HPLC Column Troubleshooting

What Every HPLC User Should Know

HPLC Components

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

All of these components can have problems and require troubleshooting.

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

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

If Column pressure is high:

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

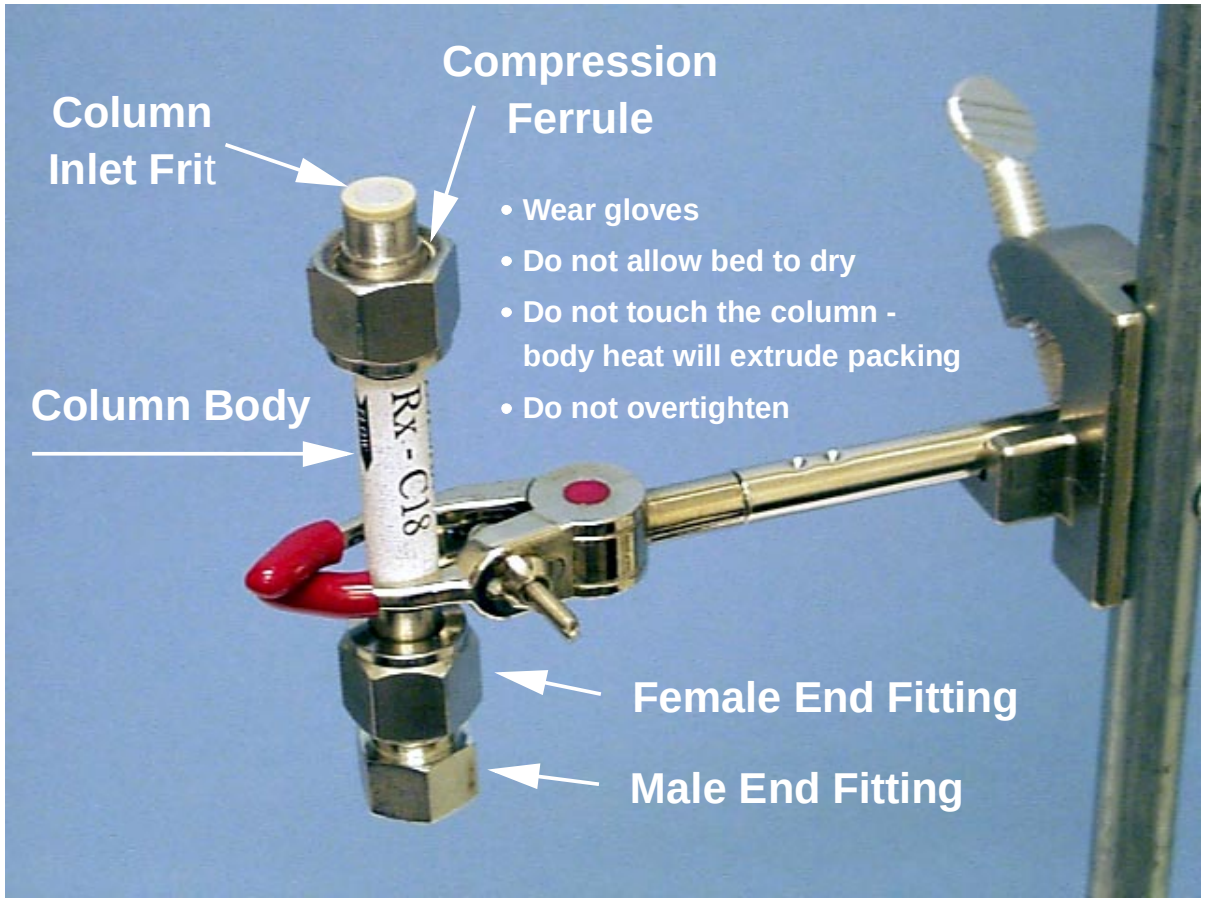
* When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.

Column Cleaning

Normal Phase Solvent Choices
in Order of Increasing Strength

- Use at least 50 mL of each solvent
- 50% Methanol : 50% Chloroform
- 100% Ethyl Acetate

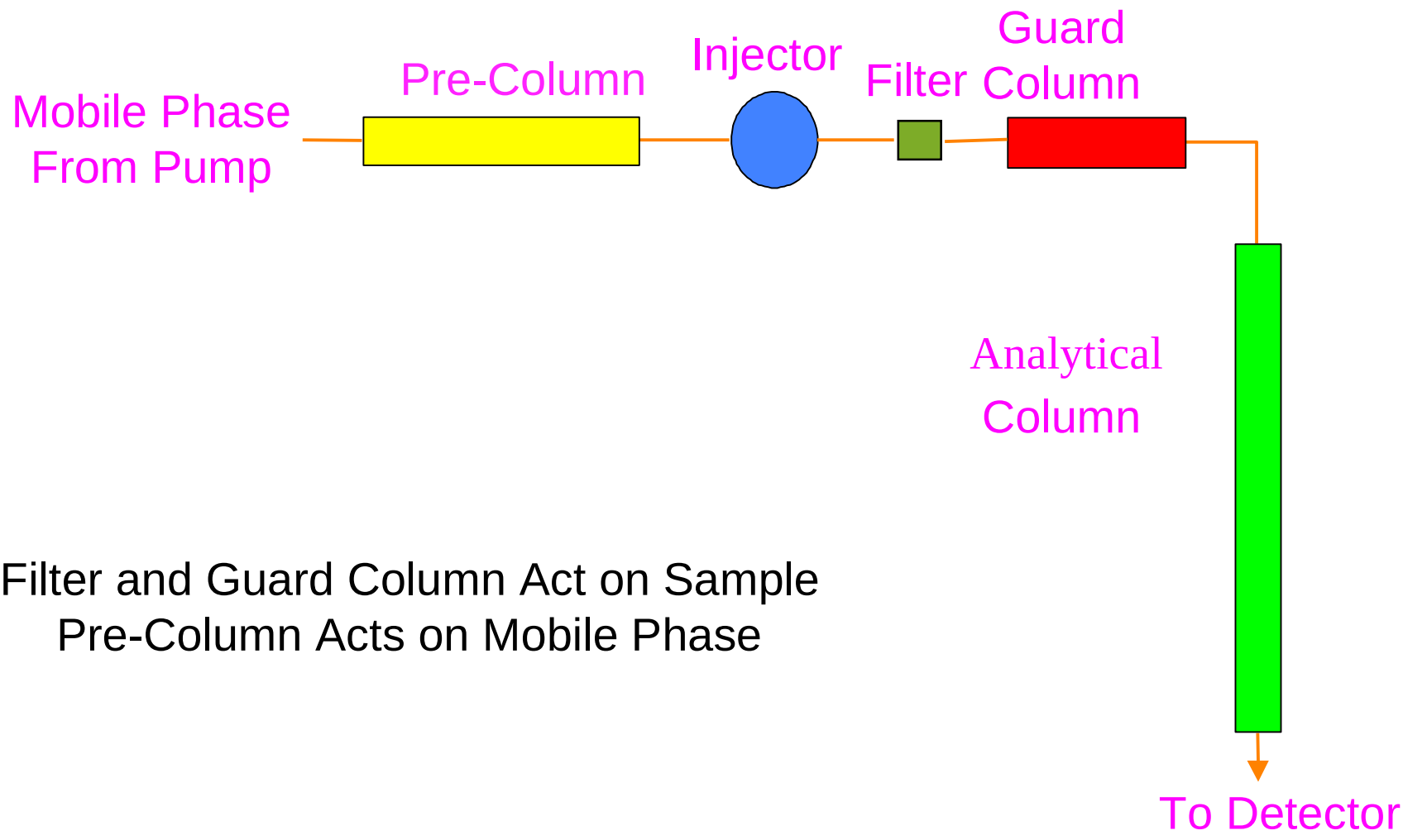
How to Change a Frit



Preventing Back Pressure Problems

- Use column protection
  - Guard columns
  - In-line filters
- Sample Preparation
- Appropriate column flushing
- Filter buffered mobile phases

Preventing Back Pressure Problems: In-Line Devices



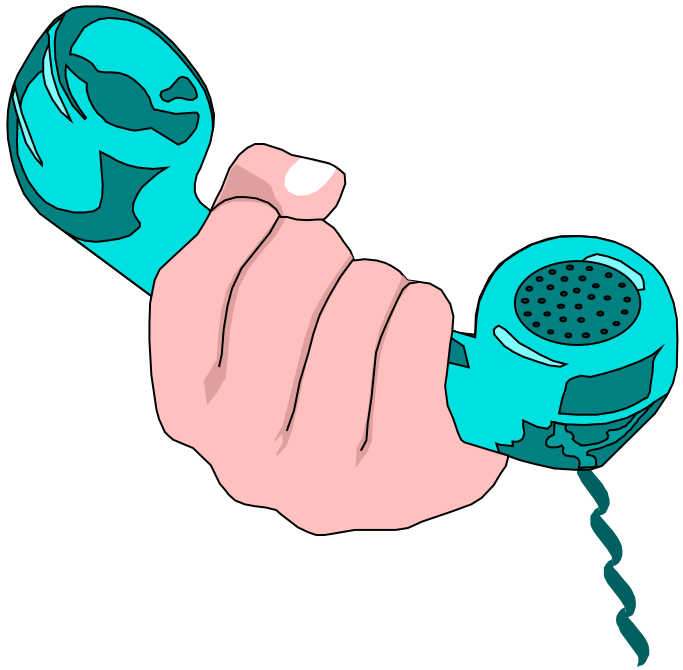
Filter and Guard Column Act on Sample
Pre-Column Acts on Mobile Phase

Preventing Back Pressure Problems: Sample Preparation

- Solvent/Chemical Environment
- Particulate/Aggregate Removal
    - Filter samples
    - Centrifugation
- Solid Phase Extraction (S.P.E.)
    - Cartridges or Plates
    - Disks or Membranes

Break Number 1

- For Questions and Answers
- Press *1 on Your Phone to
- Ask a Question



Peak Shape Issues

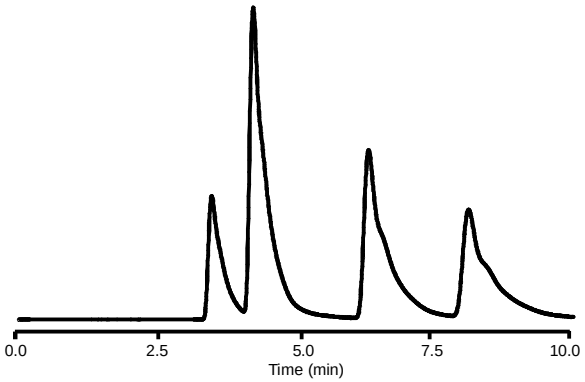
- Split peaks
- Peak tailing
- Broad peaks
- Poor efficiency

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

Split Peaks

Can be caused by:

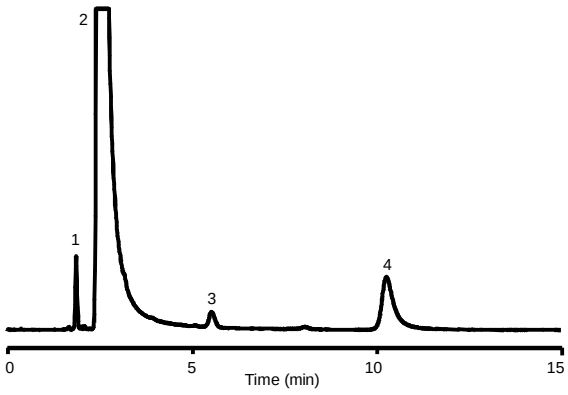
- Column contamination
- Partially plugged frit
- Column void
- Injection solvent effects



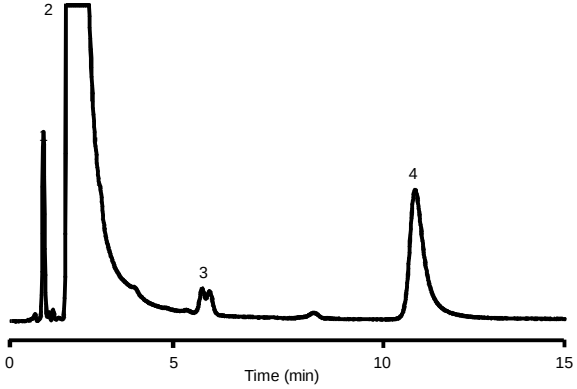
Split Peaks
Column Contamination

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

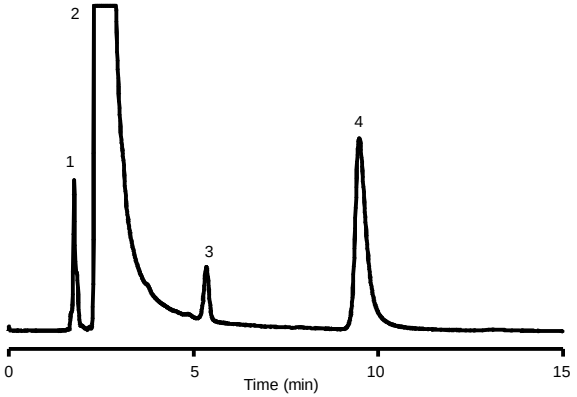
Injection 1



Injection 30



Injection 1
After Column Wash
with 100% ACN



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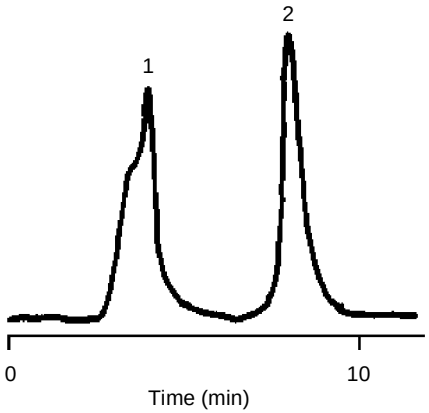
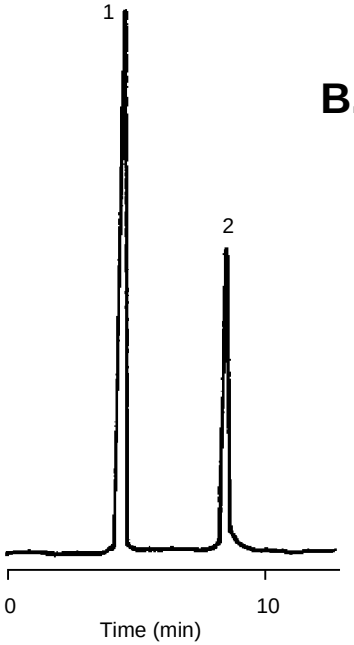
Split Peaks
Injection Solvent Effects

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



Determining the Cause of Split Peaks

1. Complex sample matrix or many samples analyzed - likely column contamination or partially plugged frit
2. Mobile phase pH ≥ 7 - likely column void due to silica dissolution (unless specialty column used)
3. Injection solvent stronger than mobile phase - likely split and broad peaks, dependent on sample volume

Peak Tailing, Broadening and Loss of Efficiency

Can be caused by:

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

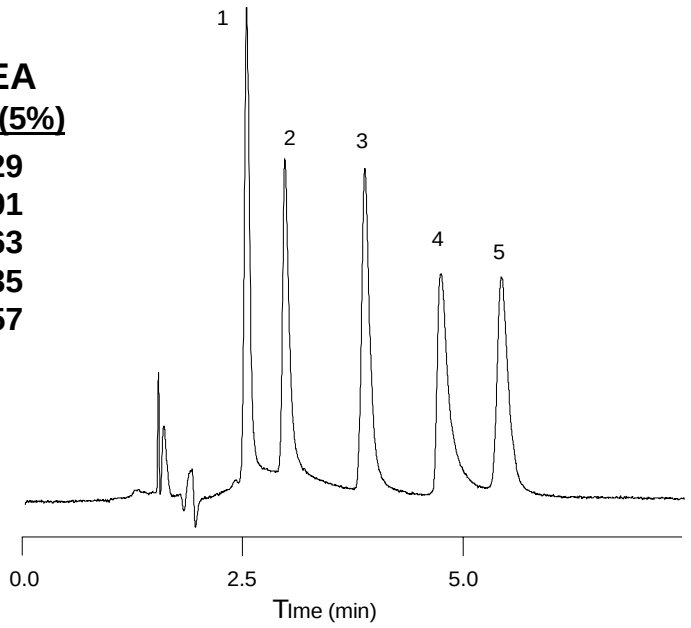
Peak Tailing Column
"Secondary Interactions"

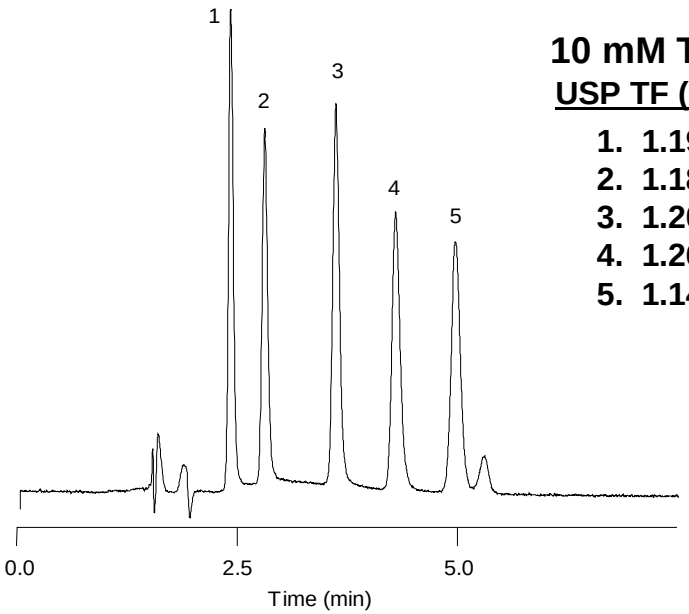
Column: Alkyl-C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 pH 7.0 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

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

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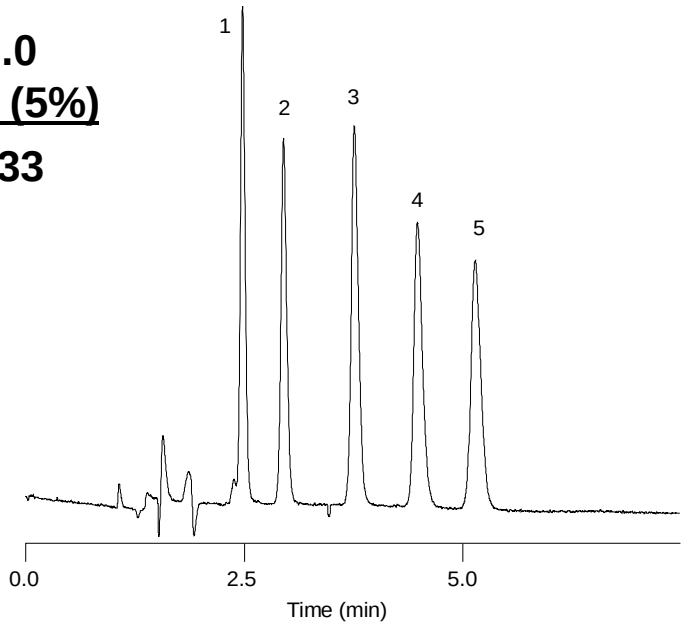
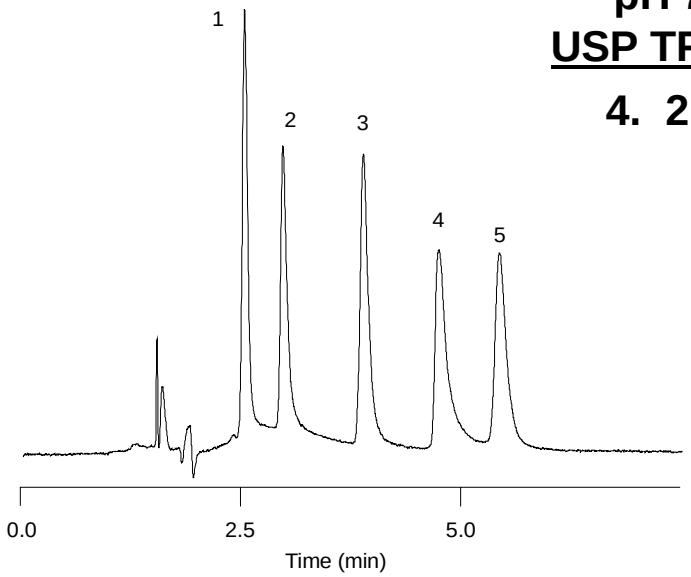
Peak Tailing Column
"Secondary Interactions"

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

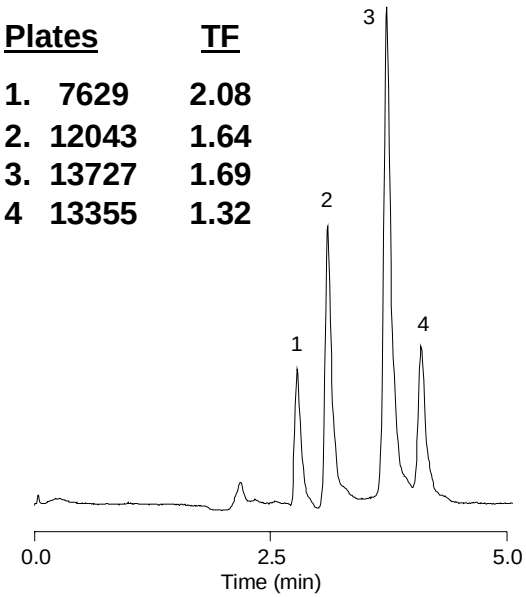


- Reducing the mobile phase pH reduces interactions with silanols that cause peak tailing.

Peak Tailing
Column Contamination

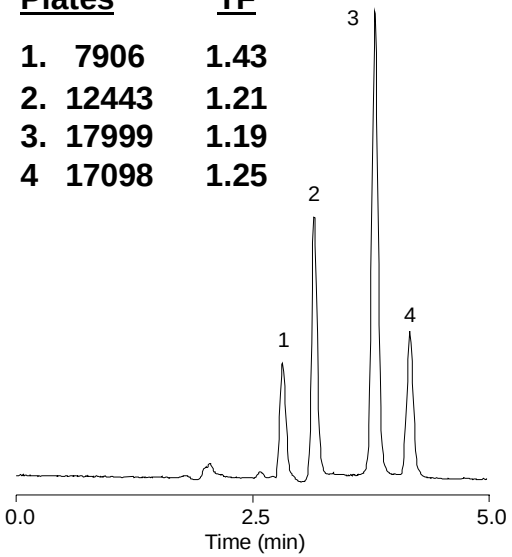
Column: StableBond SB-C8, 4.6 x 250 mm, 5µm Mobile Phase: 20% H_2O : 80% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

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



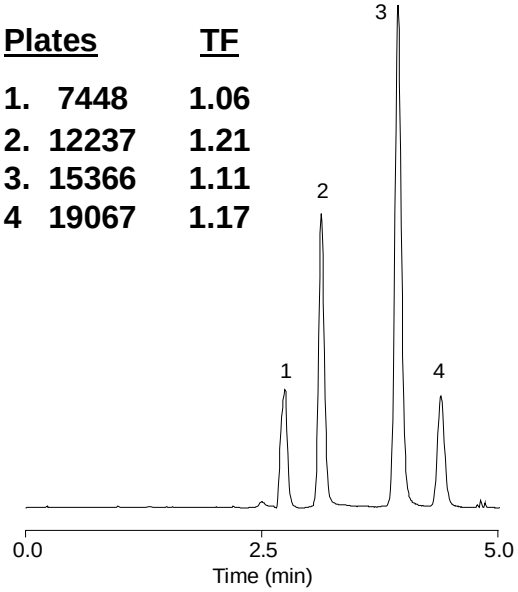
QC test forward direction

	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

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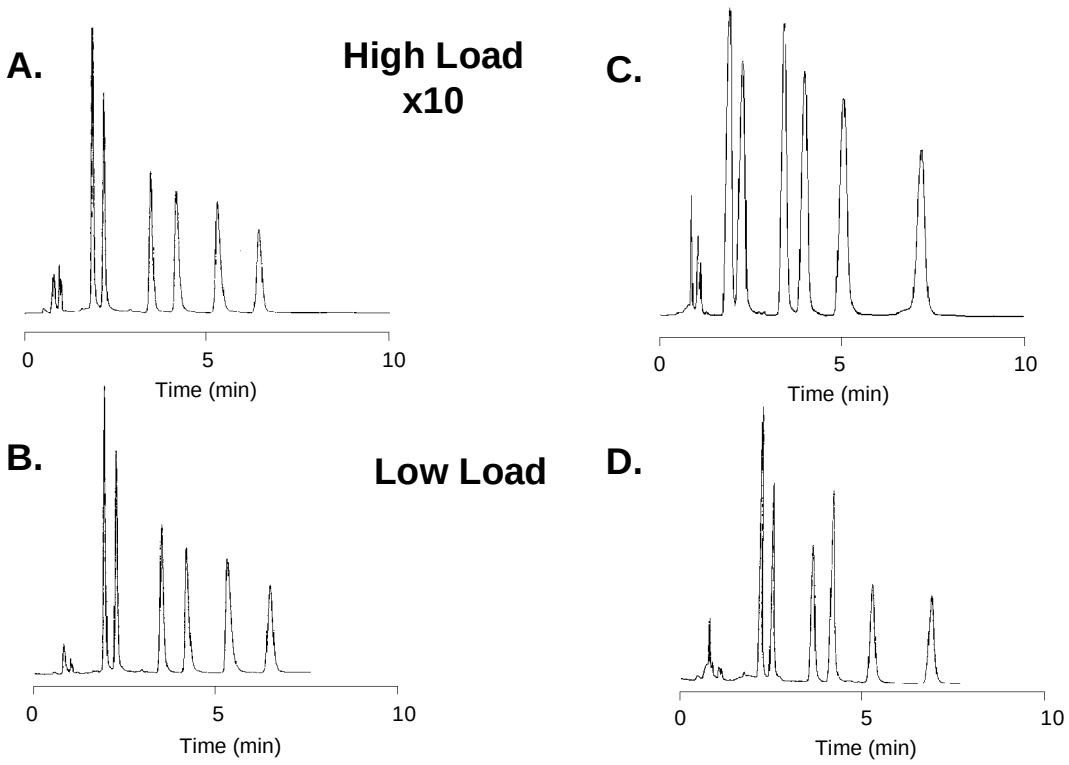
Peak Tailing/Broadening
Sample Load Effects

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



Broadening

Competitive C8
Plates

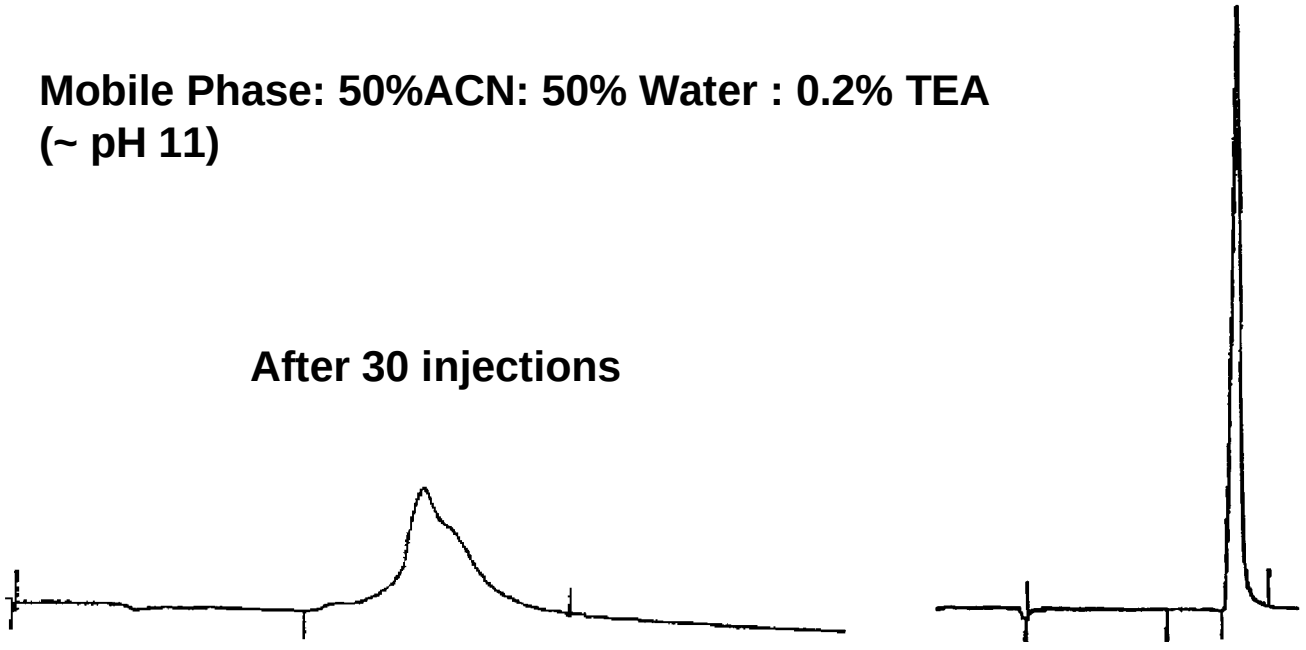
	C	D
1.	850	5941
2.	815	7842
3.	2776	6231
4.	2539	8359
5.	2735	10022
6.	5189	10725

Peak Broadening, Splitting
Column Void

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

Initial

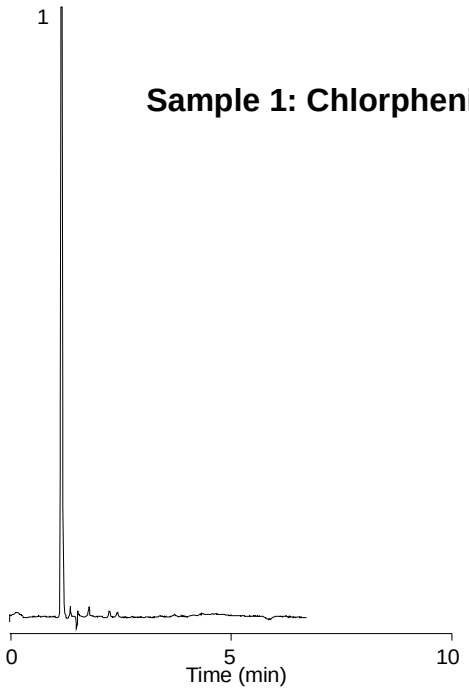
After 30 injections



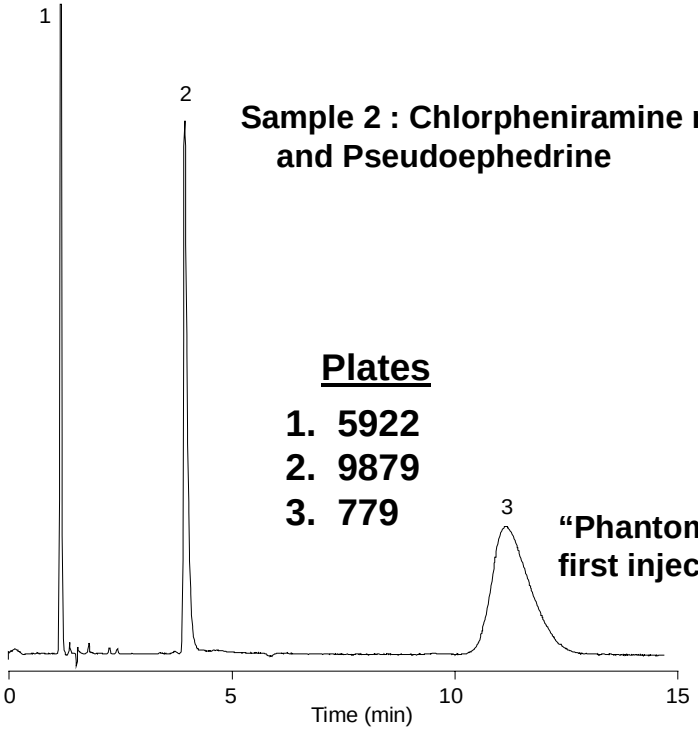
- Multiple peak shape changes can be caused by the same column problem. In this case a void resulted from silica dissolved at high pH.

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

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

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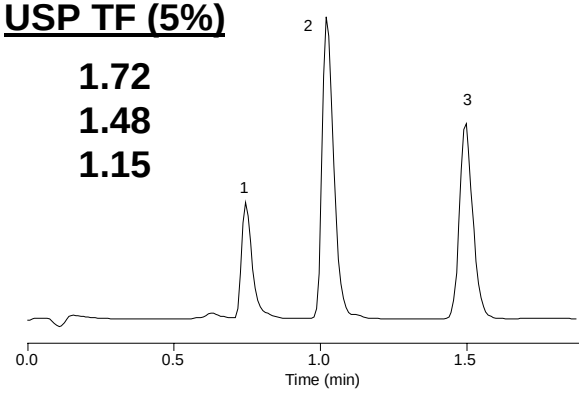
Peak Tailing
Injector Seal Failure

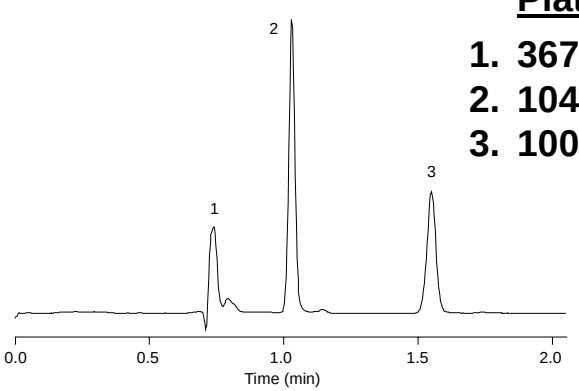
Column: Bonus-RP, 4.6 x 75 mm, 3.5 µm Mobile Phase: 30% H_2O : 70% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. N,N-Dimethylaniline

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



Before

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

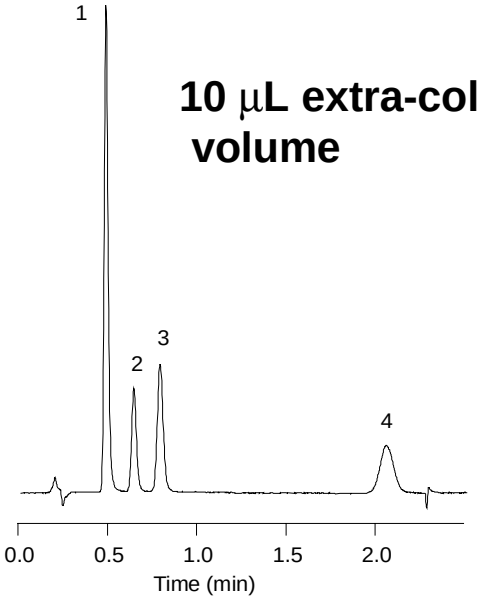


After replacing rotor seal and isolation seal

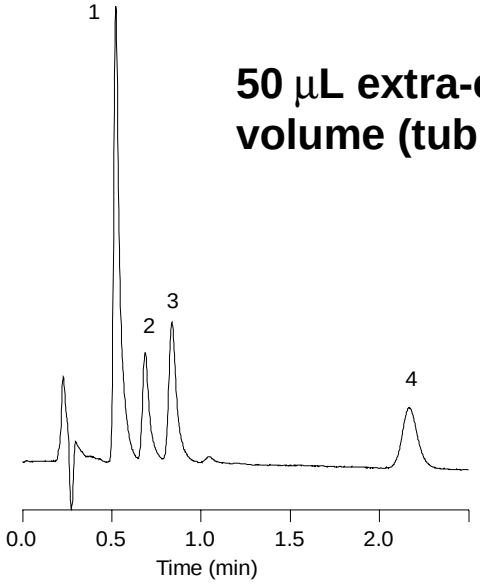
- Overdue instrument maintenance can cause peak shape problems.

Peak Tailing
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



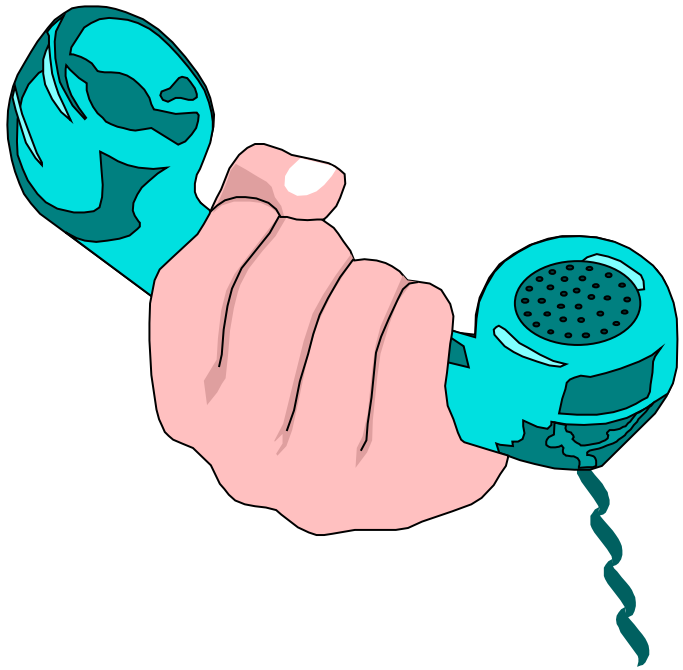
50 µL extra-column volume (tubing)

Determining the Cause 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

Break Number 2

- For Questions and Answers
- Press *1 on Your Phone to
- Ask a Question



Retention Issues

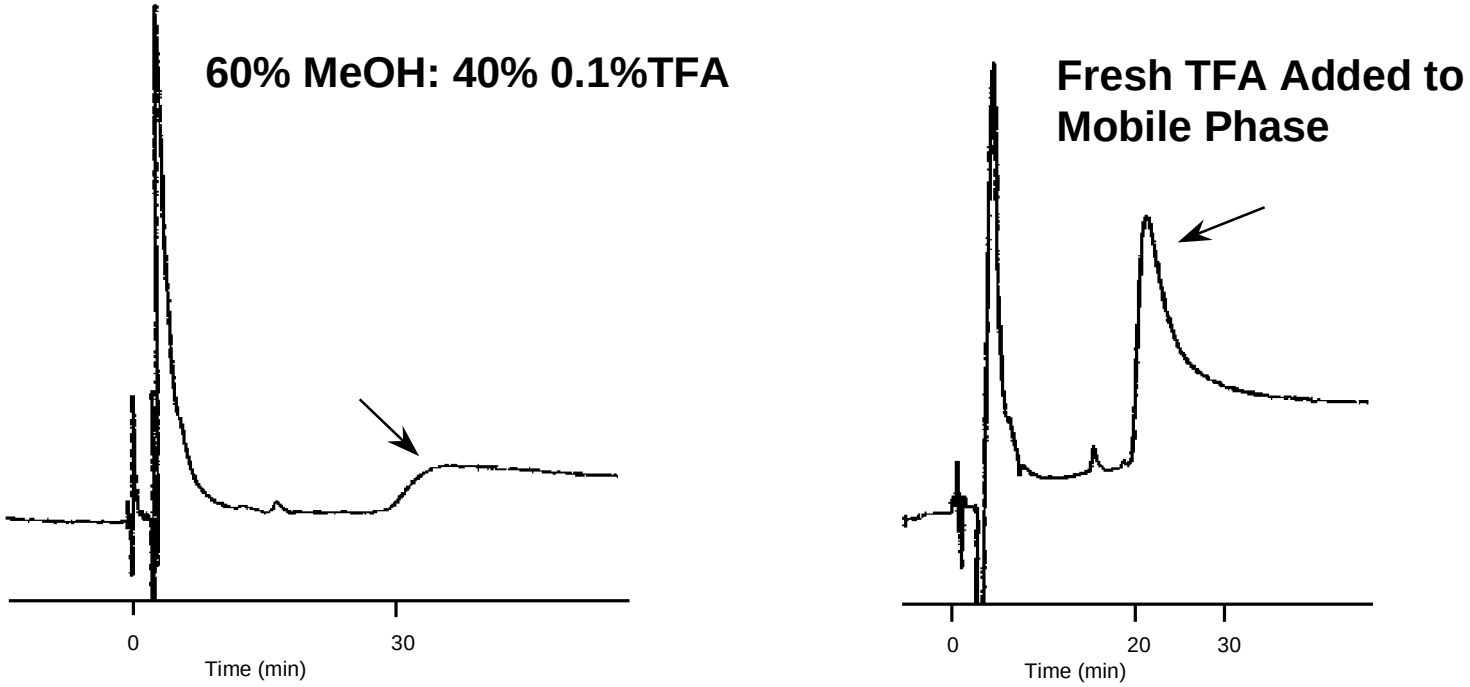
- Retention time changes (t_r)
- Capacity factor (retention) changes (k′)
- Selectivity changes (α)

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

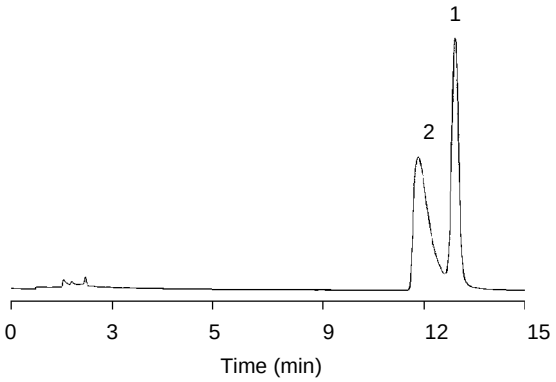
Mobile Phase Change Causes Change in Retention



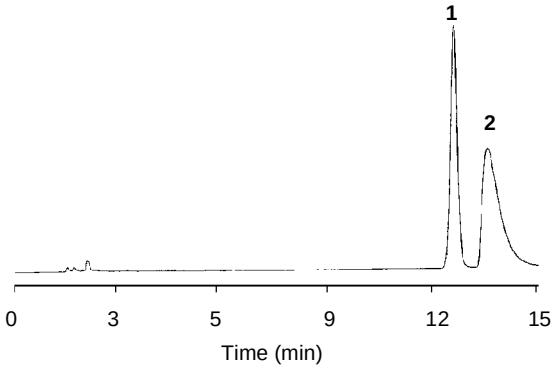
- Volatile TFA evaporated/degassed from mobile phase.
- Replacing it solved problem.

Column Aging/Equilibration Causes Retention/Selectivity Changes

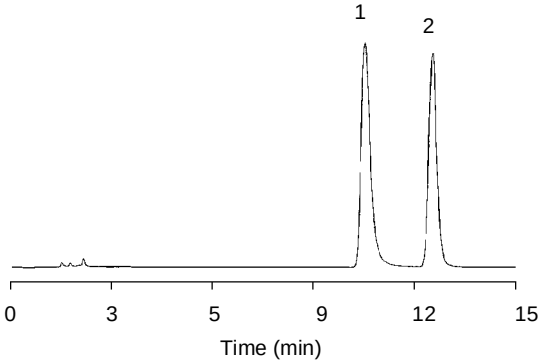
Column 1 - Initial



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 resolved by flushing the column.
- Retention and peak shape were as expected after cleaning.

Determining the 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

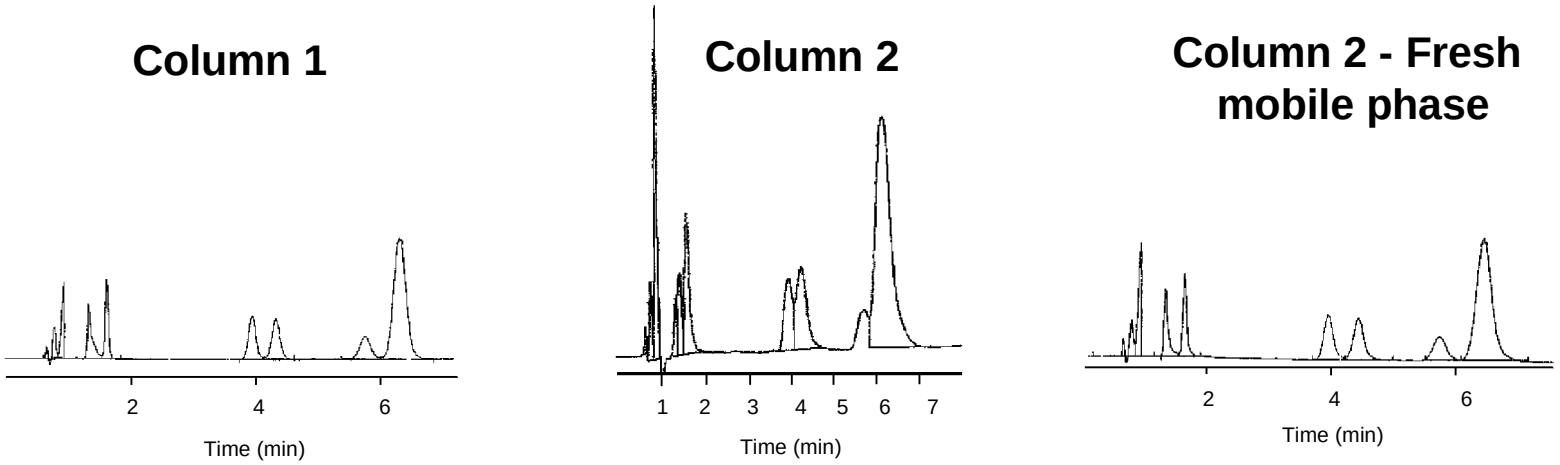
Change in Retention/Selectivity Column-to-Column

- **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



"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."

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

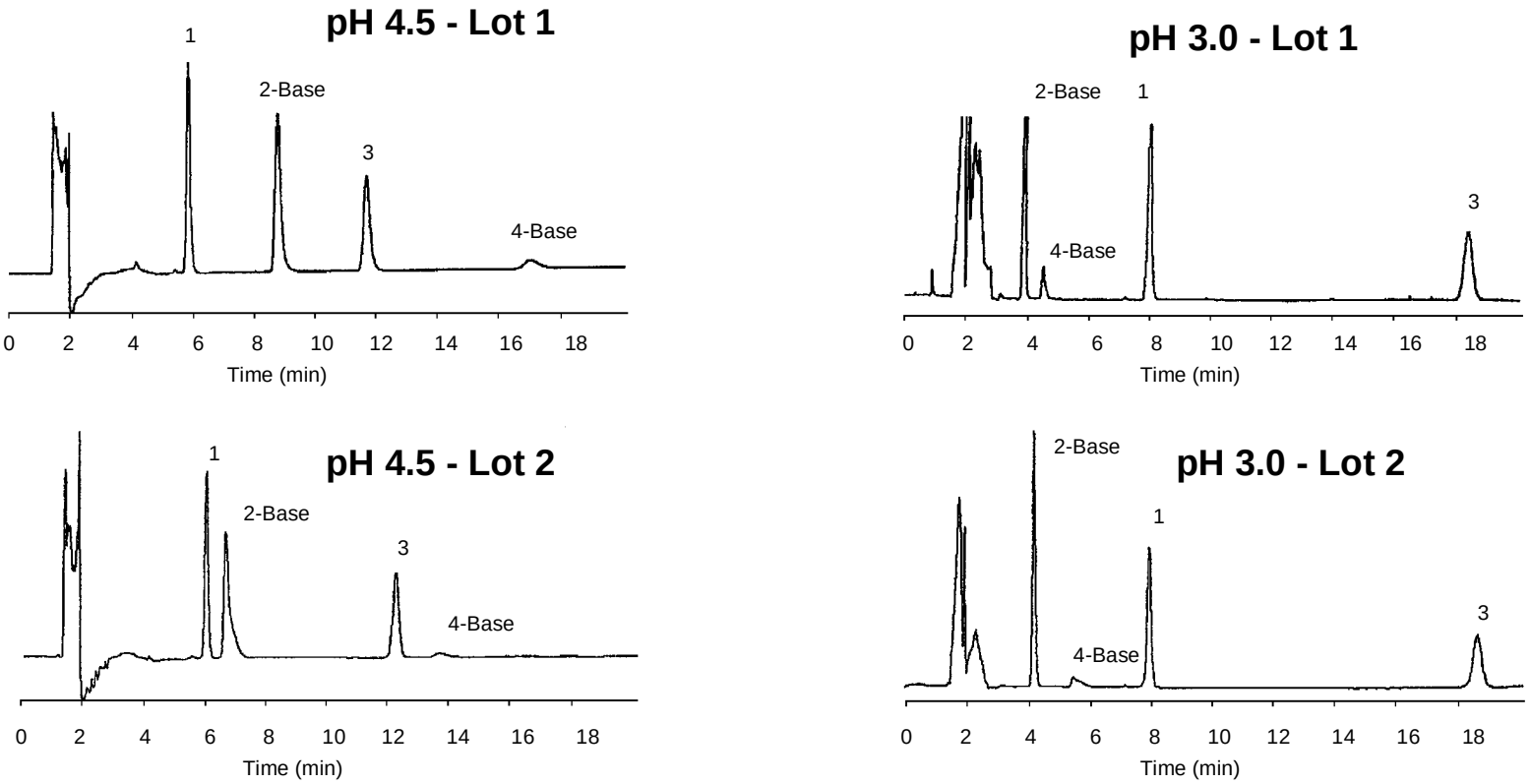
Minimize Change in Retention/Selectivity Lot-to-Lot

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



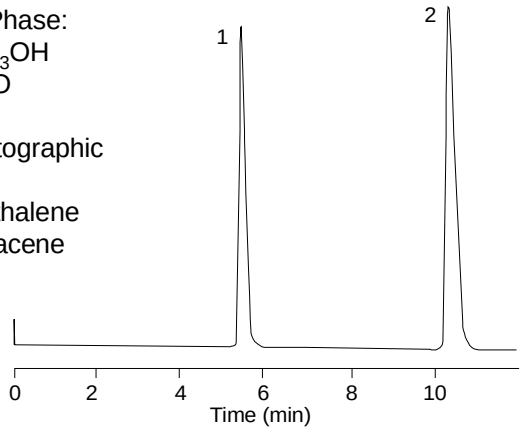
- pH 4.5 shows selectivity change from lot-to-lot for basic compounds
- pH 3.0 shows no selectivity change from lot-to-lot, indicating silanol sensitivity at pH 4.5

Experimental Conditions for Classifying Column Selectivity Changes

Bonded-Phase Test

Mobile Phase:
85% CH_3OH
15% H_2O

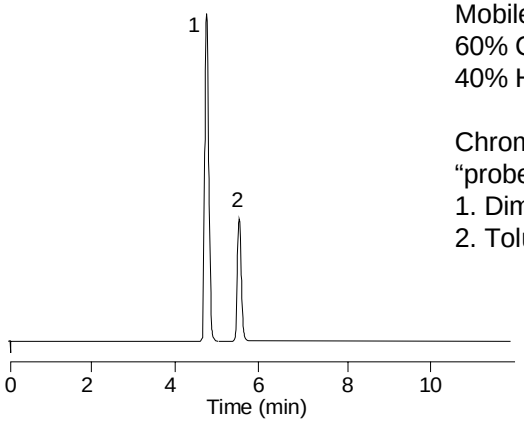
Chromatographic
"probes"
1. Naphthalene
2. Anthracene



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

Conclusions

HPLC column problems are evident as:

- High pressure
- 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.

Wrap-up E-Seminar Questions