HPLC Troubleshooting

Improving Poor Performing Separation Methods

Carl Griffin

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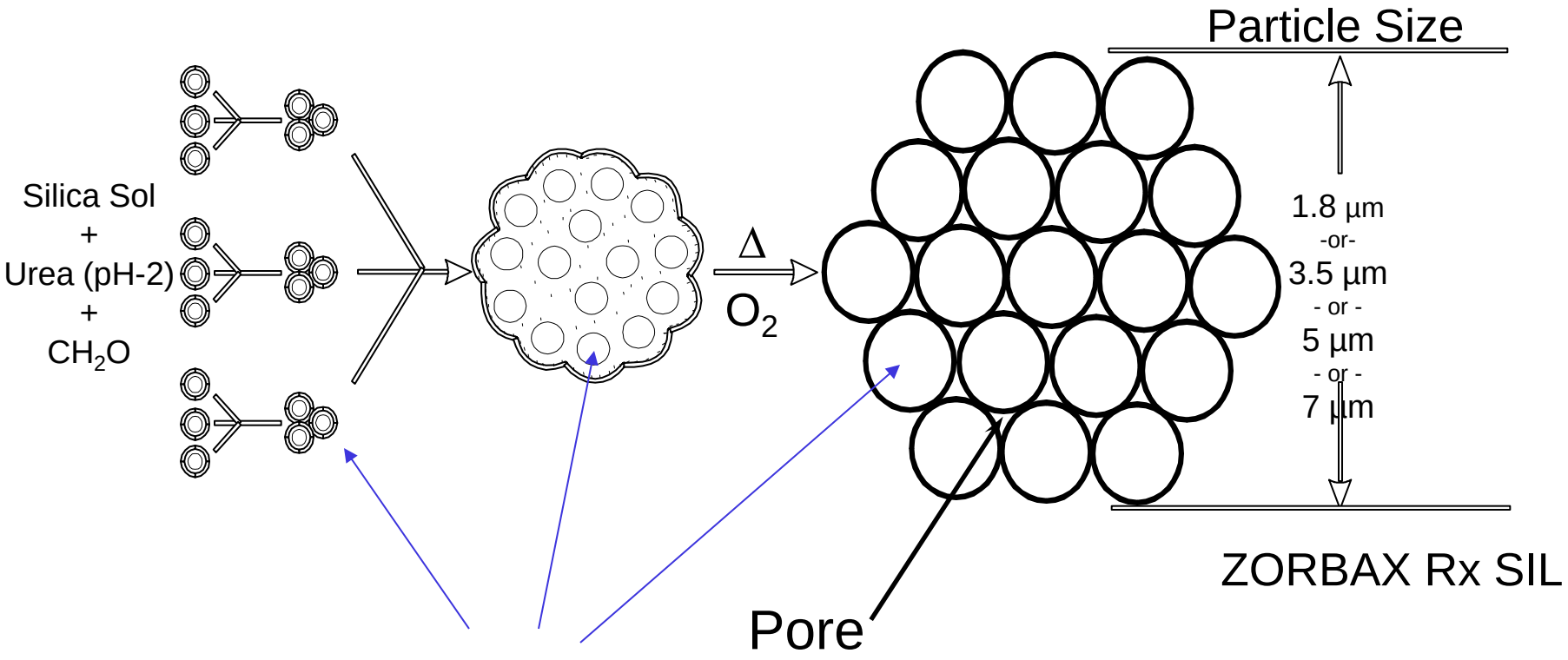
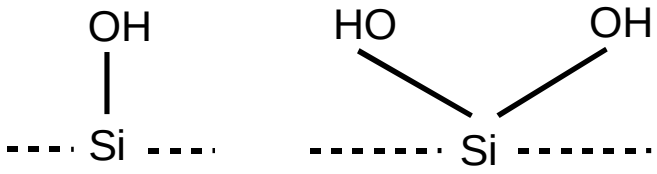
All ZORBAX Totally Porous Particles Use the Same Construction Techniques and Surface Chemistry



Pore Size Determined by Diameter of Sol-Gel

ZORBAX Surface is Thoroughly Hydrolyzed to Produce Ideal Surface Chemistry

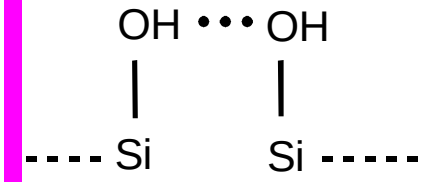
Non-Ideal Surface Re-Hydrolysis



Free Silanols

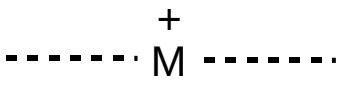
Geminol Silanols

Ideal Surface State -Product of Thorough Hydrolysis

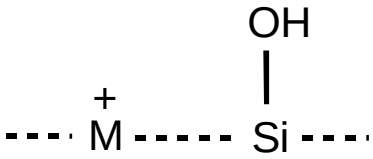


Associated Silanols

decreasing acidity



Surface Metal



Internal Metal (activated silanol) (most acidic)

Caused by Using Impure Raw Material

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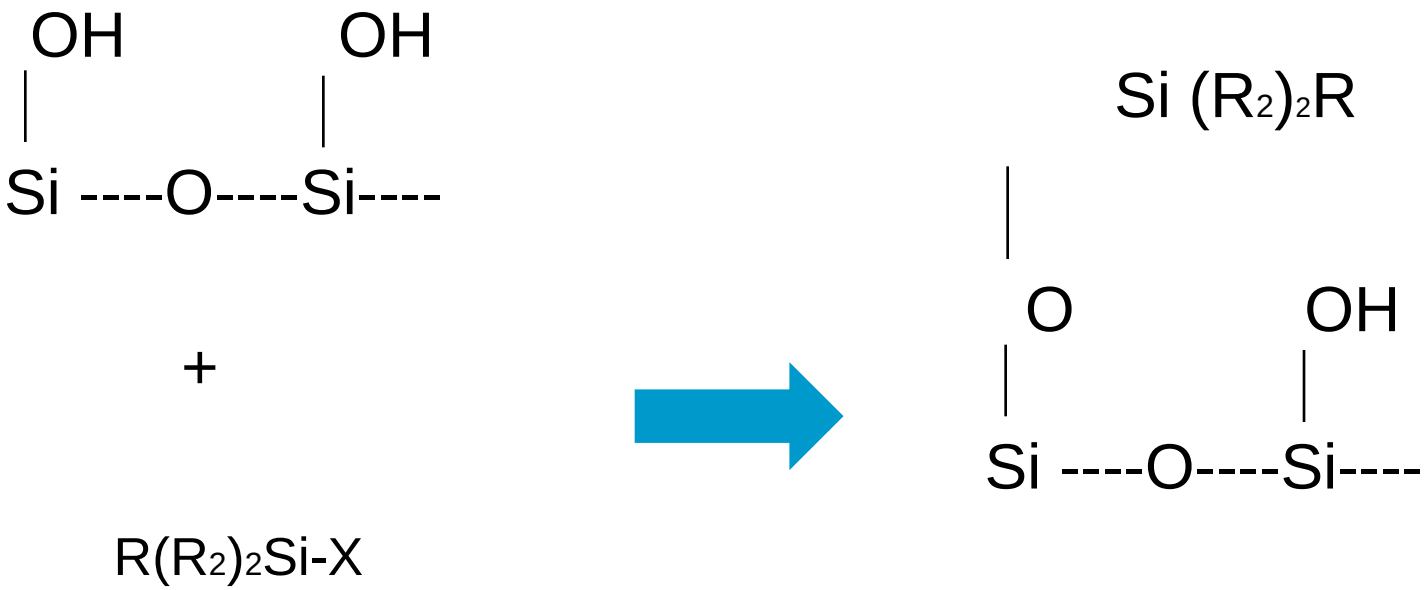
Then the Surface modifier



Storage

End of Day

Wash 10 column volumes of aq. Organic

Store in 50% aq. Acetonitrile

Long Term Storage

Wash with 10 column volumes of aq. Organic

Store in >50% aq. Acetonitrile. Preferably 100% Acetonitrile

Ask The Tough Questions:

- What kind of column is it?
- How many runs has it seen?
- What is the mobile phase and what is its pH?
- Is the Pressure High of Low?
- When was this HPLC system and column last seen working properly?
- What's the sample and what is it made up in?

Other Causes of Death

Poor Mobile Phase Conditions

Read The Manufacturers Literature
pH
Temp.

Most LC columns are good from 2 to 8

What Are Common Troubleshooting Problems?

Column didn't work for my application—need more information

Separation not the same as before (e.g. peak shape, peak doublets, retention shifts, resolution, efficiency loss)

Manufacturing Defects (e.g. leaking from column fitting, blanking nuts (end caps) missing, etc.

Pressure (e.g. too high pressure or too low pressure, fluctuating)

Baseline problems (e.g. drift, noise, spikes, pulses)

Instrument problems (e.g. leaks-pump, injector, detector)

Sample (e.g. recovery low, matrix effects, etc.)

Typical Case History: Lack of Efficiency on Poroshell 300

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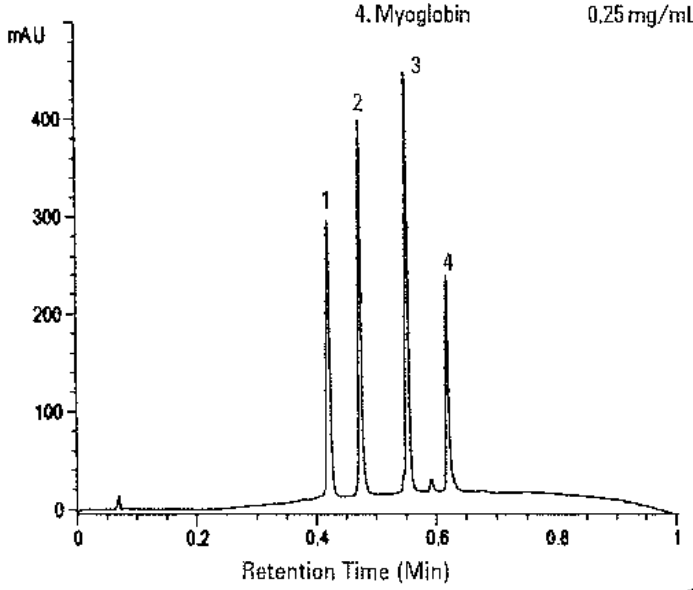
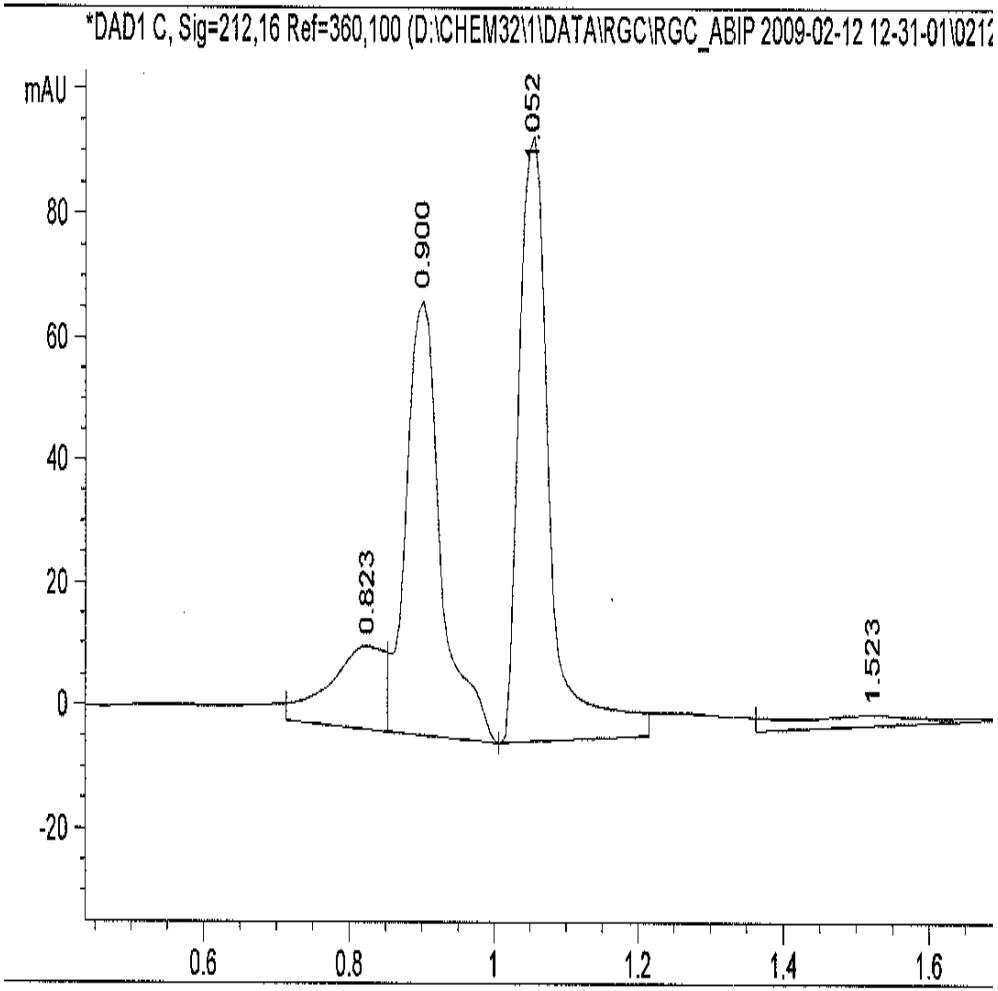
FIGURE 1
POROSHELL 300SB-C18 QA Chromatogram

OPERATING CONDITIONS
Column: POROSHELL 300SB-C18 2.1 mm ID x 75 mm (5µm)
Mobile Phase A: Water with 0.1% TFA
Mobile Phase B: Acetonitrile with 0.07% TFA
Gradient: 0 Minutes 5%B
1.0 Minutes 100%B
Flow Rate: 2.0 mL/min
Temperature: 70°C
Detector: UV (212nm)
Sample Volume: 2 µL
Peak Identity:

1. Neurotensin	0.25 mg/mL
2. Ribonuclease A	0.25 mg/mL
3. Lysozyme	0.25 mg/mL
4. Myoglobin	0.25 mg/mL



Customer Results



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Looked Bad – What Did We Do?

1. Customer needed to understand that the column used – 2.1 x 75mm, Poroshell 300 column---is a low volume column, and therefore, low column volume guidelines should be followed

2. What about the instrument and sample ?

3. Turns out that instrument was quaternary pump 1100, not set up appropriately for low volume use (difficult to optimize completely)

4. Customer provided with details on how to achieve a better low volume set-up, based on 1100-RRHT Kit documents

5. Customer now achieving acceptable results

Categories of Column and System Problems

A. Pressure

B. Peak shape

C. Retention

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Pressure Issues

Column Observations	Potential Problems
High pressure	- Plugged frit
	- Column contamination
	- Plugged packing
Low Pressure	- Leak
	- Flow Incorrect
Fluctuating Pressure	- Pump not operating correctly
	- Check valves failing

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Determining the Cause and Correcting High Back Pressure

Check pressure with/without column - many pressure problems are due to blockages elsewhere in the system.

If Column pressure remains high:

Rinse column (remove detector from flow path!)

- Eliminate column contamination and plugged packing
- high molecular weight/adsorbed compounds
- precipitate from sample or buffer

Back flush column – may clear plugged column inlet frit

Install New column

Eliminate pressure issues – add a disposable 0.2, 0.5 or 2 um in-line filter to system.

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.

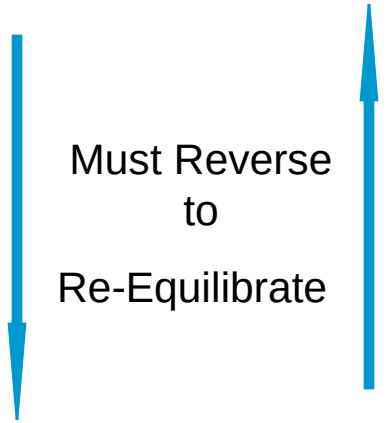
This Is Time Consuming

Often Performed Offline

- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile

- 75% Acetonitrile:25% Isopropanol

- 100% Isopropanol
- 100% Methylene Chloride*
- 100% Hexane*



Must Reverse to

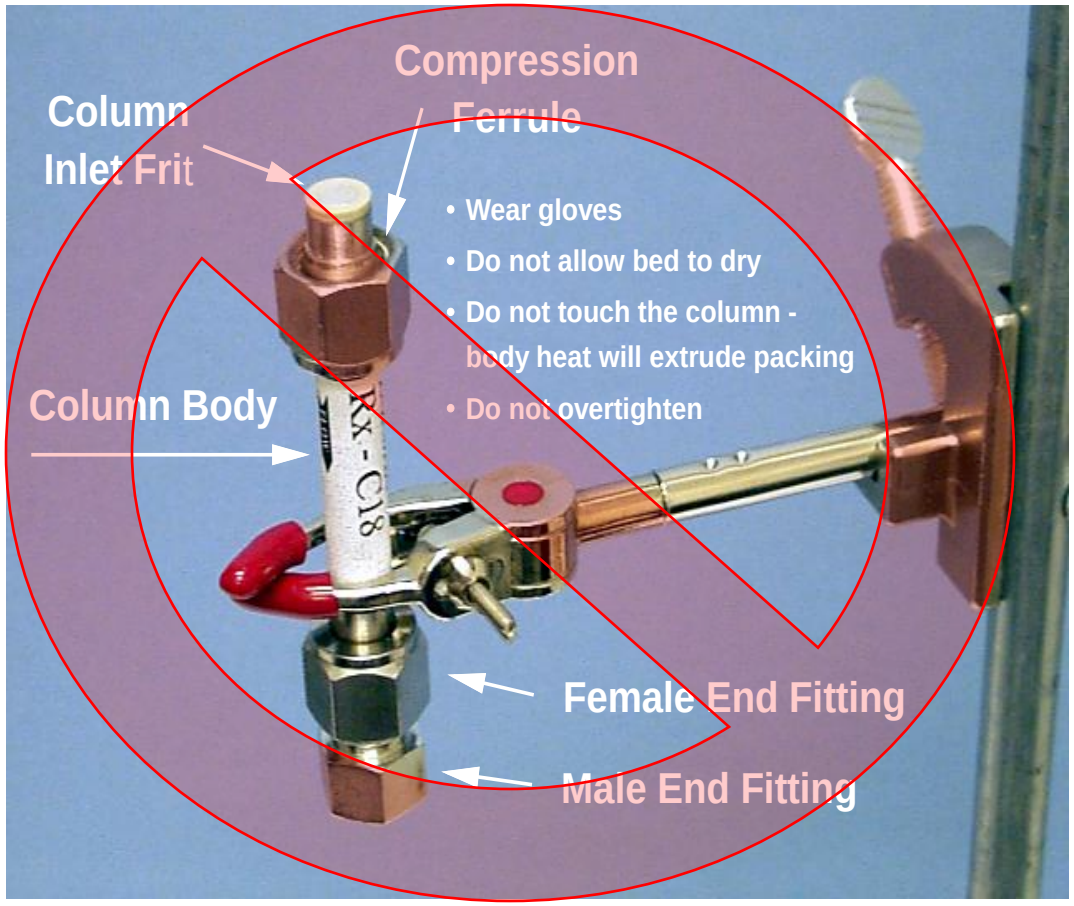
Re-Equilibrate

*Tip: When using either Hexane or Methylene Chloride the column with Isopropanol before returning to your reversed-phase mobile

Changing a Frit May Not Be a Good Idea

May not be possible with new generation columns

May damage high performance columns



Tip: Prevention is a Much Better Idea!

The Trick:
Prevention Techniques - A Better Choice!

- Use column protection
  - In-line filters
    - Guard columns
- Filter samples
- Filter buffered mobile phases

Easy

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

Not As Easy

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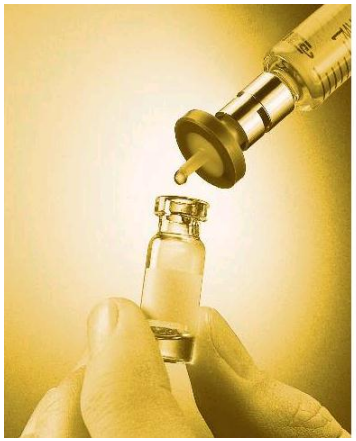
Inexpensive Filters Prevent Column Frit Plugging



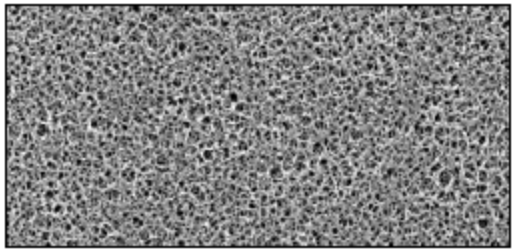
Regenerated Cellulose (RC) Recommended

•Universal hydrophilic membrane, compatible with most solvents - aqueous and organic

•High purity, extremely low extractables and binding

•More Uniform Surface

•*<u>Different than Other Cellulose Filters!!</u>*

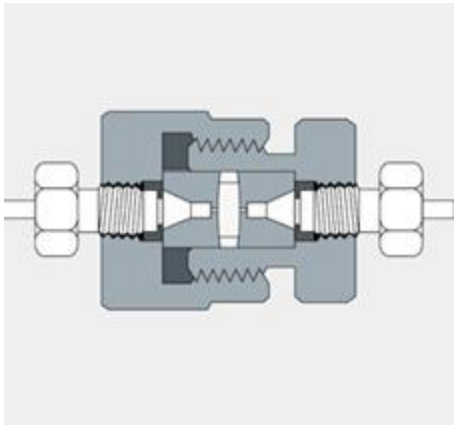


In-line Filters Easy to Use and replace

Frits Available in 0.2,0.5 and 2.0µ Porosity

Much Less expensive than a Column

Easier and Faster to Replace than a Column Frit



What Are Common Peak Shape Issues?

1. Split peak(s)
2. Peak tailing/fronting
3. Broad peak

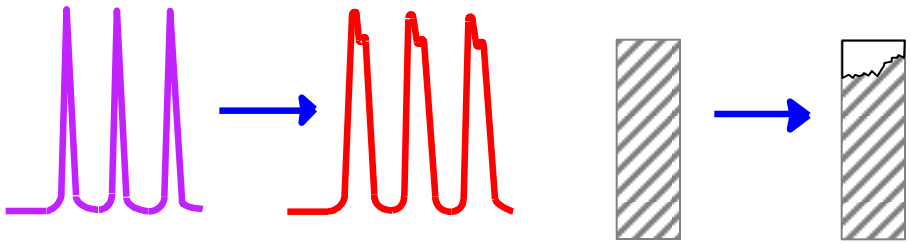
• Many peak shape issues are also combinations - i.e. broad and tailing or tailing with increased retention

•Symptoms do not necessarily affect all peaks in the chromatogram

•Each of these problems can have multiple causes

Peak Splitting Caused By Disrupted Sample Path

- **Flow Path Disrupted by Void**
- **Sample Allowed to Follow Different Paths Through Column**
- **Poorly Packed Bed Settles in Use**
- **High pH Dissolves Silica**



Normal **Double Peaks**

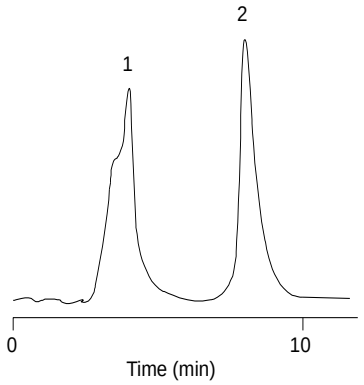
Split or Double Peaks

Tip: Similar Effect Can be Caused by Partially Plugged Frit

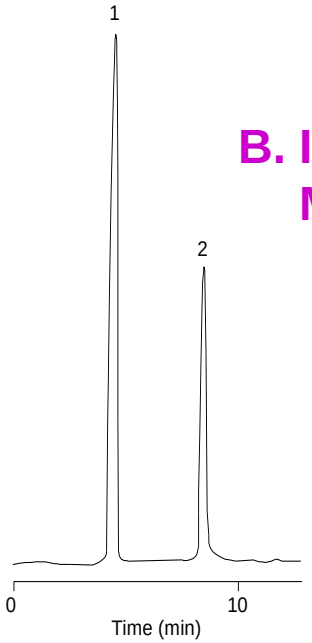
Split Peaks from Injection Solvent Effects

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



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

Trick: Keep Organic Concentration in Sample Solvent $\leq$ Mobile Phase

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Peak Tailing, Broadening and Loss of Efficiency

May be caused by:

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

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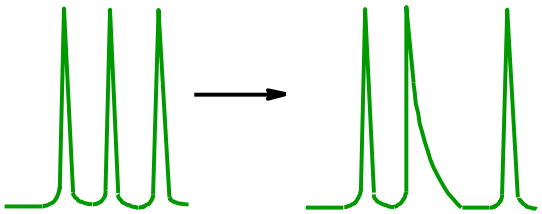
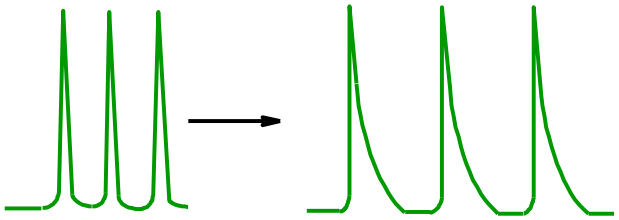
Peak Shape: Tailing Peaks

Symmetry > 1.2



Normal Tailing



Normal Tailing

Causes

Some Peaks Tail:

- Secondary - Retention Effects.
- Residual Silanol Interactions.
- Small Peak Eluting on Tail of Larger Peak.

All Peaks Tail:

- Extra-Column Effects.
- Build up of Contamination on Column Inlet.
- Heavy Metals.
- Bad Column.

Peak Tailing

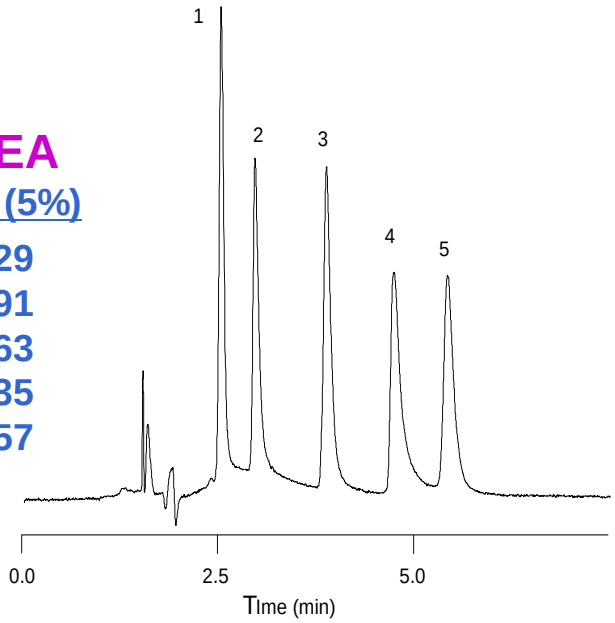
Identifying Column "Secondary Interactions"

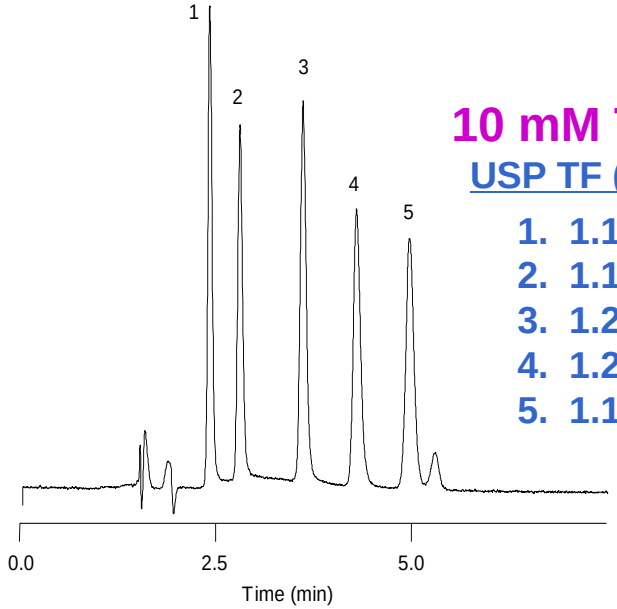
Column: Alkyl-C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 pH 7.0 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35° C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

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

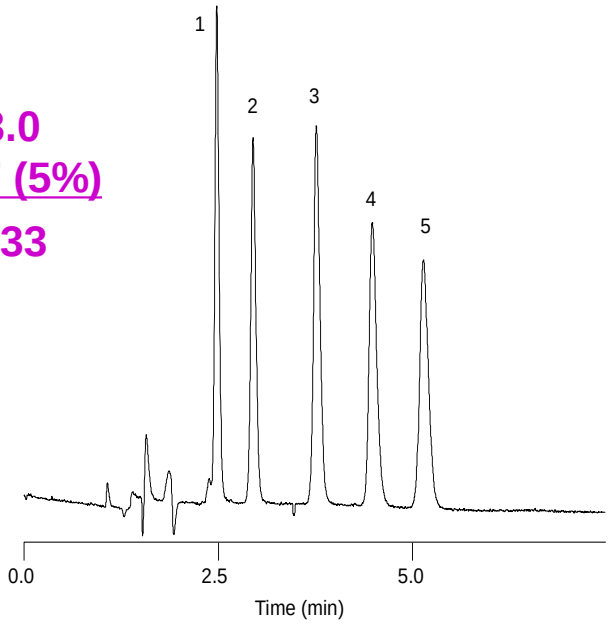
Tip: Mobile phase modifier (TEA = triethylamine) competes with sample molecule for surface ion exchange sites at mid-range pH values

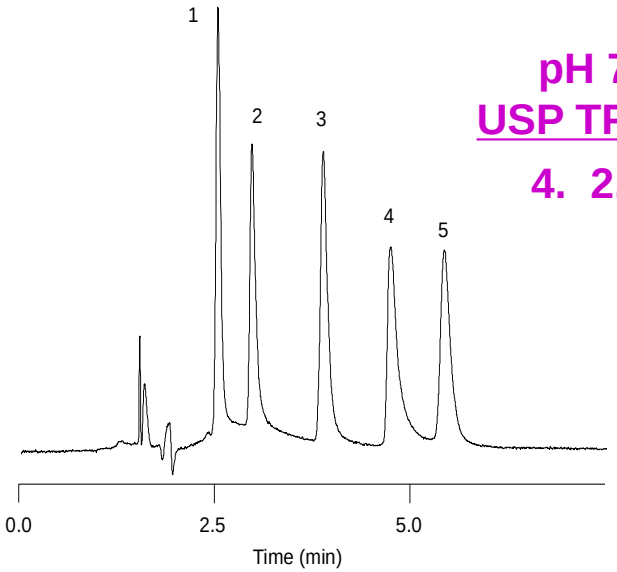
Peak Tailing

Low pH Minimizes "Secondary Interactions" for Amines

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

Tip: Reducing mobile phase pH reduces interactions with silanols and peak tailing.

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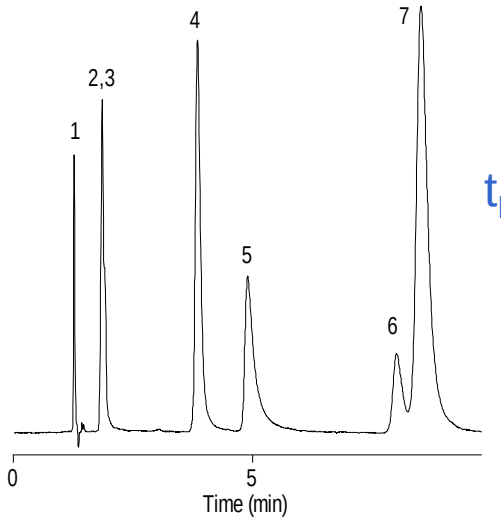
Peak Tailing

High pH Eliminates "Secondary Interactions" for Amines

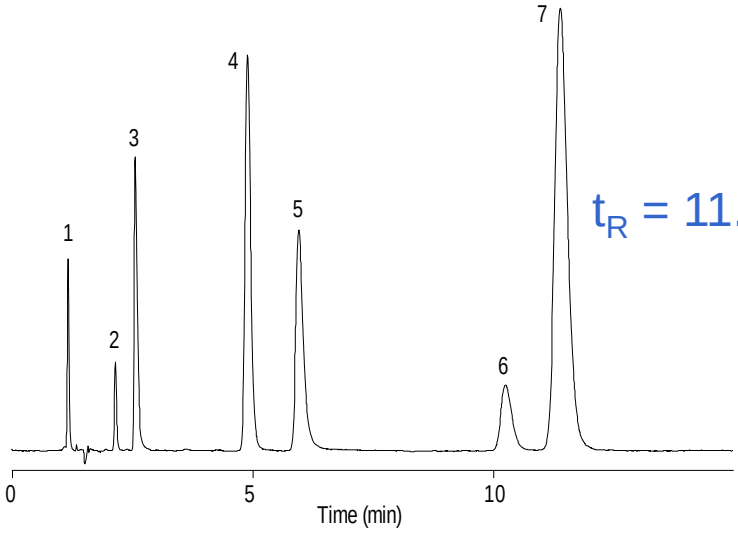
Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 m m Mobile Phase: See Below Flow Rate: 1.0 mL/min Temperature: RT
Detection: UV 254 nm
Sample 1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7
30% 20 mM Na_2HPO_4
70% MeOH



t_R = 8.5

pH 11
30% 20 mM TEA
70% MeOH



t_R = 11.4

Peak Shape and Retention of this sample of basic compounds improves at high pH where column has high IEX activity. *Why?*

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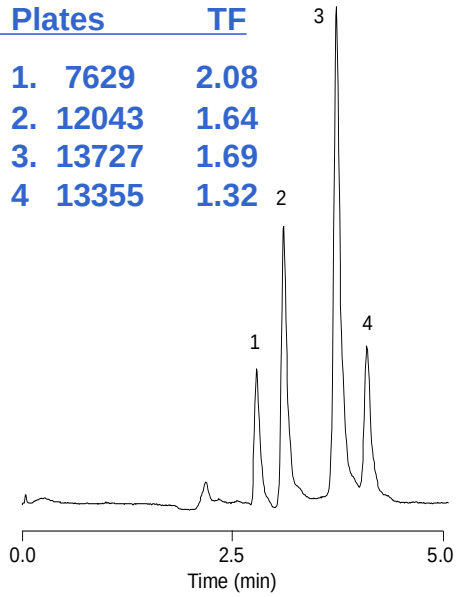
Peak Tailing - Column Contamination

Tip: Quick Test to Determine if Column is Dirty or Damaged

Trick: Reverse Column and Run Sample –If Improved, Possible Cleaning Will Help -No improvement-Column Damaged and Needs to be Replaced

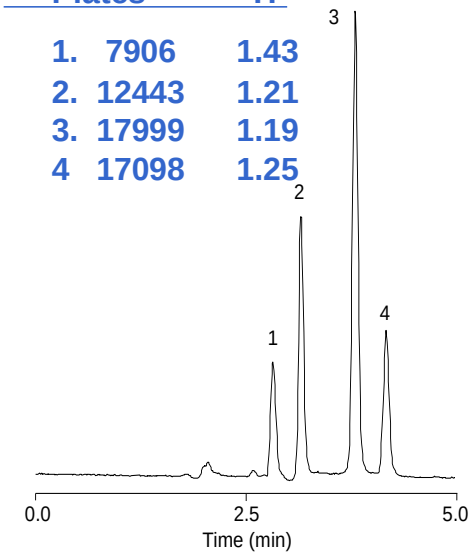
QC test forward direction

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



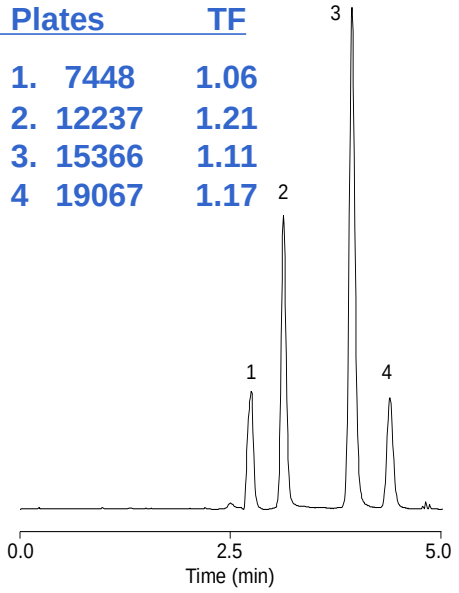
QC test reverse direction

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



QC test after cleaning 100% IPA, 35˚ C

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



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

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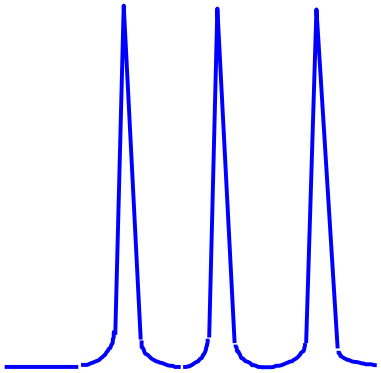
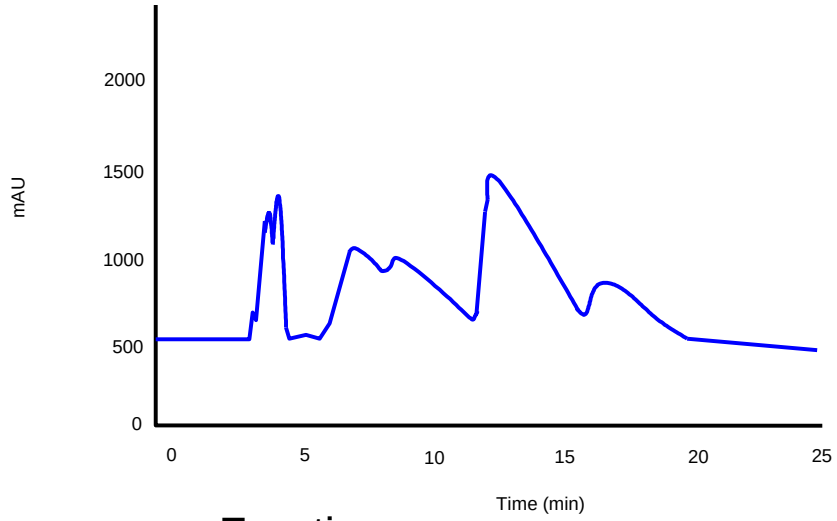
Peak Shape: Fronting Peaks



Normal



Fronting

Symmetry < 0.9

Causes:

- Column Overload-reduce sample size

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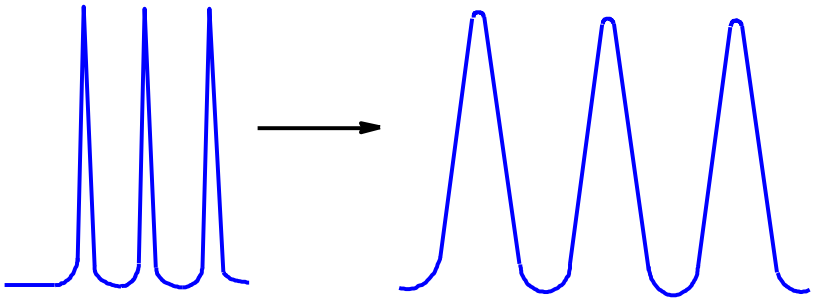
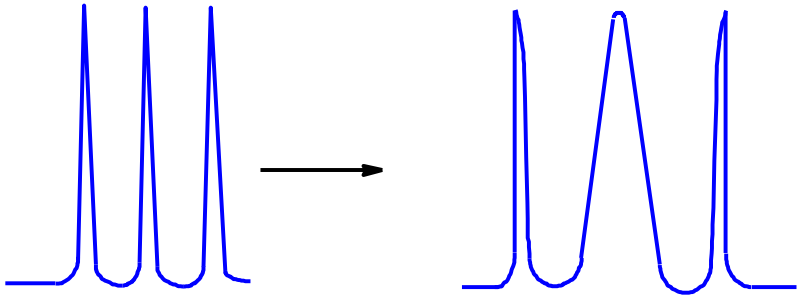
Peak Shape: Broad Peaks



All Peaks Broadened:

- Loss of Column Efficiency.
- Column Void.
- Large Injection Volume.



Some Peaks Broadened:

- Late Elution from Previous Sample (Ghost Peak).
  - High Molecular Weight.
  - Sample - Protein or Polymer.

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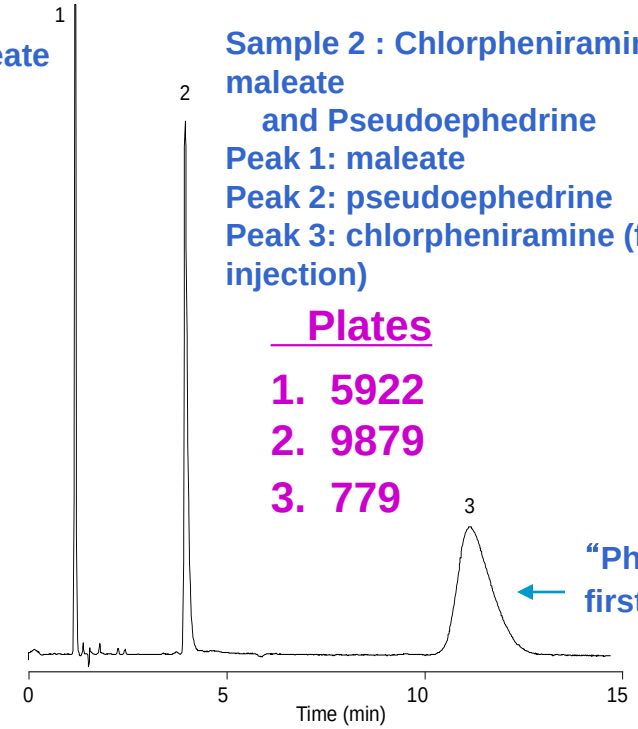
Unknown “Phantom” Peaks

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

Tip: The extremely low plates for moderately retained peaks are an indication of a very late eluting peak from a preceding run.

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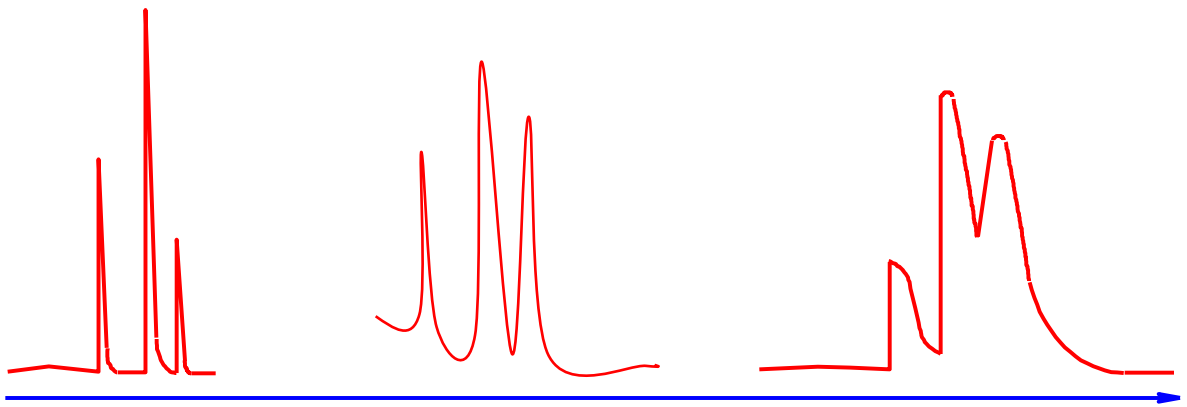
Extra-Column Dispersion (Volume)



- Use short, small internal diameter tubing between the injector and the column and between the column and the detector.
- Make certain all tubing connections are made with matched fittings.
- Use a low-volume detector cell.
- Inject small sample volumes.

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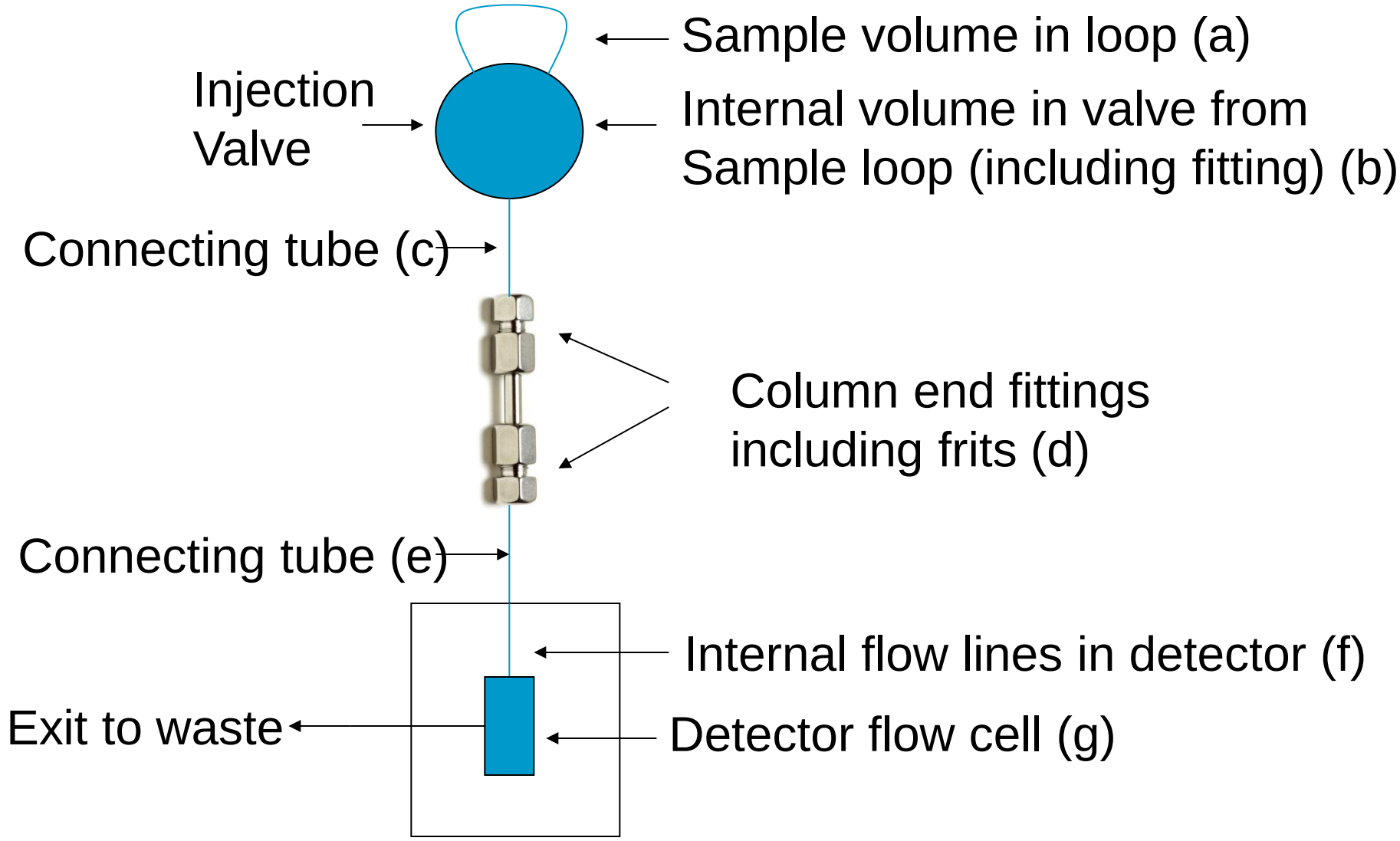
Extra-Column Volumes in HPLC Sample Flow System



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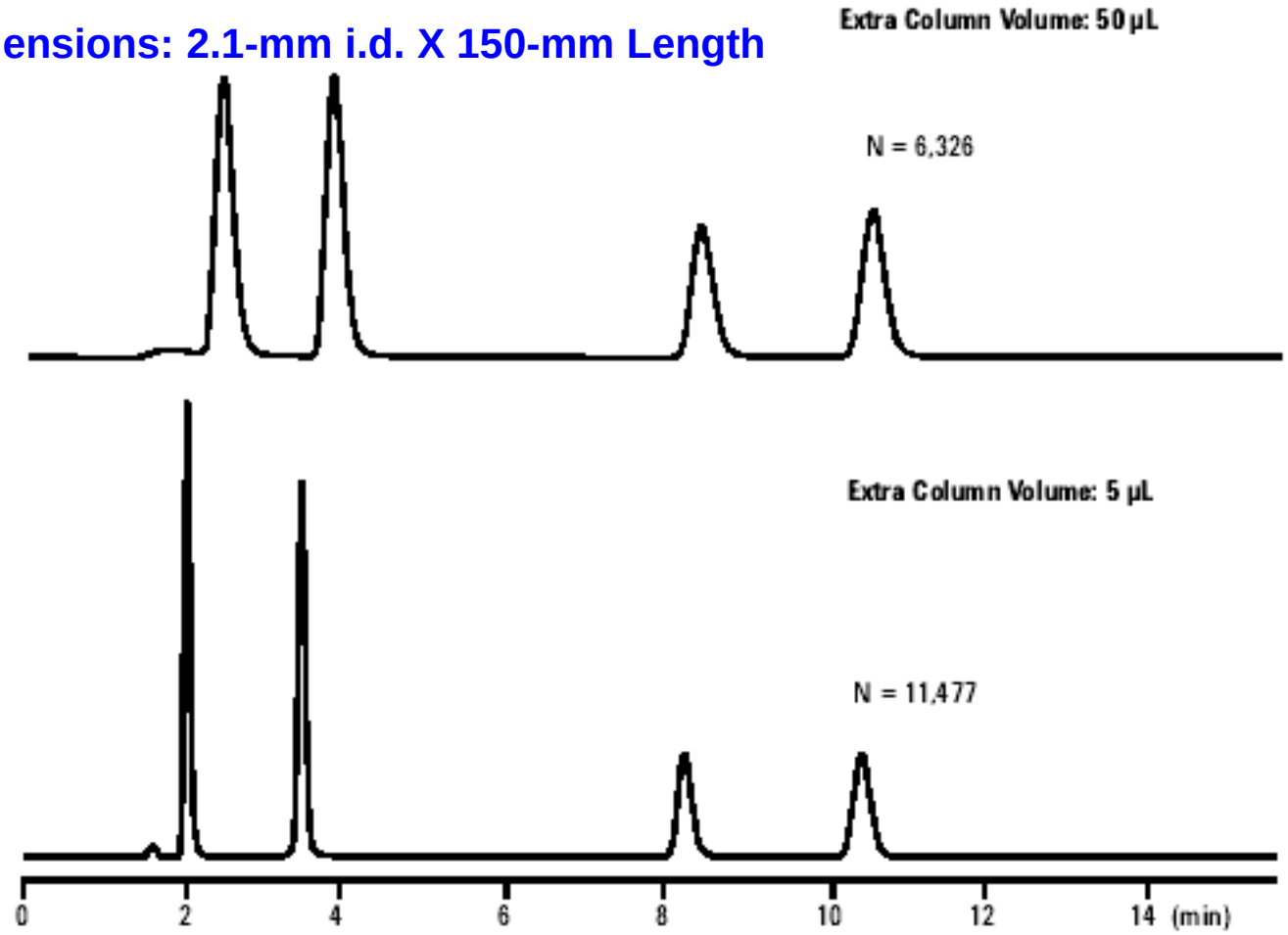
The Effects of Extra-Column Dispersion (Volume) on Narrowbore Column Performance

Column Dimensions: 2.1-mm i.d. X 150-mm Length

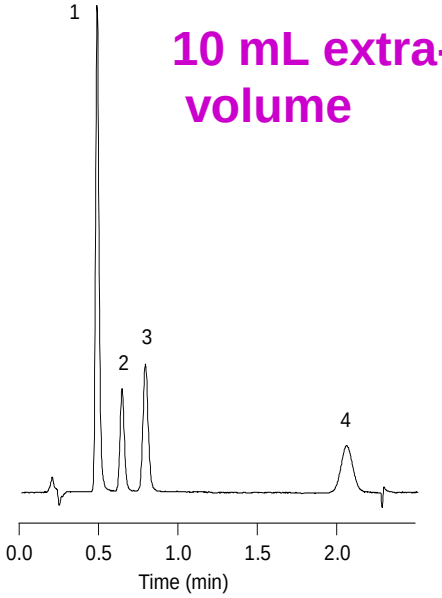


another example....

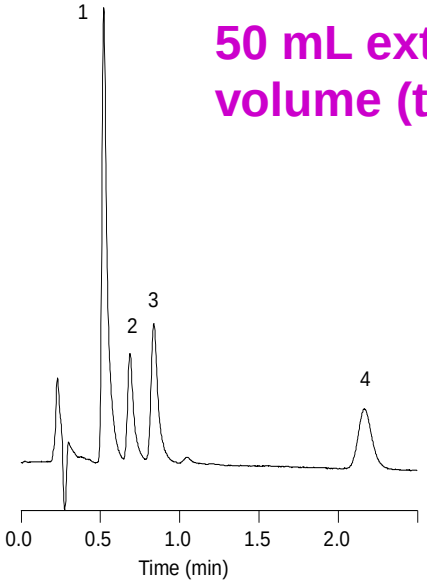
Peak Broadening
Extra-Column Volume

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 mL extra-column volume



50 mL extra-column volume (tubing)



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Tip: Poorly Made HPLC System Connections Can Cause Peak Broadening

The System Has Been Optimized and :

- All Tubing Lengths Are Minimum
- Smallest Diameter Tubing Used
- Proper Flow Cell Volume

Symptom Still Seems to Have Too Much Extra-Column Volume

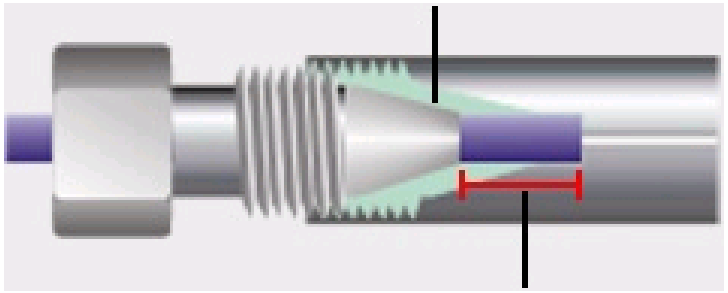
What Is Wrong?

Have You Made the Connections Properly?

What Happens If the Connections Are Poorly Made?

Wrong … too long

Ferrule cannot seat properly

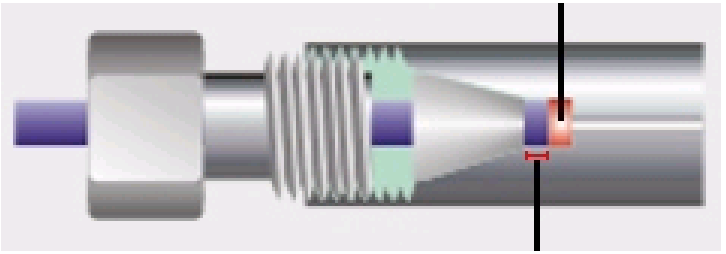


X

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

These poor connections cause:

- Poor efficiency
- Peak tailing
- Leaking

Changes in Retention (k) - Same Column, Over Time

May be caused by:

1. Column aging
2. Column contamination
3. Insufficient column equilibration
4. Poor column/mobile phase combination
5. Change in mobile phase
6. Change in flow rate
7. Change in column temperature
8. Other instrument issues (e.g. different Gradient Delay Volumes)

Separation Conditions That Cause Changes in Retention*

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

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

Determining the Cause of Retention Changes

Same Column

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

Change in Retention/Selectivity

Column-to-Column

1. Different column histories (aging)
2. Insufficient/inconsistent equilibration
3. Poor column/mobile phase combination
4. Change in mobile phase
5. Change in flow rate
6. Other instrument issues
7. Slight changes in column bed volume (t_r only)

Case History: Problem – Selectivity Does Not Appear the Same from Column-to-Column

Details:

- 3 Columns with the Same Bonded Phase were used
- They were the same dimensions, just different particle sizes (and therefore different lots of material)
- They were tested on the same day on the same instrument and with the same mobile phase
- Isocratic elution; binary (two channel) pump generated mobile phase

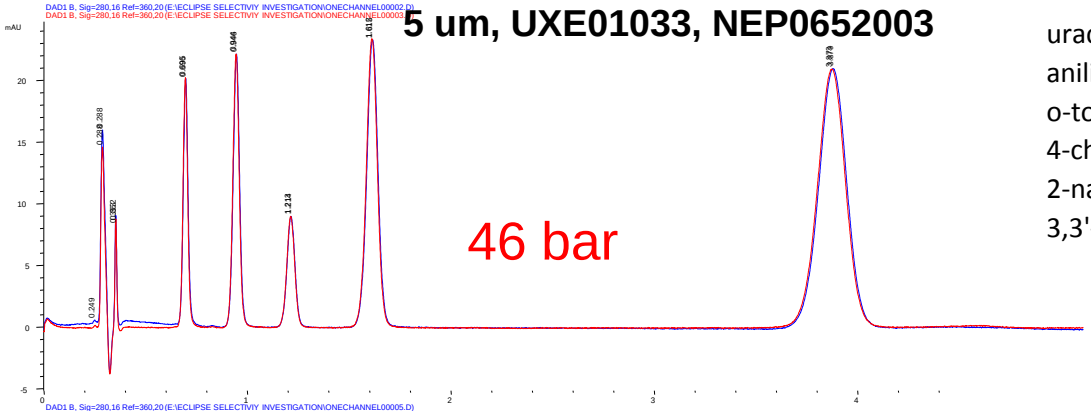
Problem:

- The selectivity was different on each of the columns

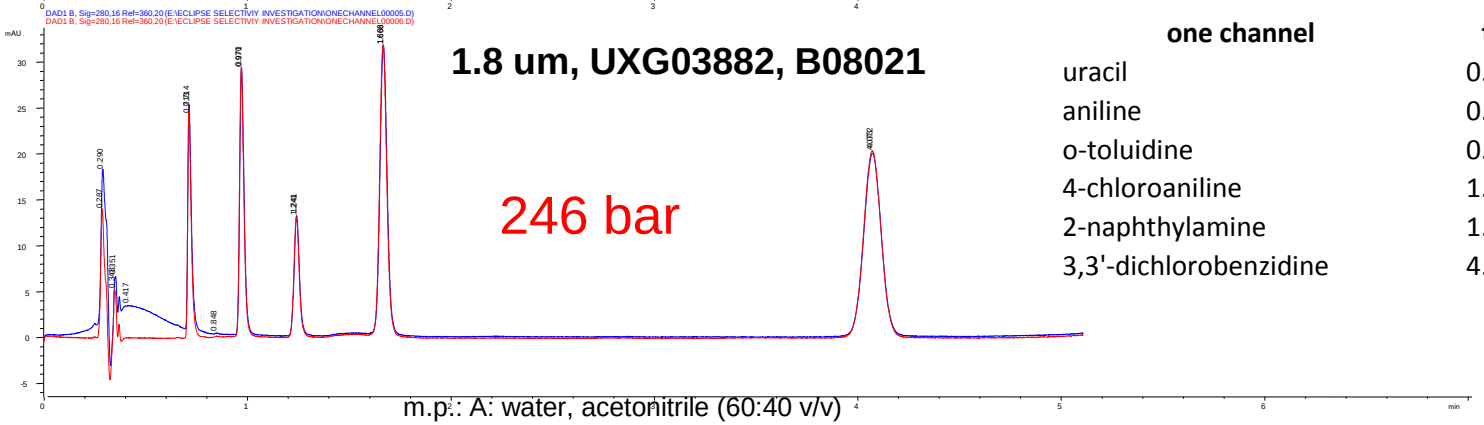
What Questions Should We Ask and What Should We Try to diagnose problem?

- Compare more columns – see if they are different—is this practical?
- Try another LC
- Premix the mobile phase

One channel premixed mobile phase shows similar α whereas previous two channels doesn't show similar α!



one channel	tr	k	alpha
uracil	0.29	0.00	
aniline	0.69	1.38	#DIV/0!
o-toluidine	0.94	2.24	1.63
4-chloroaniline	1.21	3.17	1.42
2-naphthylamine	1.61	4.55	1.43
3,3'-dichlorobenzidine	3.87	12.34	2.71

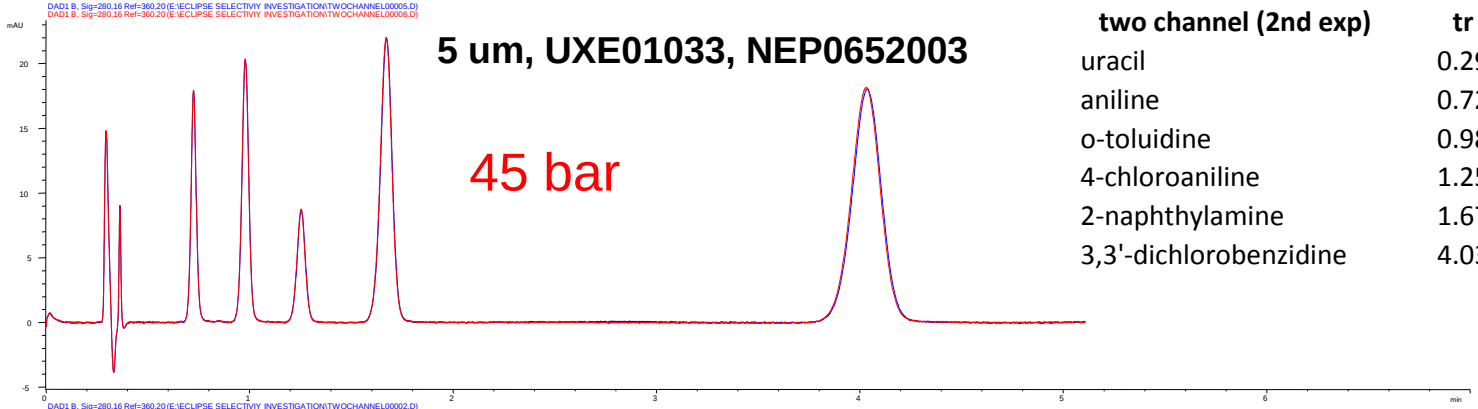


one channel	tr	k	alpha
uracil	0.29	0.00	
aniline	0.71	1.45	#DIV/0!
o-toluidine	0.97	2.34	1.62
4-chloroaniline	1.24	3.28	1.40
2-naphthylamine	1.67	4.76	1.45
3,3'-dichlorobenzidine	4.07	13.03	2.74

m.p.: 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

Repeat experiment of two channel isocratic mobile phase confirms different selectivity

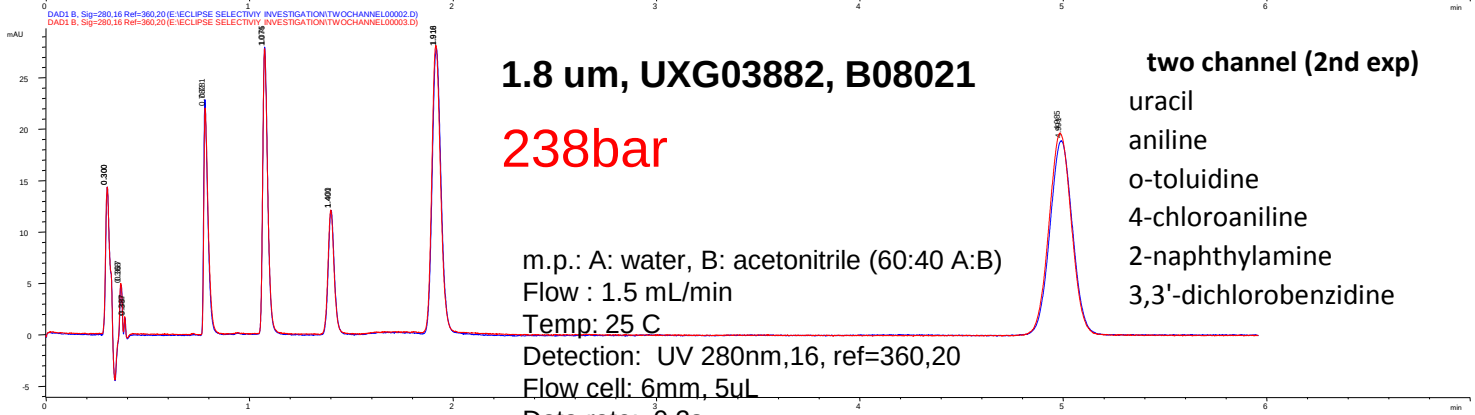
probably due to wrong composition of mobile phase being delivered



5 um, UXE01033, NEP0652003

45 bar

two channel (2nd exp)	tr	k	alpha
uracil	0.29	0.00	
aniline	0.72	1.48	#DIV/0!
o-toluidine	0.98	2.38	1.60
4-chloroaniline	1.25	3.31	1.39
2-naphthylamine	1.67	4.76	1.44
3,3'-dichlorobenzidine	4.03	12.90	2.71



1.8 um, UXG03882, B08021

238bar

m.p.: A: water, B: acetonitrile (60:40 A:B)
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

two channel (2nd exp)	tr	k	alpha
uracil	0.3	0.00	
aniline	0.78	1.60	#DIV/0!
o-toluidine	1.07	2.57	1.60
4-chloroaniline	1.4	3.67	1.43
2-naphthylamine	1.92	5.40	1.47
3,3'-dichlorobenzidine	4.98	15.60	2.89

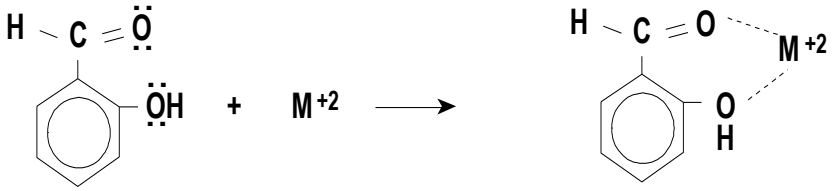
Causes of Retention Changes

...other parameters to explore

1. **Chelation properties of solute**
2. **Column “aging”**
3. **Determine pH sensitivity for ionizable compounds– use of buffers**
4. **Dwell volume (gradient delay volume) of instrument in gradient elution**
5. **Method and detection issues**
6. **Contact your supplier for additional support**

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

a-benzoinoxomine
5-membered ring complex

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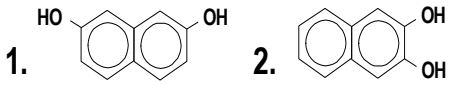
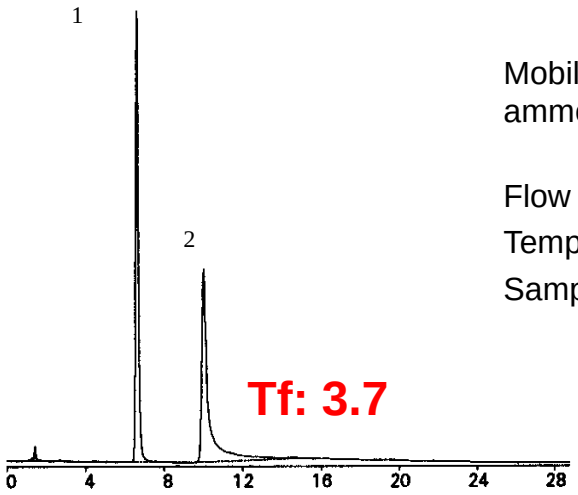
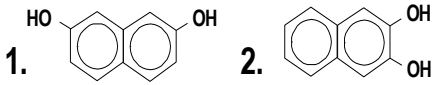
Acid Wash Can Improve Peak Shape

Before Acid Wash





After Acid Wash
50 – 100 mLs 1% H_3PO_4



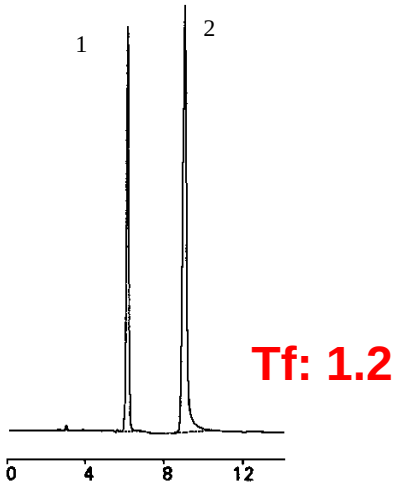
Columns: **ZORBAX SB-Phenyl 4.6 x 150 mm**

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

Flow Rate: 1.0 mL/min.

Temperature: RT

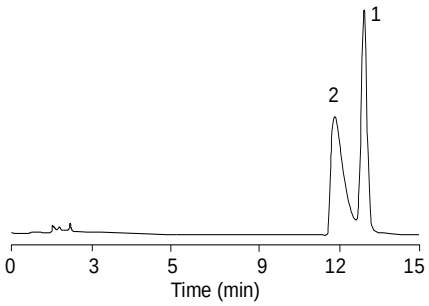
Sample Size: 5 mL



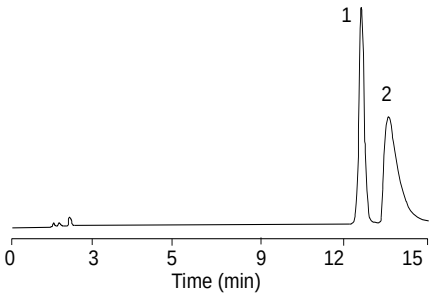
- A 1% H_3PO_4 solution is used on SB columns, 0.5 % can be used on endcapped columns.

Column Aging/Equilibration Causes Retention/Selectivity Changes

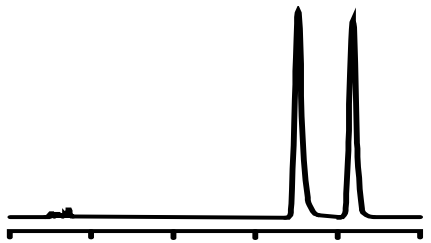
Column 1 - Initial



Column 1 - Next Day



Column 1 - After Cleaning with 1% H_3PO_4 /Equilibration



- The primary analyte was sensitive to mobile phase aging/ conditioning of the column
- The peak shape was a secondary issue (metal chelating compound) resolved by “de-activating” the active metal contamination

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

- pH Effects Ionization
  - Silica Surface of Column
  - Sample Components of Interest

- Buffers
  - Resist Changes in pH and Maintain Retention
  - Improve Peak Shape for Ionizable Compounds

- Effects Column Life
  - Low pH strips Bonded Phase
  - High pH Dissolves Silica

Use of Buffers Can Minimize Change in Retention/Selectivity

Evaluate:

- Method ruggedness
  - Type of buffer used
  - Ionic strength of buffer
  - Buffer capacity

- pH sensitivity
  - Of sample
  - Of column

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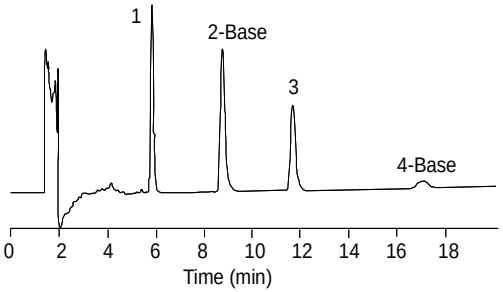
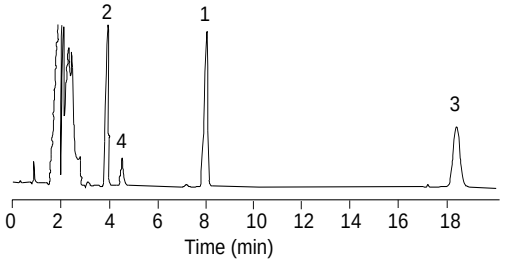
Case History: Lot-to-Lot Selectivity Change Related to pH Choice

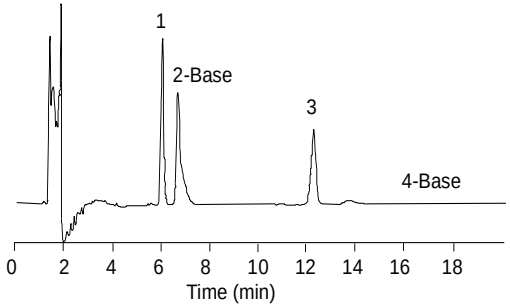
pH 4.5 - Lot 1



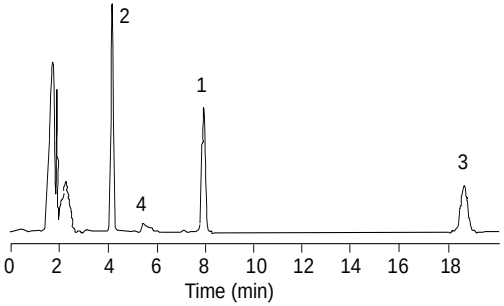
pH 3.0 - Lot 1



pH 4.5 - Lot 2



pH 3.0 - Lot 2

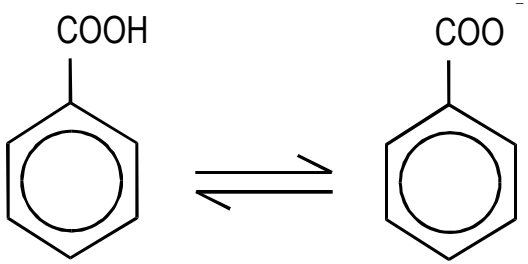


- pH 4.5 shows selectivity change from lot-to-lot for basic compounds
- pH 3.0 shows no selectivity change from lot-to-lot
- Indication of poorly controlled ionization

Why Worry About pH?
pH, pKa and Weak Acids

$$RCOOH \rightleftharpoons RCOO^- + H^+$$

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



$$+ \quad H^+$$

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

$pK_a = 4.2$

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

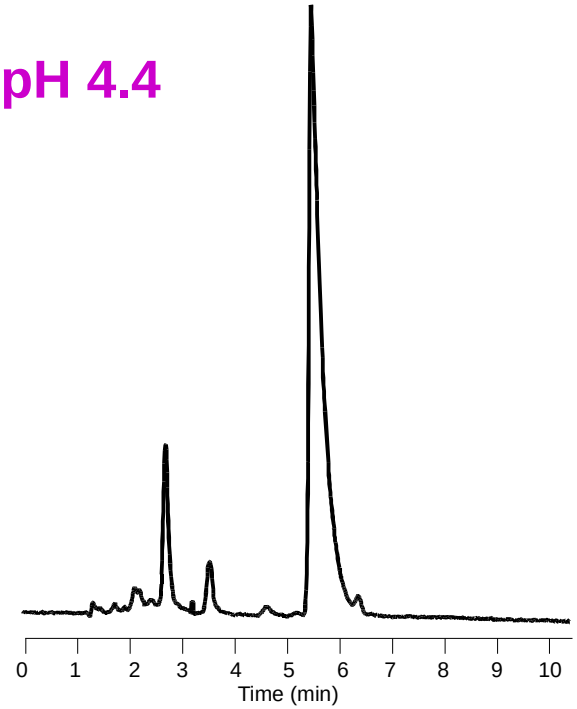
At pH 5.2 – 91% of the sample exists as the benzoate ion. RP retention decreases.

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

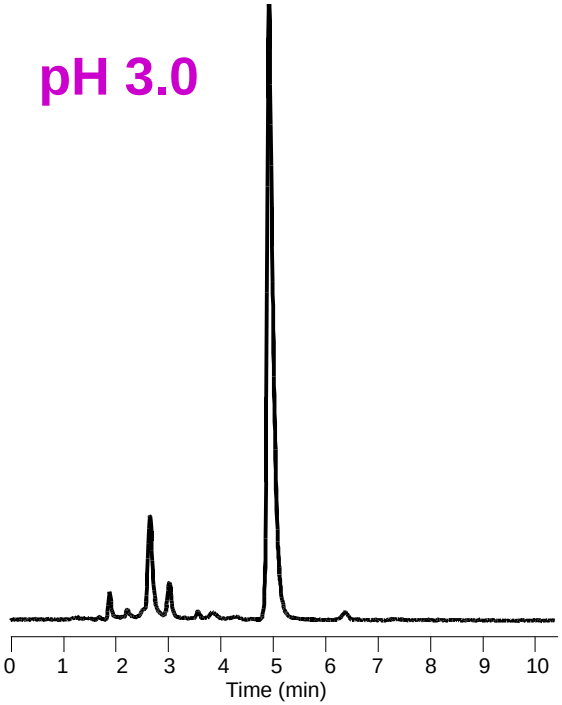
Effect of pH on Peak Shape at or Near the Sample pK_a

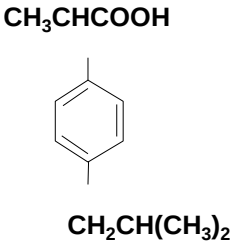
Column: ZORBAX SB-C8 4.6 x 150 mm, 5 mm Mobile Phase: 40% 5 mM KH_2PO_4: 60% ACN
Flow Rate: 1.0 mL/min. Temperature: RT

pH 4.4



pH 3.0





Ibuprofen
pK_a = 4.4

- Inconsistent and tailing peaks may occur when operating close to an analyte pKa and should be avoided.

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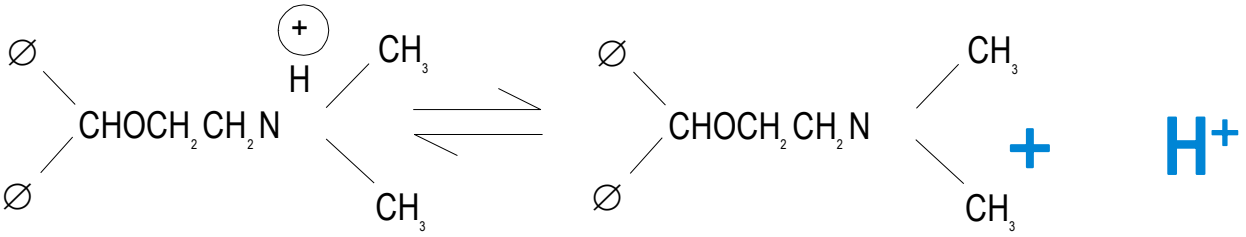
Why Worry About pH?
pH, pKa and Weak Bases

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

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



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

$pK_a = 9$

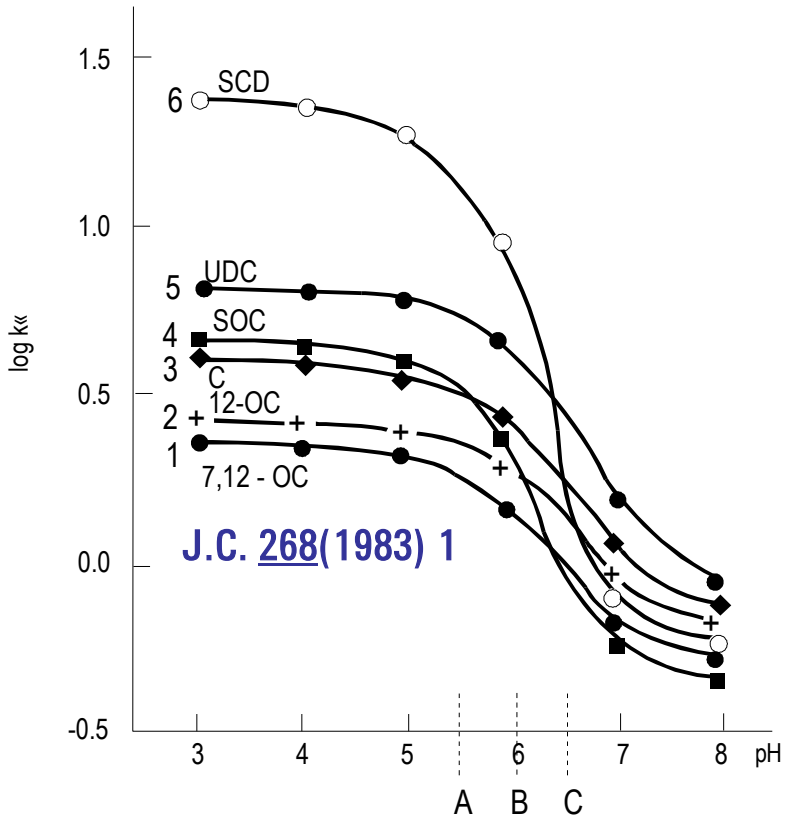
At pH 9 – the sample exists as protonated and unprotonated diphenhydramine in a ratio of 1:1. Peak shape can be poor.

At pH 10 – 91% of the sample exists as unprotonated diphenhydramine.

At pH 8 – 91% of the sample exists as protonated diphenhydramine.

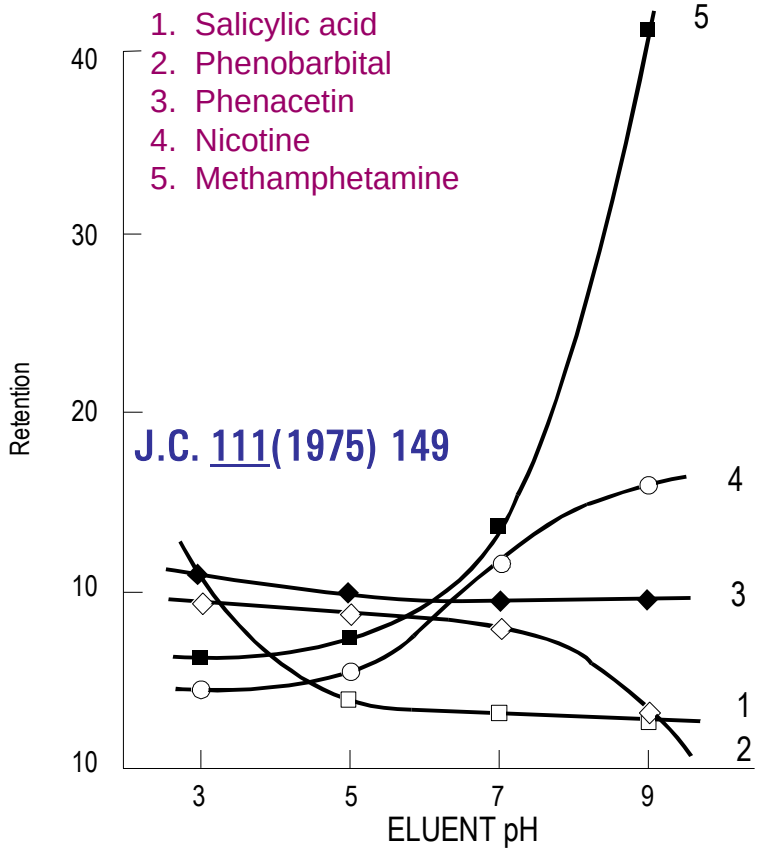
pH vs. Selectivity for Acids and Bases

Column: Nucleosil-C18
Mobile Phase: 45% ACN/55% phosphate buffer
Sample: Bile Acids



Column: mBondapak-C18
Mobile Phase: 60% 25 mM phosphate buffer
40% Methanol

1. Salicylic acid
2. Phenobarbital
3. Phenacetin
4. Nicotine
5. Methamphetamine



- **Retention and selectivity can change dramatically when pH is changed.**

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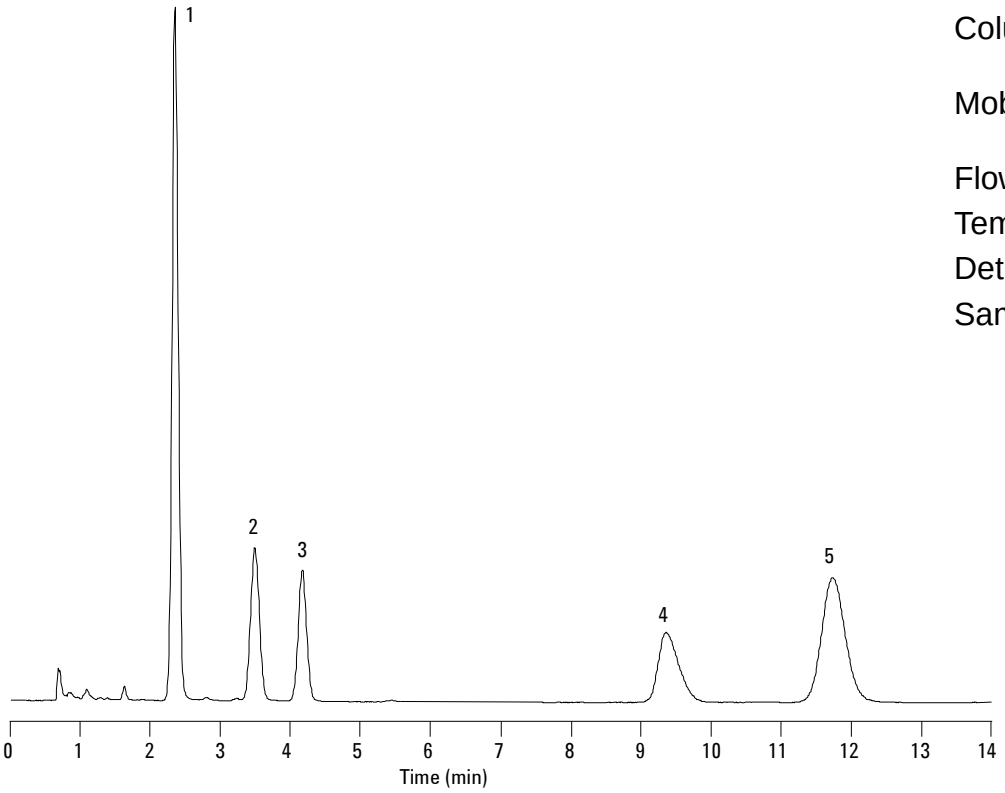
Importance of pH and Buffers
A Practical Example

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

Importance of pH and Buffers - A Practical Example Optimized Isocratic Conditions for Cardiac Drugs



Column: StableBond SB-C18, 4.6 x 150 mm, 5 mm
Mobile Phase: 45% 25 mM NaH_2PO_4, pH 3.0
55% 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

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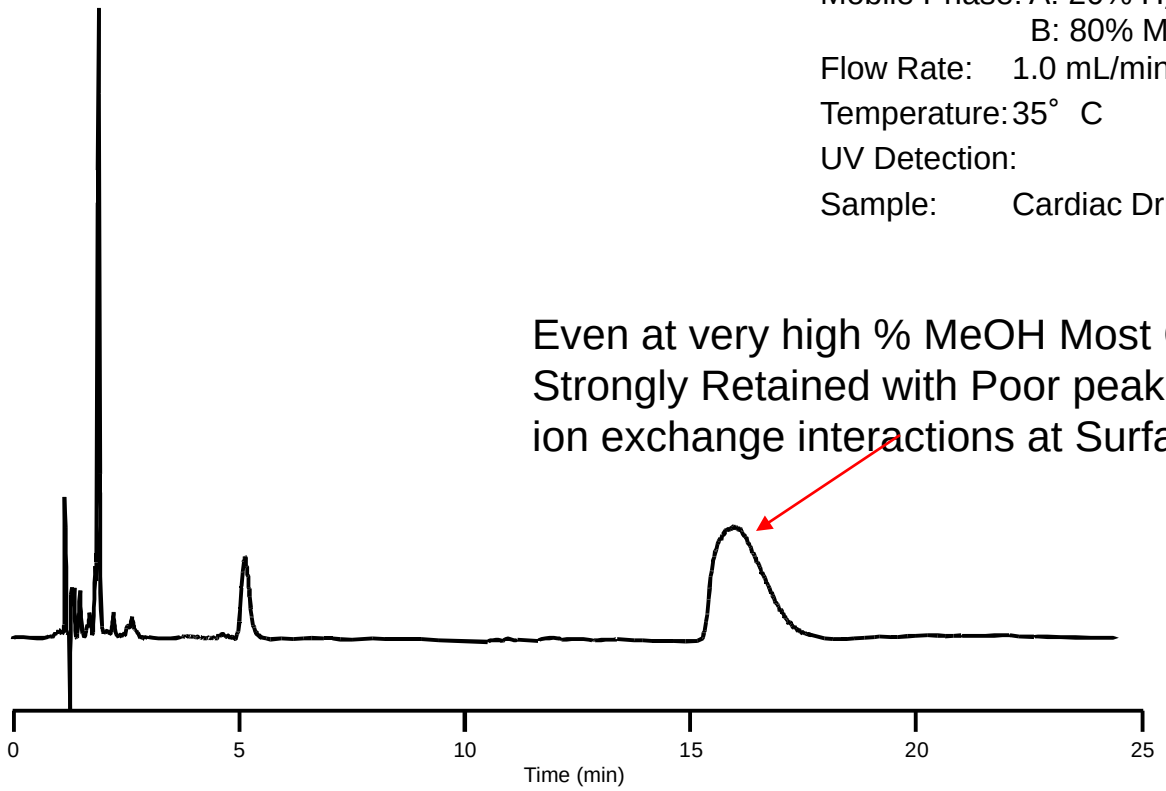
I Don't Have Time to Make Buffers or Adjust pH …

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

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

Even at very high % MeOH Most Components Strongly Retained with Poor peak Shape Due to ion exchange interactions at Surface



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

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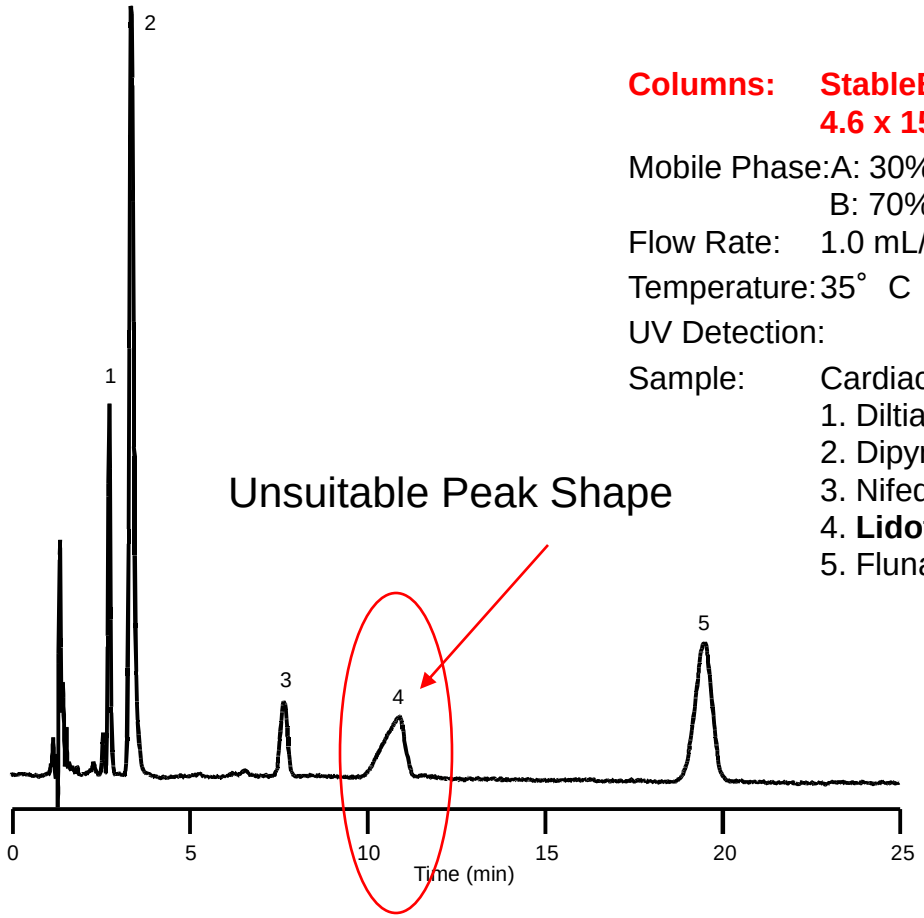
What If You Work Outside the Buffer Range?



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

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

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Buffers for Reversed-Phase HPLC

Buffer	pK_a	Buffer Range
Phosphate		
pK_1	2.1	1.1 - 3.1
pK_2	7.2	6.2 - 8.2
pK_3	12.3	11.3 - 13.3
Citrate		
pk_1	3.1	2.1 - 4.1
pK_2	4.7	3.7 - 5.7
pK_3	5.4	4.4 - 6.4

Buffer	pK_a	Buffer Range
Formate	3.8	2.8 - 4.8
Acetate	4.8	3.8 - 5.8
Tris(hydroxymethyl) aminomethane	8.3	7.3 - 9.3
Ammonia	9.2	8.2 - 10.2
Borate	9.2	8.2 - 10.2
Pyrrolidine	10.5	9.5 - 11.5

pH Stabilizers at pH < 2.5

Phosphoric acid, 0.1%

TFA, 0.1%

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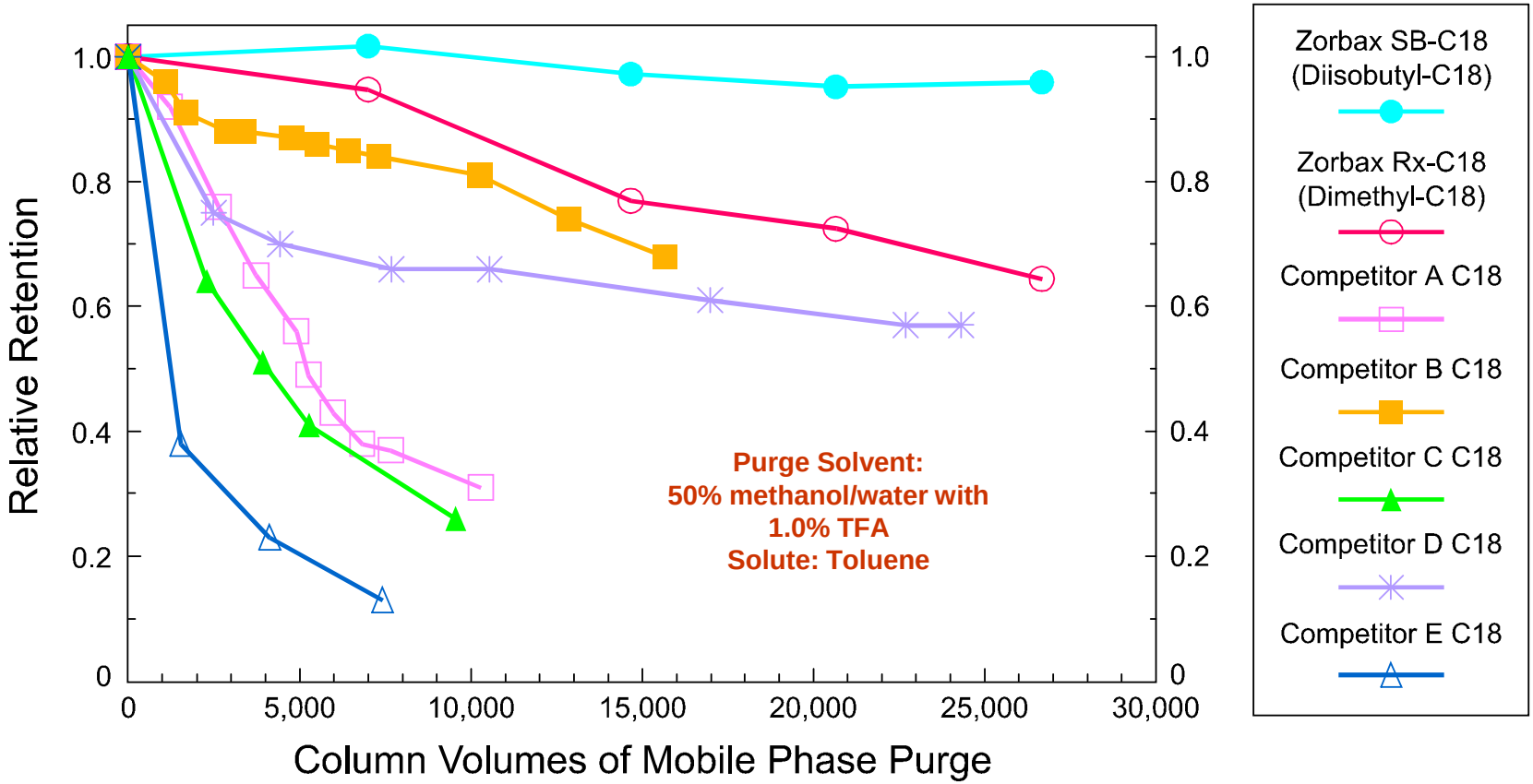
Don't Forget - Match Column to pH of Mobile Phase for Maximum Column Lifetime

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.

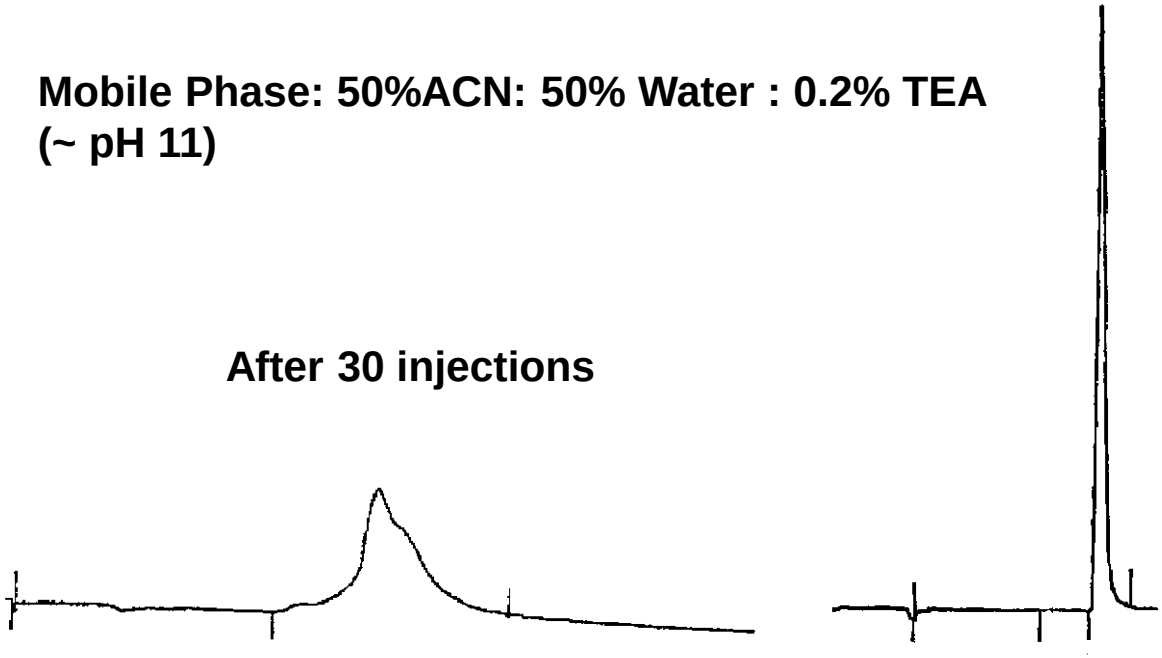
Don't Forget - Match Column to pH of Mobile Phase for Maximum Column Lifetime

High pH and Room Temperature (pH 11 RT)

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

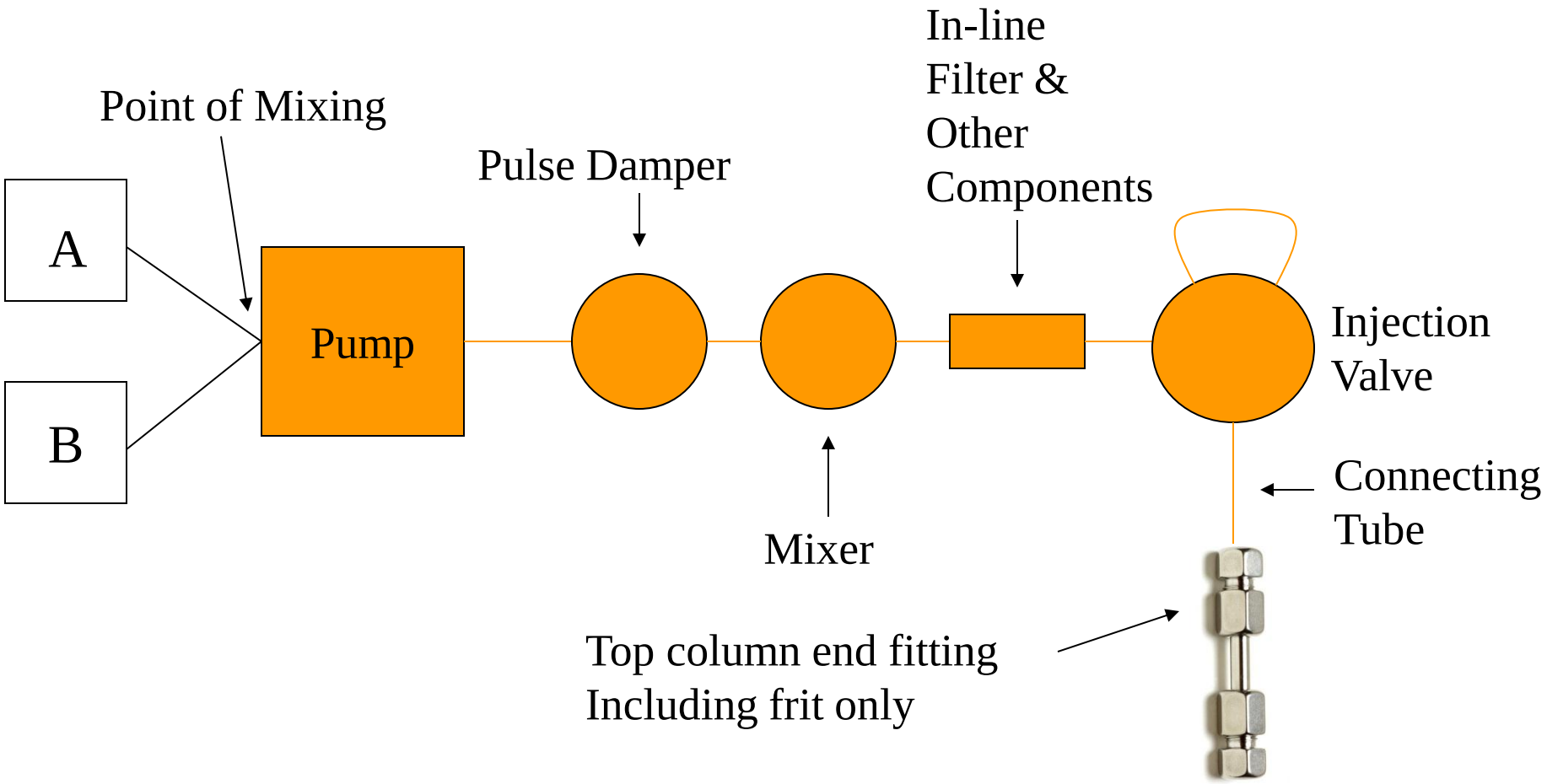
Initial

After 30 injections

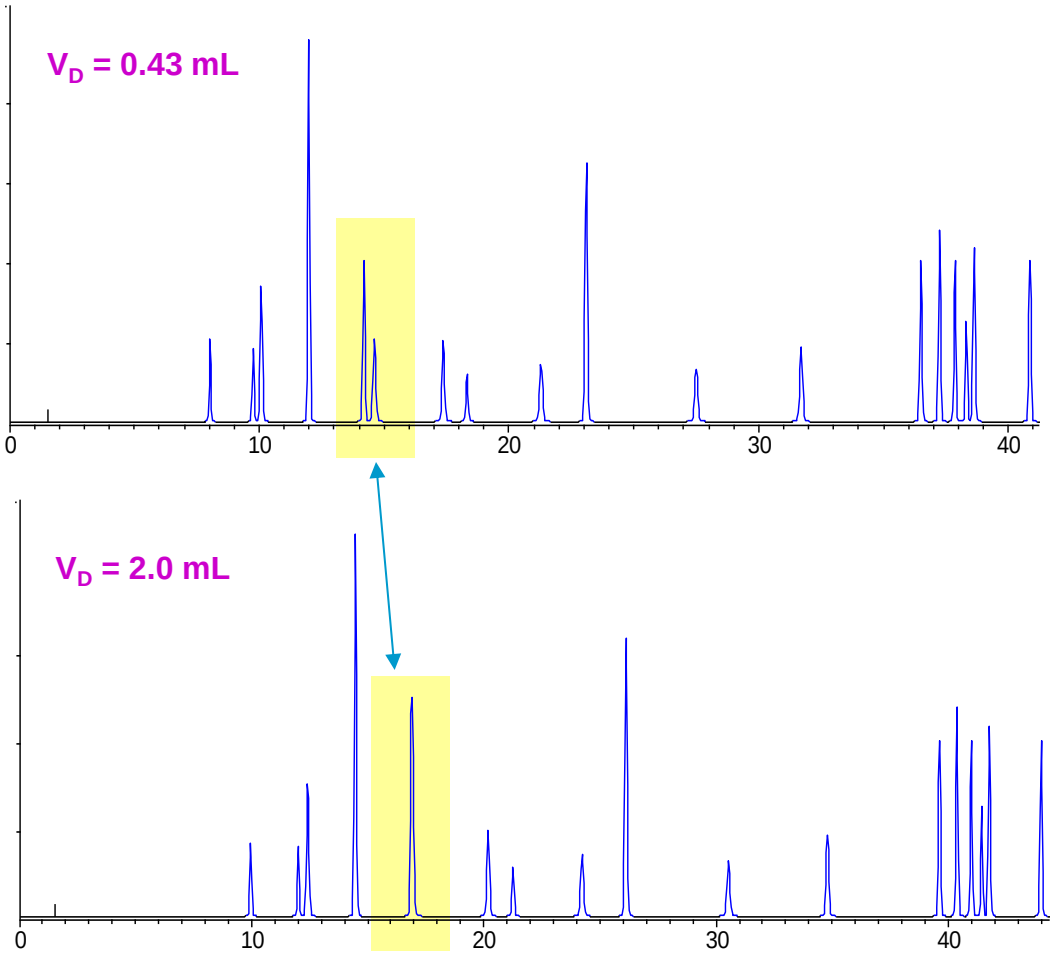


Tip: This column was not recommended for pH values above 9.0; Use Columns Designed for chosen pH (i.e. 11)

Dwell Volume of HPLC System with Low Pressure Mixing: Important for Gradient Elution



Tip: Dwell Volume Differences Between Instruments Can Cause Changes in Retention and Resolution



Column: **ZORBAX Rapid Resolution Eclipse XDB-C8 4.6 x 75 mm, 3.5 µm**

Mobile Phase: Gradient, 0 - 100 %B in 52.5 min.
A: 5/95 methanol/ 25 mM phosphate pH 2.50
B: 80/20 methanol/25 mM phosphate pH 2.50

Flow Rate: 0.5 mL/min

Temperature: 25° C

Injection: 5 µL

Detection: 250 nm

Sample: Mixture of antibiotics and antidepressants

Upper trace simulates actual run data entered into DryLab® 3.0 software

Lower trace is simulated chromatogram for larger V_D

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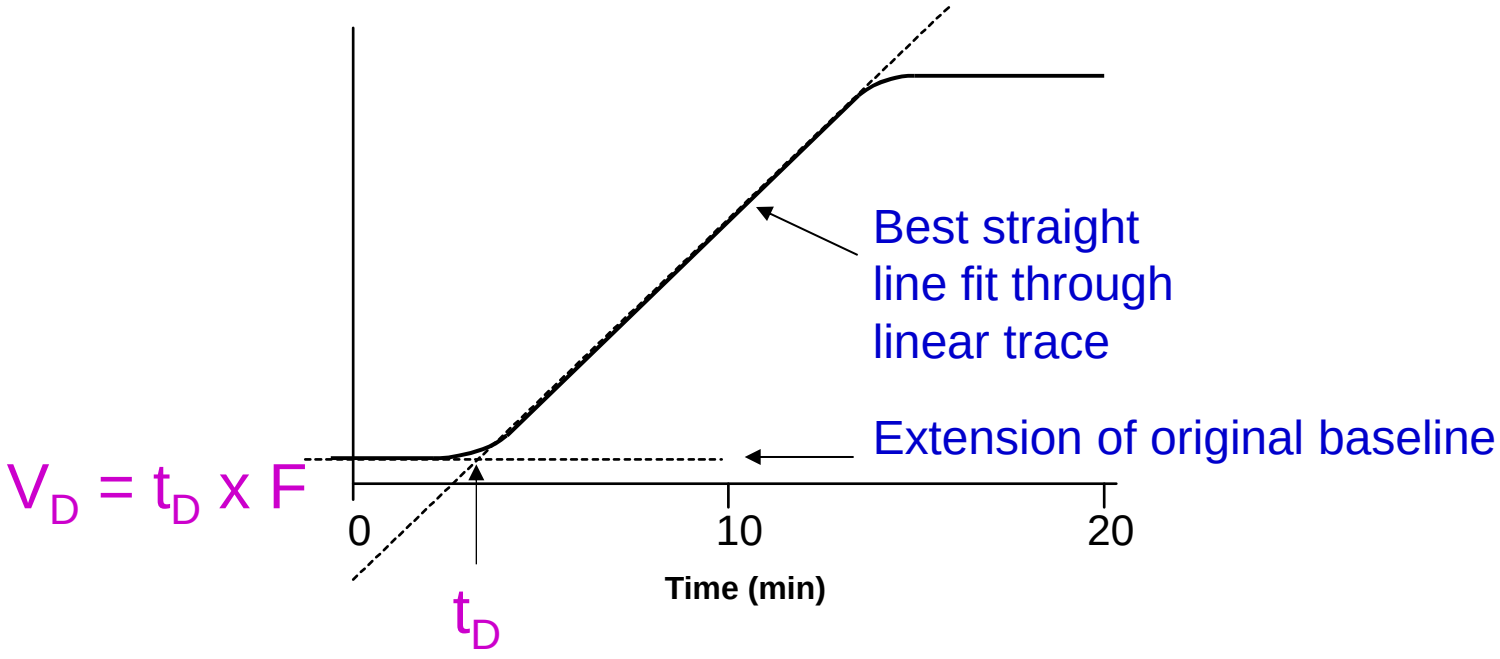
Measuring System Dwell Volume (V_D)



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

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Trick: Measure and Correct for Dwell Volume (V_D)

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

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

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

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

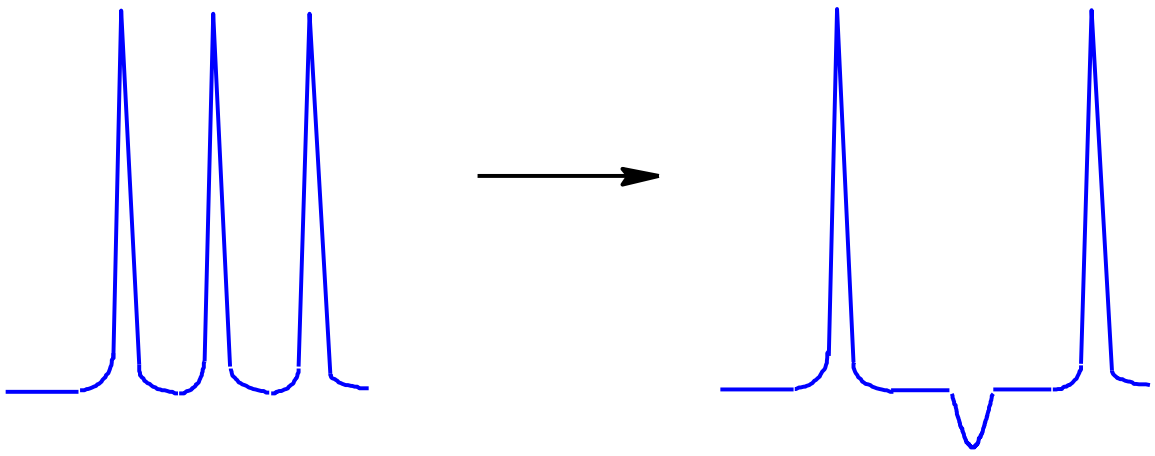
Detection Issues

Recognize Where the Problem Originates

- Is it a consequence of technique?
- Is it expected because of certain mobile phase components?
- Can it be corrected by adjusting detector parameters?
- Answers to these questions will help find a solution!

Let's Explore Some Problems and Solutions

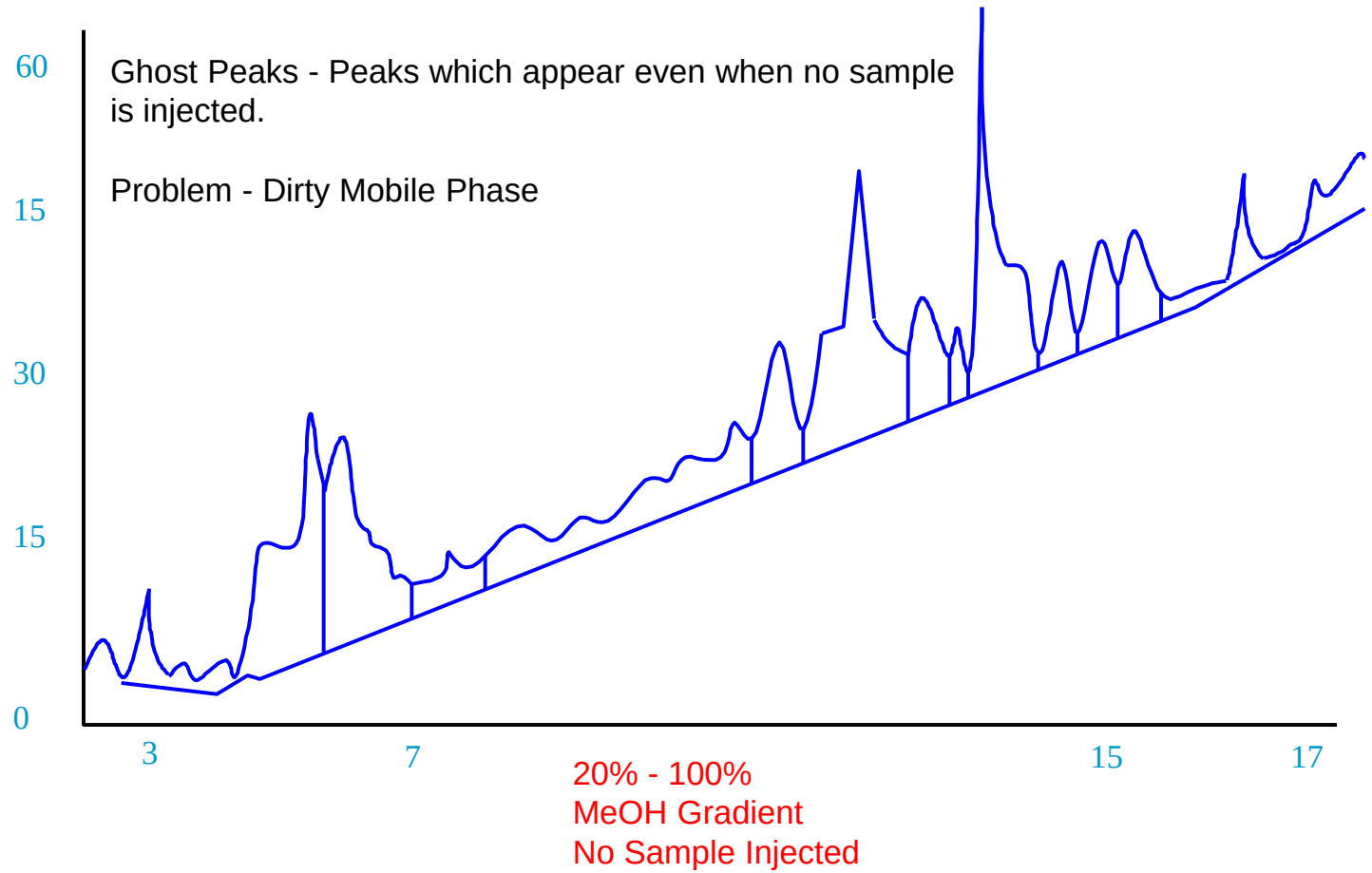
Peak Shape: Negative Peaks



Causes:

- Absorbance of sample is less than the mobile phase.
- Equilibrium disturbance when sample solvent passes through the column.
- Normal with Refractive Index Detectors.

Ghost Peaks



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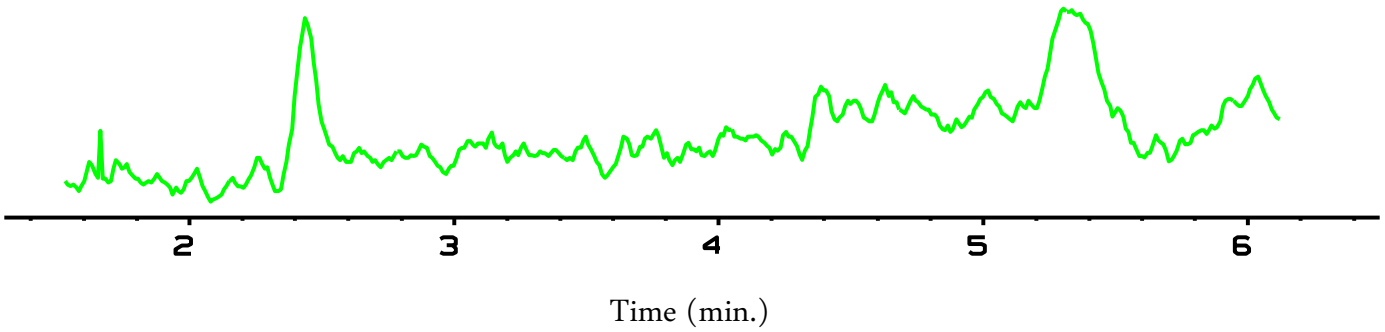
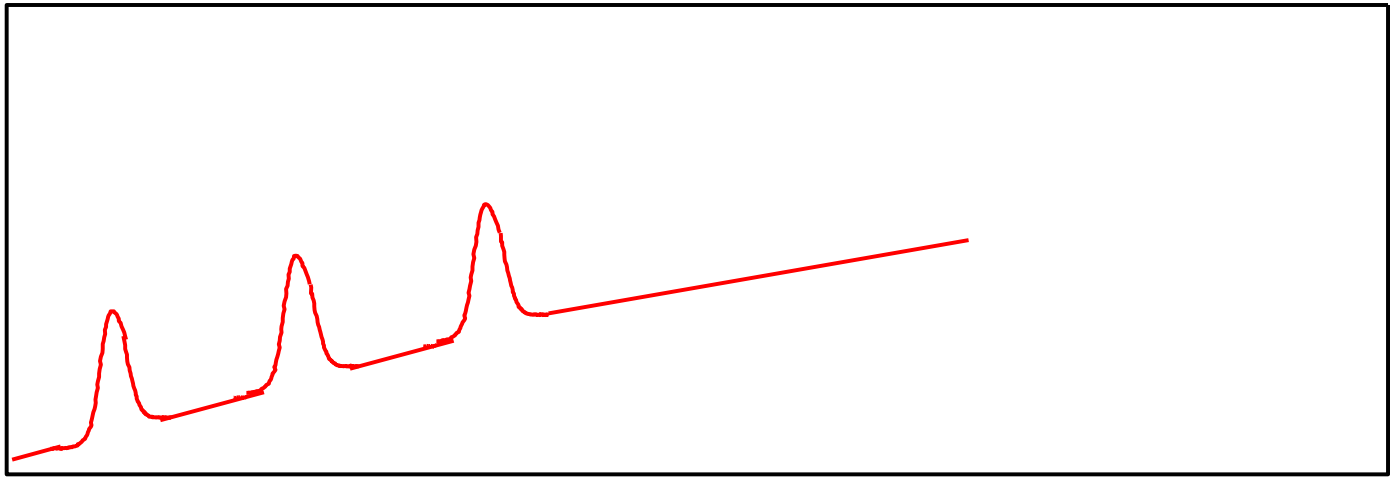
Noisy Baselines



Possible Causes:

- Dirty Flow Cell
- Detector Lamp Failing
- Pulses from Pump if Periodic
- Temperature Effects on Detector
- Air Bubbles passing through Detector

Drifting Baselines



- Gradient Elution
- Temperature Unstable (Refractive Index Detector)
- Contamination in Mobile Phase
- Mobile Phase Not in Equilibrium with Column
- Contamination Bleed in System

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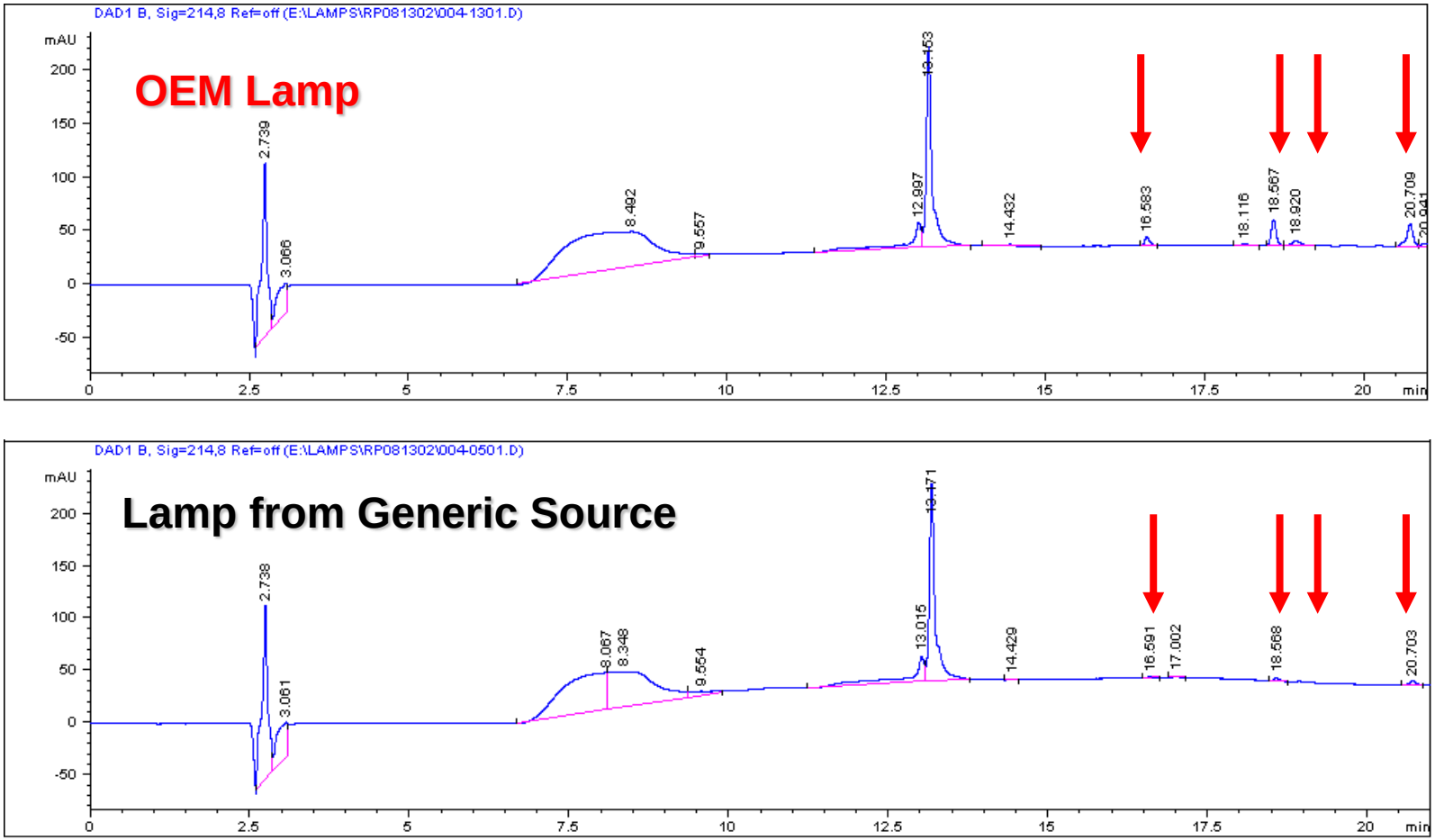
Chromatographic Results with “Wrong” Lamp at 214 nm Wavelength



Tip: Could also be a symptom of aging lamp

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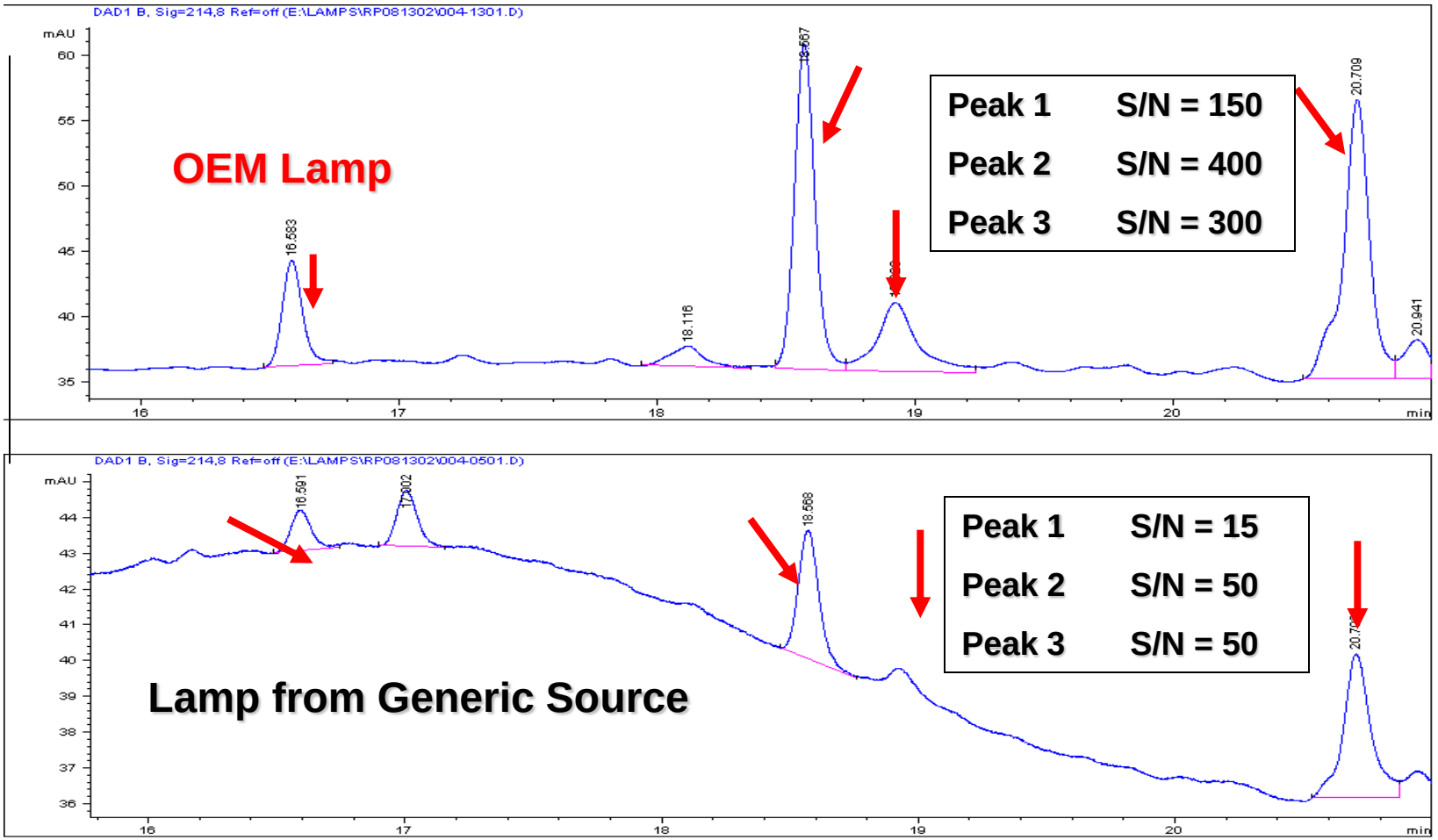
Expanded View of Chromatographic Results Generic Source Lamp at 214 nm Wavelength



Tip: Poor S/N makes it difficult to detect low level impurities

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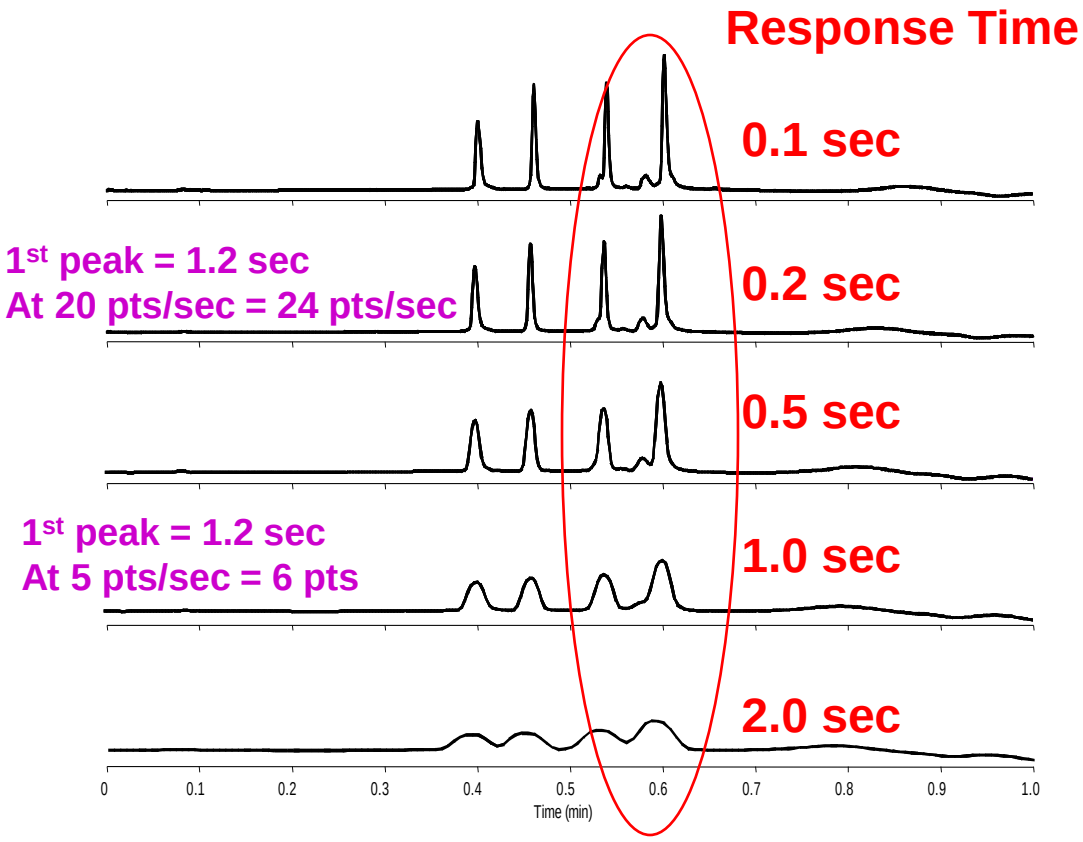
Effect of Detector Response Time

The System is operating well-the settings were poorly made!

Slow Data Rates Can Hinder Impurity Detection and Reduce Sensitivity



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

- Tip: Adjust the response rate of your detector for best peak detection.

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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 chemistry (method) issues.

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

Start With the Correct Questions

- Find the Answers
- The Answers will Lead to Solutions