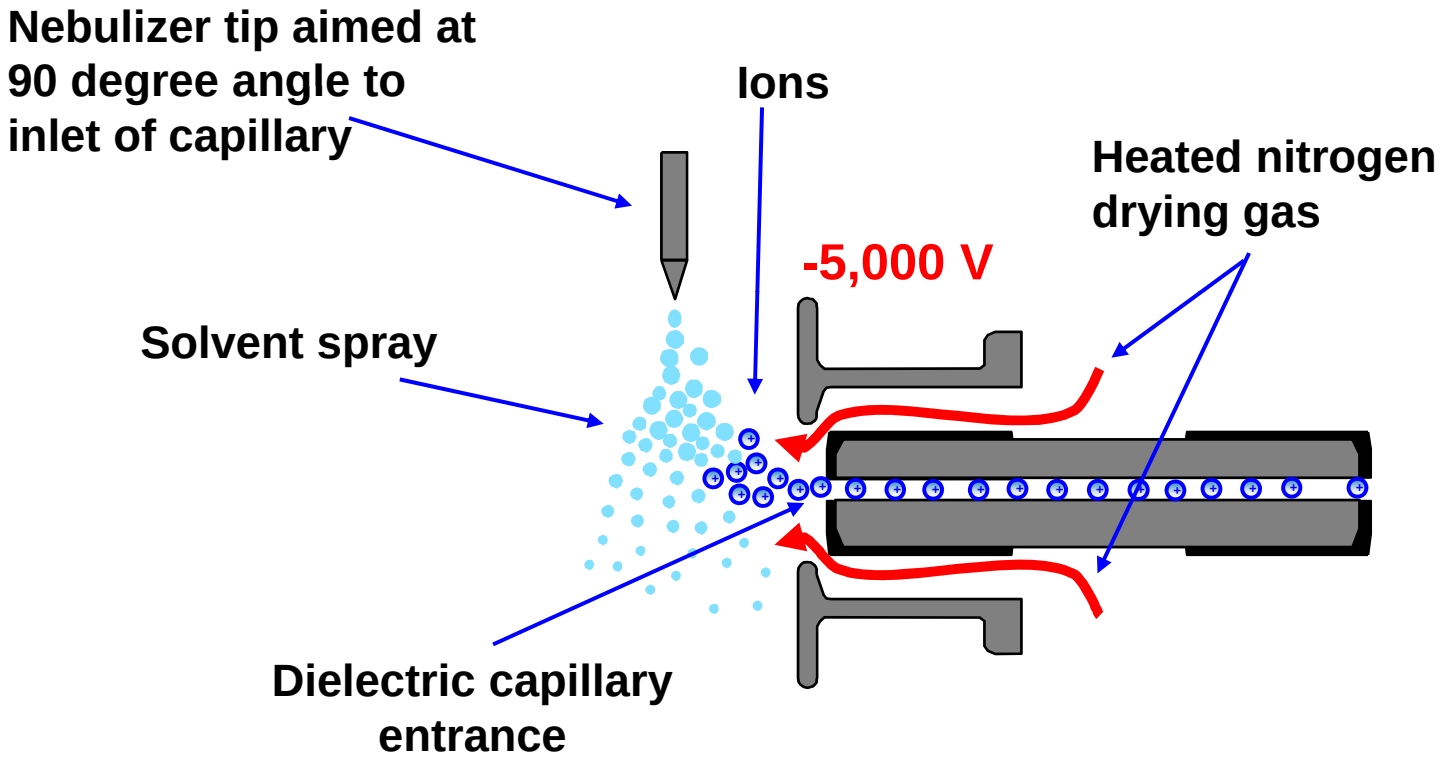


Kirkland, J.J. and J.W. Henderson, Journal of Chromatographic Science, 32 (1994) 473-480.

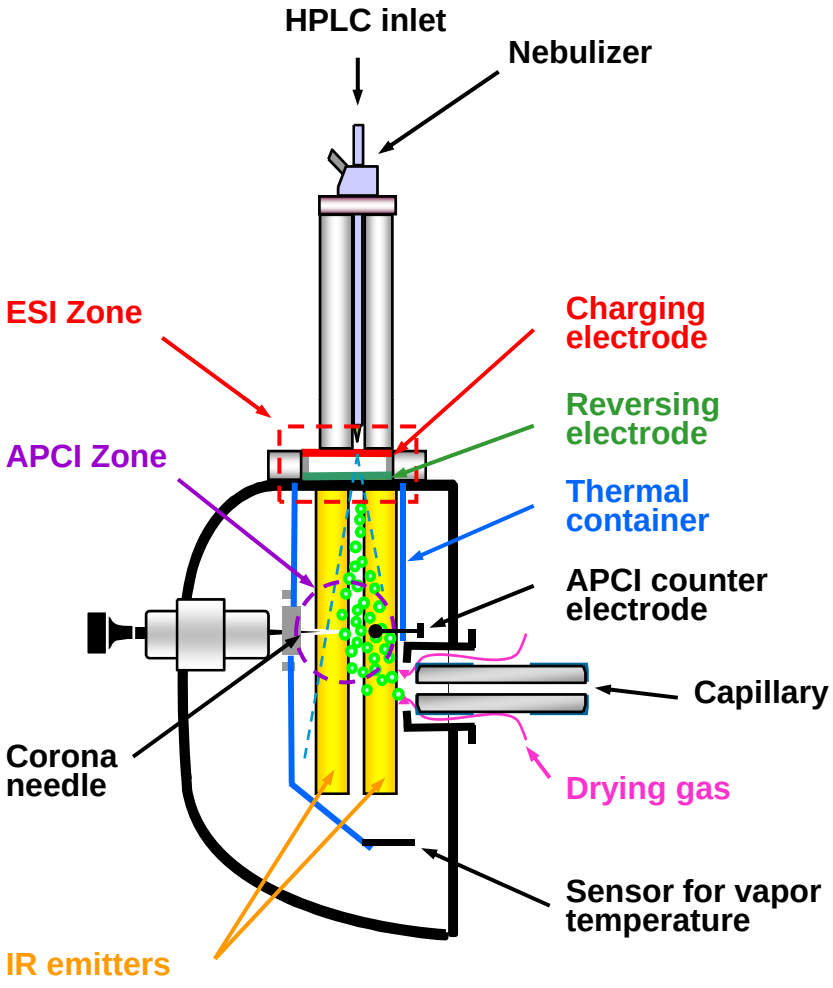
Why Are Volatile Buffers Important?

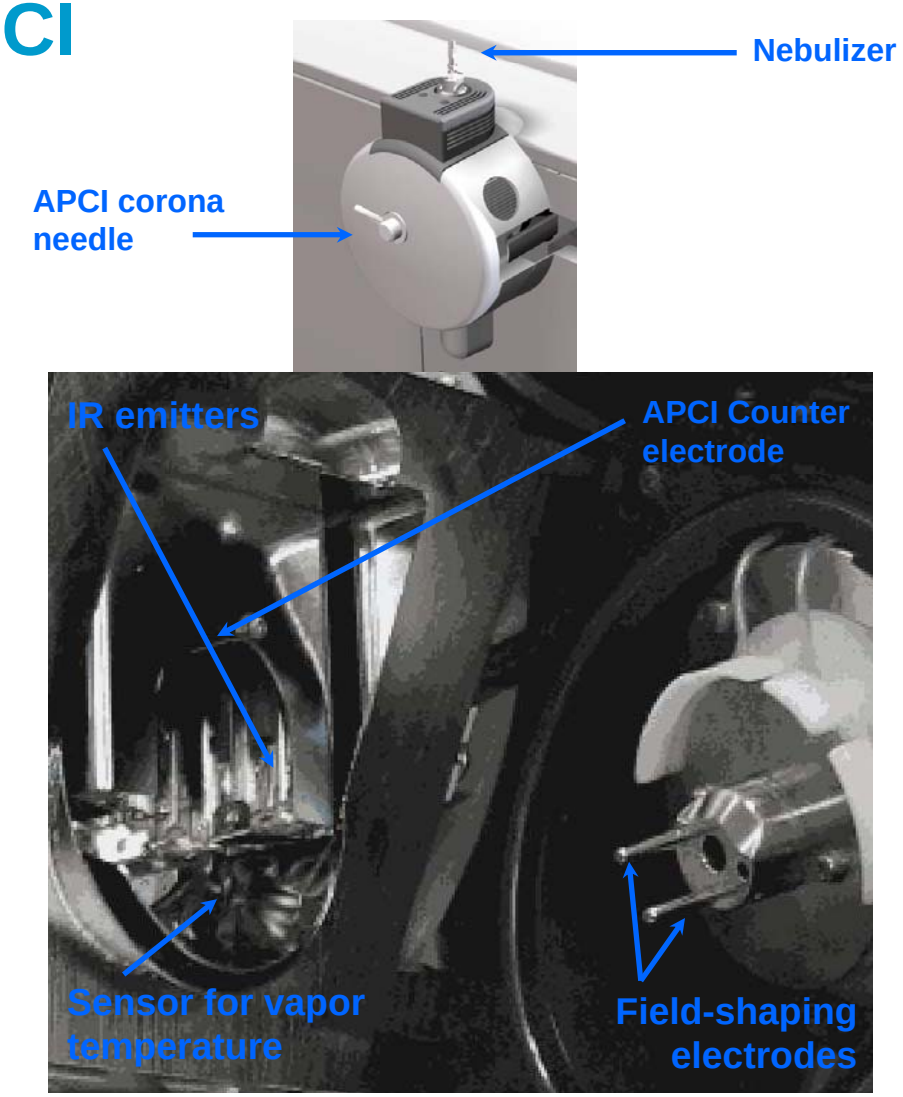
- Prevent Contamination of Sensitive Detectors
- Reduce Build up of Solids in Evaporative Process Detectors

Agilent 6000 Series: Based on Innovation
Orthogonal Introduction and Electrospray Ionization



The Agilent Multimode Source: Innovation
Simultaneous ESI and APCI

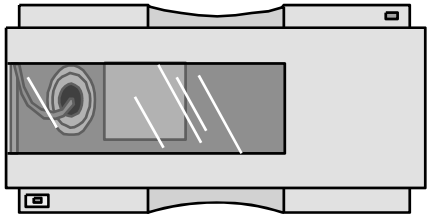


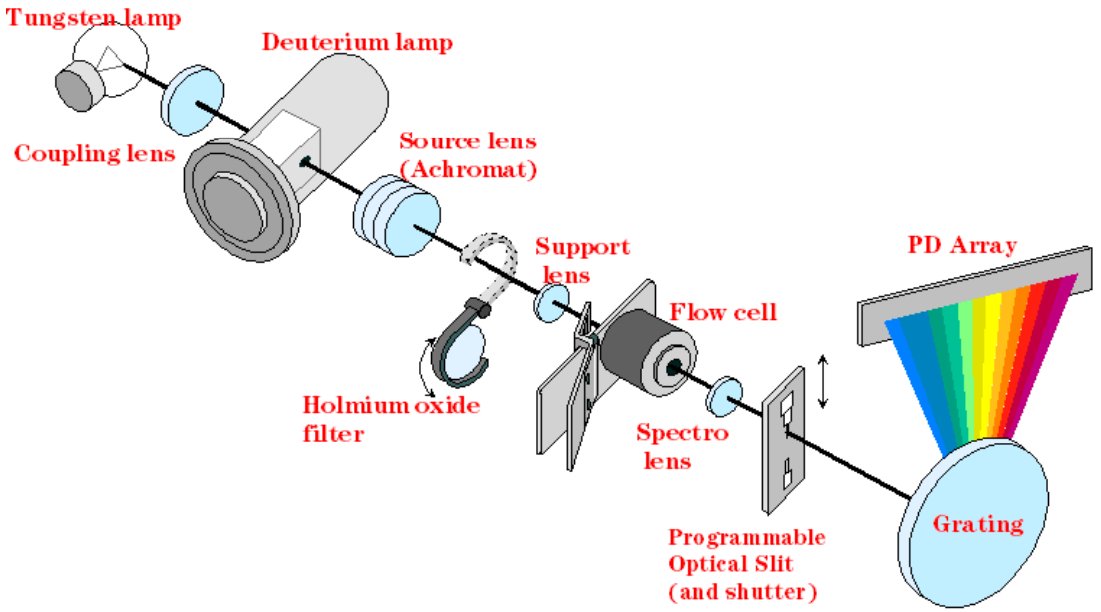


Detection Issues

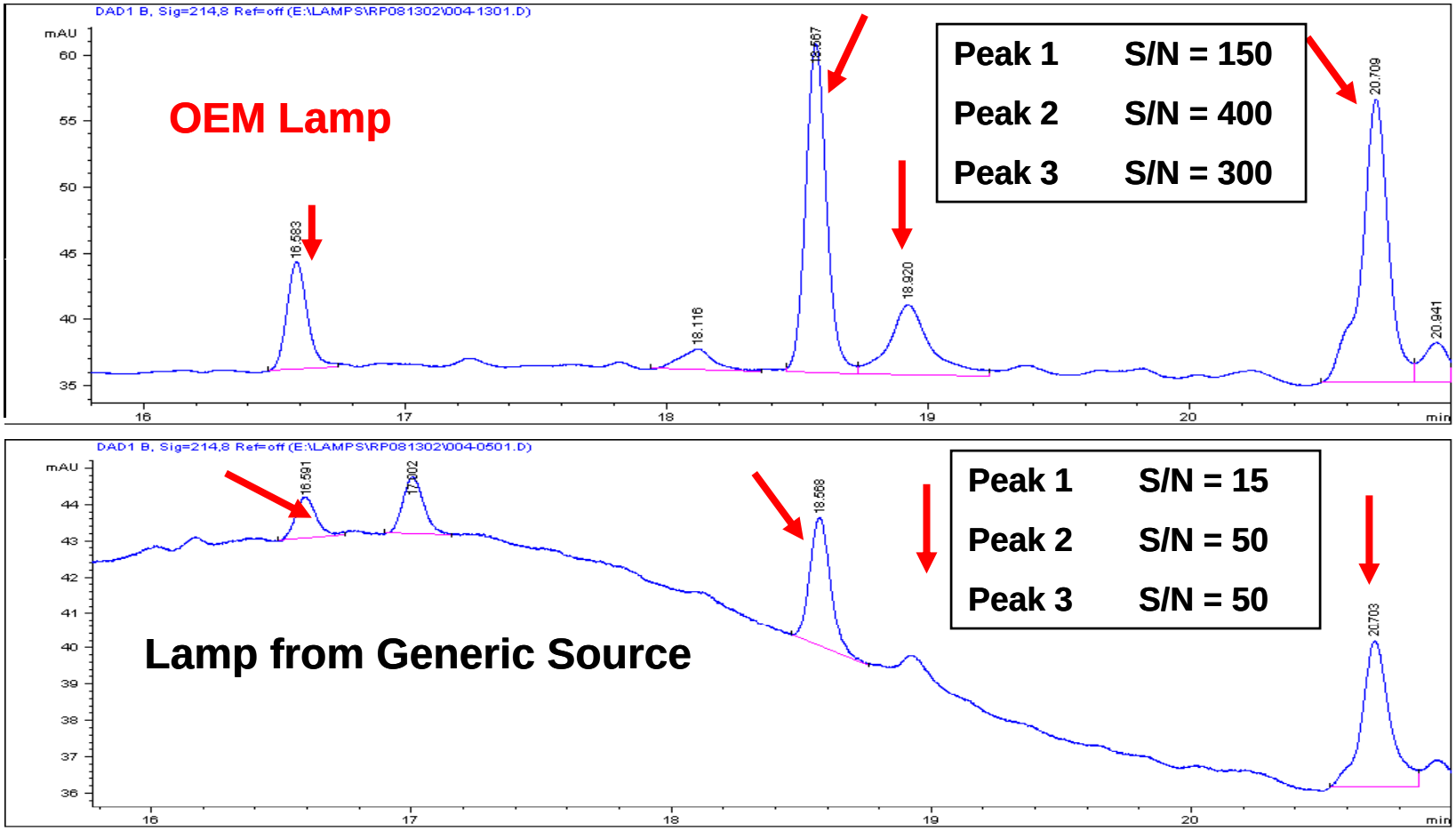
- Poor Detection Due to Incorrect Specification Detector Lamp
- Optimizing Data Collection Rate

Example of Detector Light Path



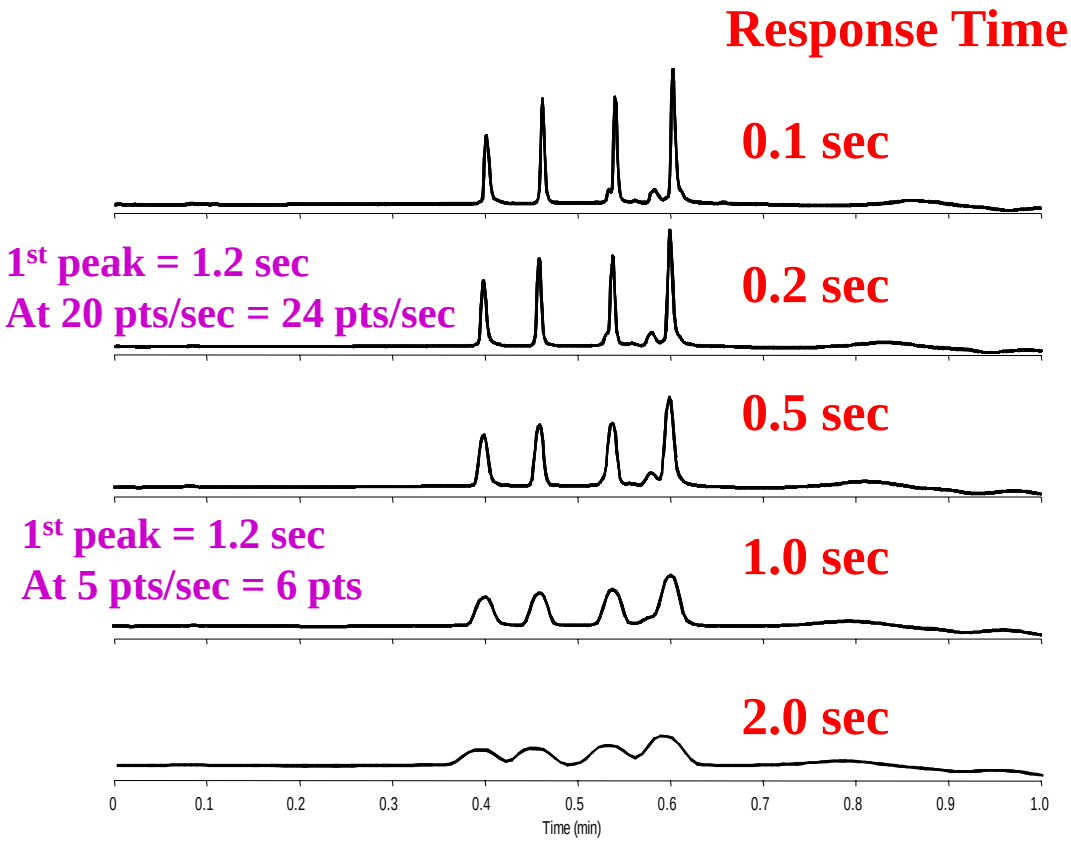


Expanded View of Chromatographic Results
Generic Source Lamp at 214 nm Wavelength



Agilent Technologies

Effect of Detector Response Time on Ultra-Fast Gradient Analyses



Agilent 1100 DAD
Agilent 1100 WPS with ADVR

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

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

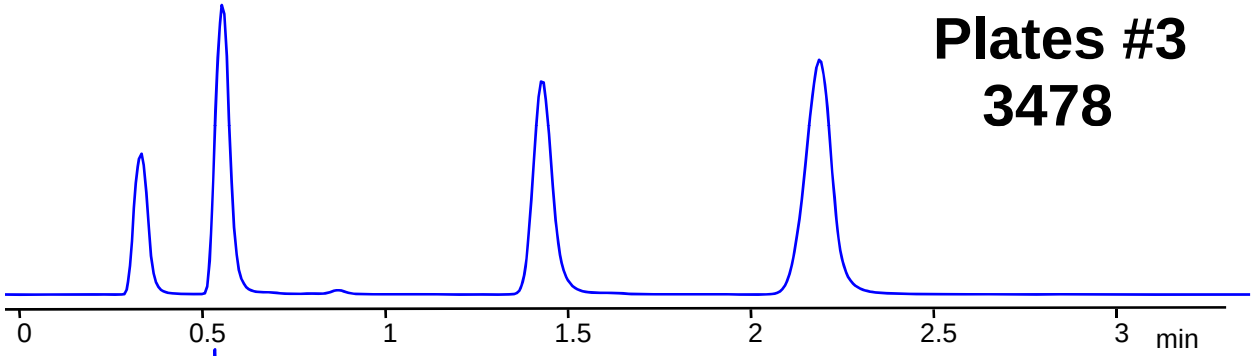
You may have to adjust the response rate of your detector for rapid peak detection.

Optimization of Results with Rapid Resolution HT Columns - Data Acquisition Rate Comparison

Column: ZORBAX Rapid Resolution HT SB-C18 4.6 x 30 mm, 1.8 μm Mobile Phase: 60% Methanol: 40 Water Flow Rate: 1mL/min Temperature: RT Detection: UV 254 nm Sample: QC Test 1. Uracil 2. Phenol 3. 4-Cl-Nitrobenzene 4. Toluene

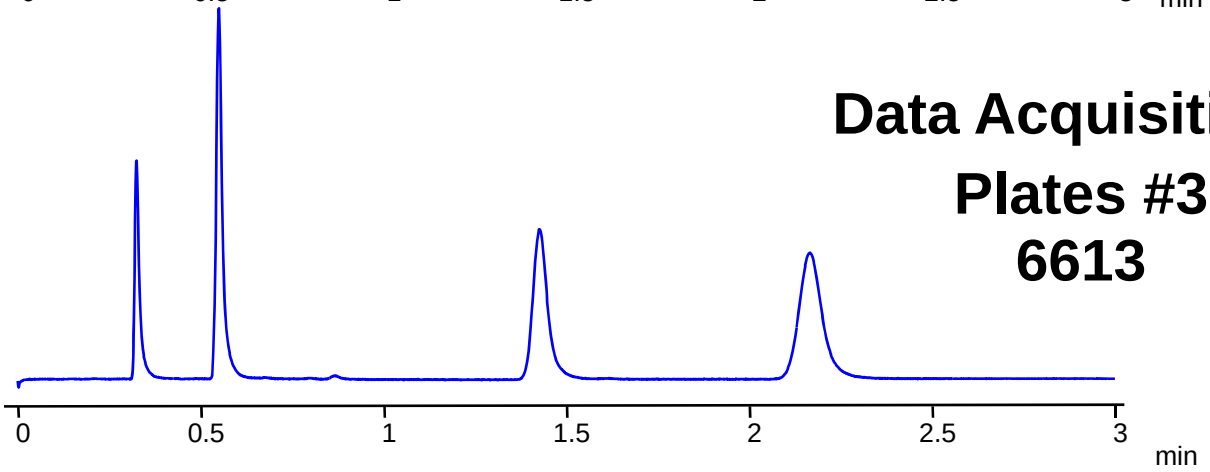
Data Acquisition Rate = 2 sec

Plates #3 **Plates #4**
3478 **4384**



Data Acquisition Rate = 0.1 sec

Plates #3 **Plates #4**
6613 **6138**

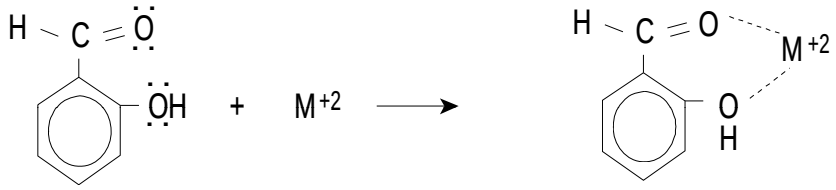


Metal Complexation May Cause Poor Peak Shape

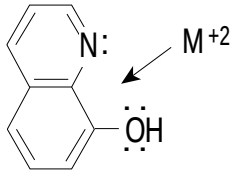
- Analytes that may complex with metals may show poor peak shape
- Both tailing and fronting may result from metal complexation
- Metals are present in LC systems from solvents, tubing, and stainless steel frits
- High purity silica eliminates silica as a source of metals

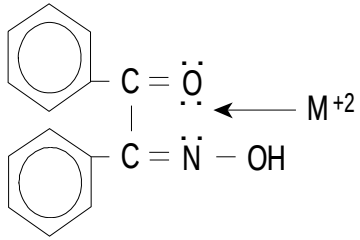
Metal Sensitive Compounds Can Chelate

Hint: Look for Lone Pair of Electrons on :O: or N Which Can Form 5 or 6 Membered Ring with Metal



Salicylaldehyde **6-membered ring complex**



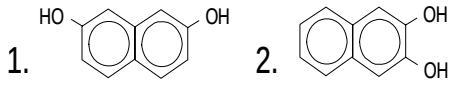


8-hydroxyquinoline
5-membered ring complex

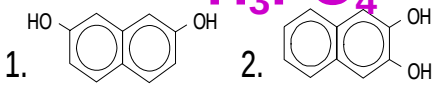
α-benzoinoxomine
5-membered ring complex

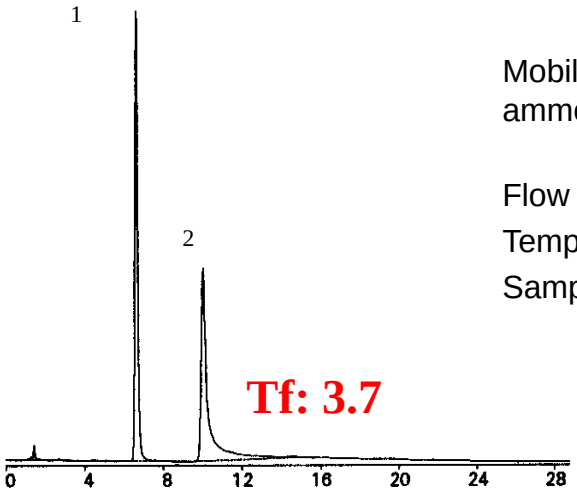
Acid Wash Can Improve Peak Shape

Before Acid Wash



After Acid Wash 50 – 100 mLs 1% H_3PO_4





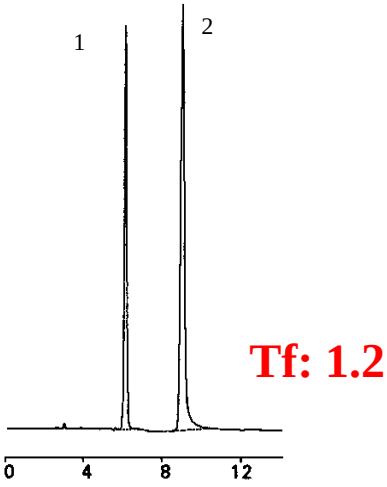
Columns: ZORBAX SB-Phenyl 4.6 x 150 mm

Mobile Phase: 75% 25 mM ammonium phosphate buffer
25% ACN

Flow Rate: 1.0 mL/min.

Temperature: RT

Sample Size: 5 µL



A 1% H_3PO_4 solution is used on SB columns, 0.5 % can be used on endcapped columns.

Conclusions

HPLC column problems are evident as:

- High pressure (prevention better than the cure)
- Undesirable peak shape
- Changes in retention/selectivity

Often these problems are not associated with the column and may be caused by instrument and experimental condition issues.

- pH of mobile Phase
- Instrument Connections
- Detector Settings
- Metal Contamination

APPENDIX

Recommended Buffer Preparation

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.

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).

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).

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

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 3150-057

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.