Tips and Tricks of HPLC Separation

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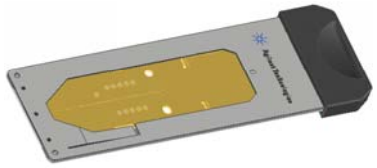
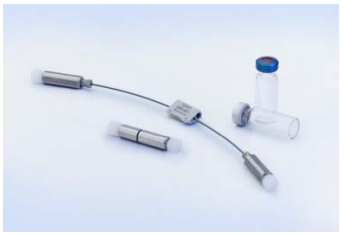
Goals for this presentation:

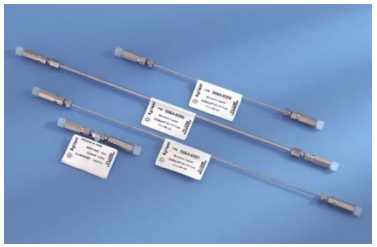
1. Introduce the most commonly observed column related problems in HPLC.
2. Explore the reasons for these column problems.
3. Propose preventative maintenance and method development/optimization approaches to minimize HPLC column problems and increase column lifetimes.



Chip LC



Nano LC

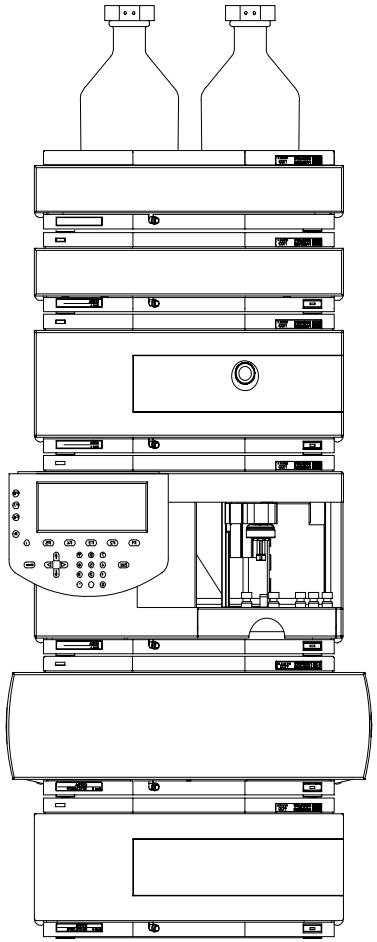


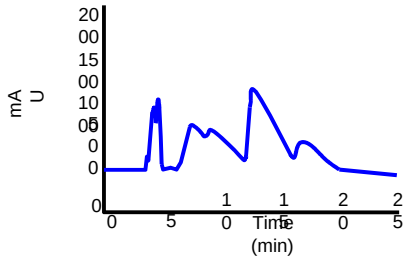
Capillary LC

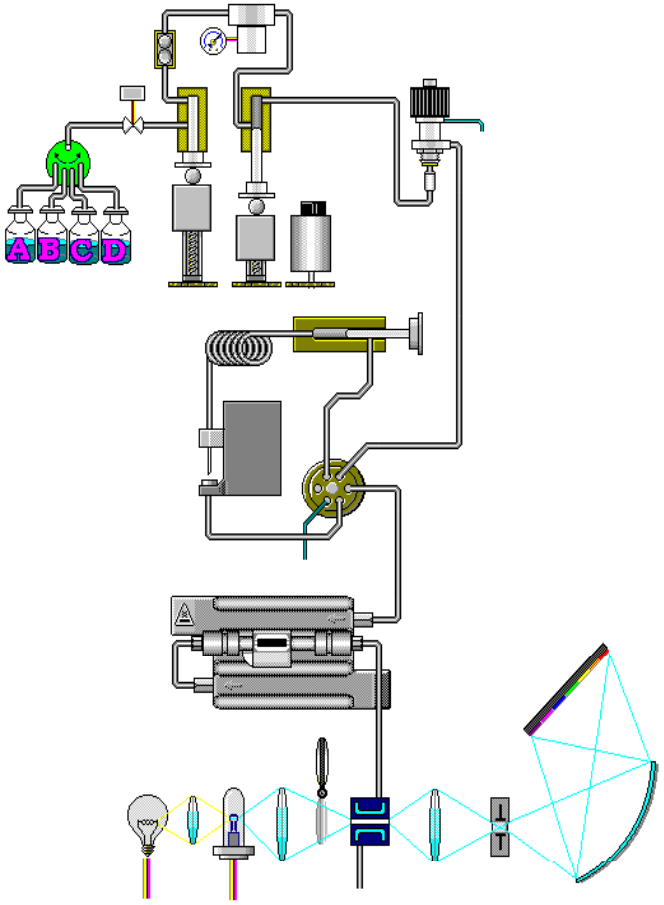
Analytical LC

Prep LC

Troubleshooting in HPLC







Major Areas of Column Problems - Dramatic Changes in 3 Key Areas:

1. **HPLC System Pressure**
2. **Chromatogram - Peak Shape**
3. **Chromatogram - Peak Retention/Selectivity**

1. Pressure Issues

Column Observations	Potential Problems
Large pressure change	Plugged inlet 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 elsewhere in the system.**

If Column pressure remains high:

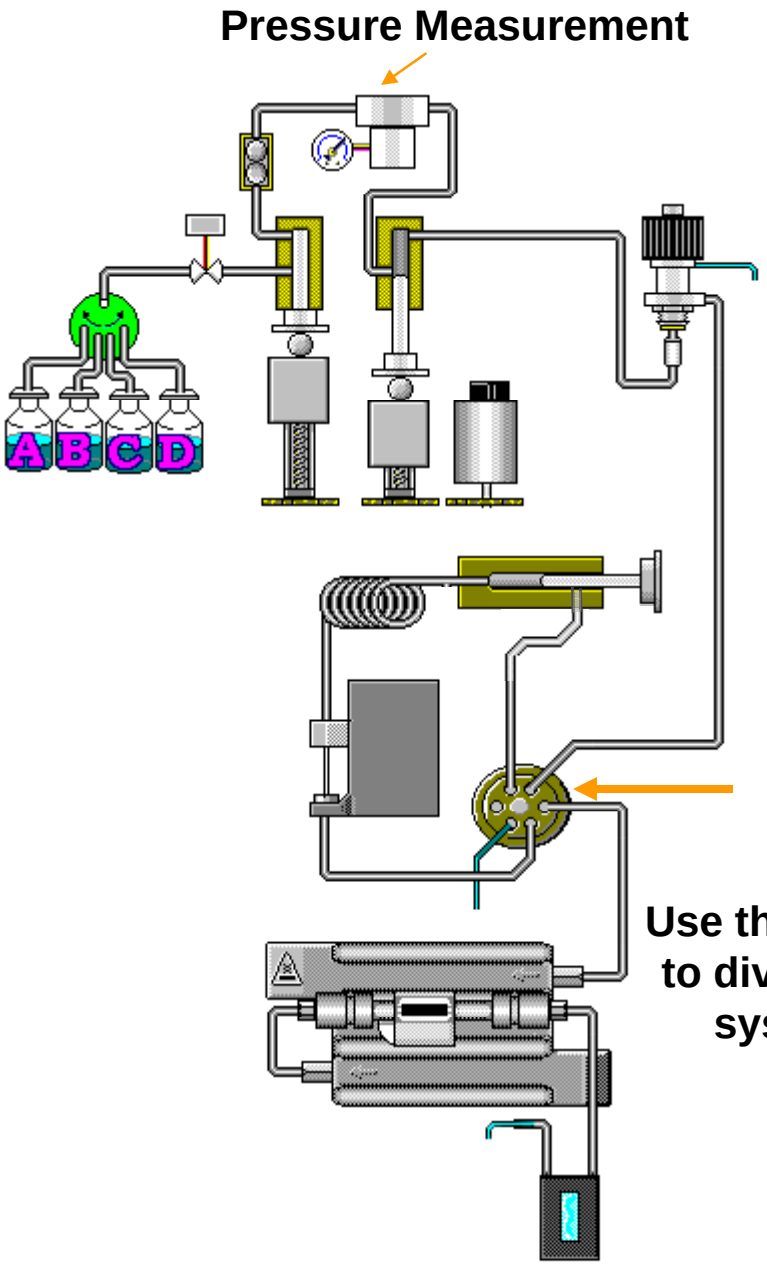
- **Rinse column (remove detector from flow path!)**
  - **Eliminate column contamination and plugged packing**
  - **high molecular weight/adsorbed compounds**
  - **precipitate from sample or buffer**
- **Back flush column – may clear plugged column inlet frit**
- **Change column inlet frit (… or discard column)**

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

Pressure Problem I

Pressure Too High

- Column inlet frit contaminated
- Frit in purge valve contaminated
- Column contaminated
- Blockage in a capillary, particularly needle seat capillary
- Rotor in injection valve plugged
- Injection needle or needle seat plugged

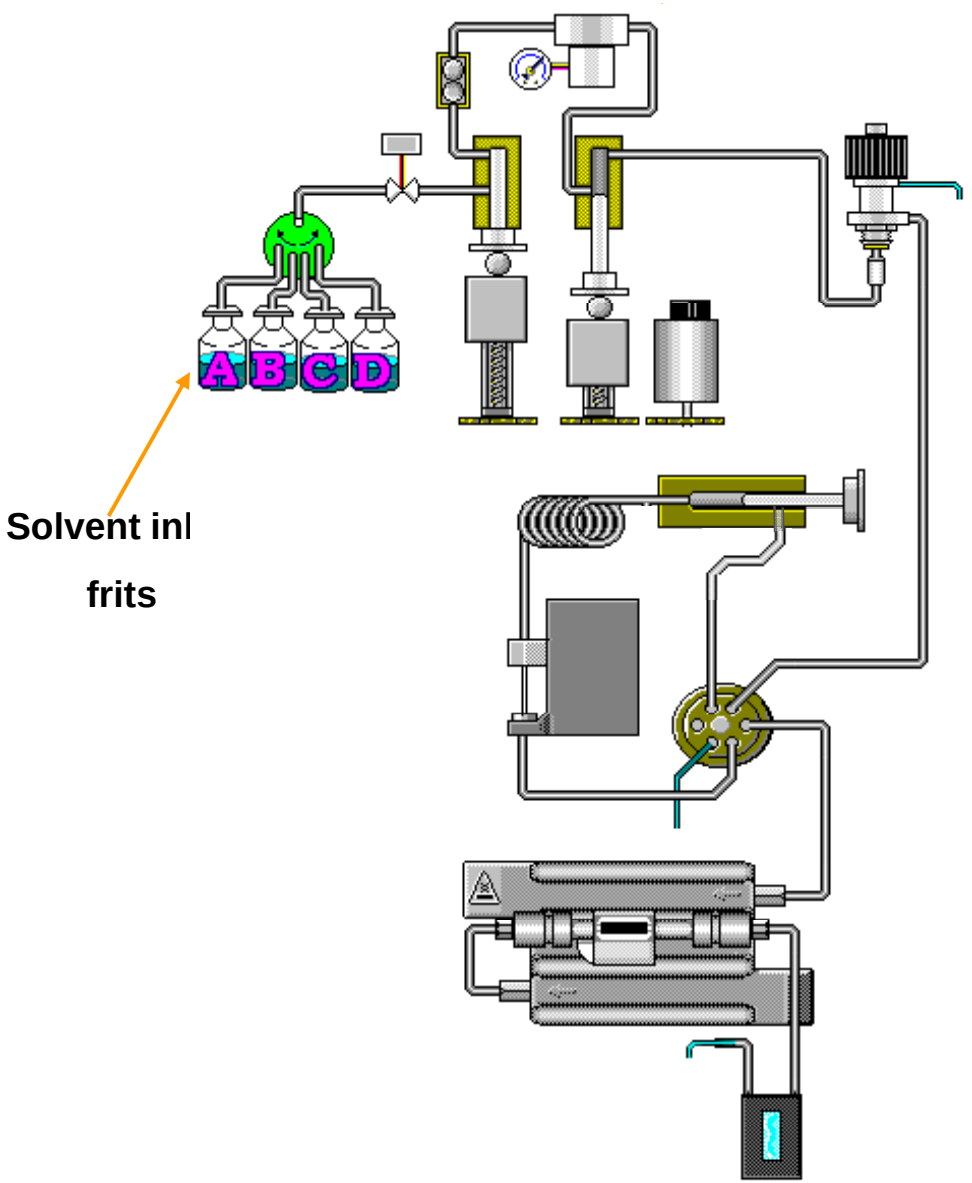


Pressure Problem II

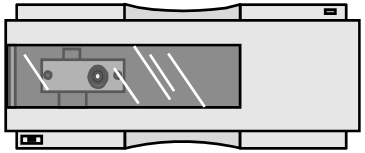
Pressure Too Low

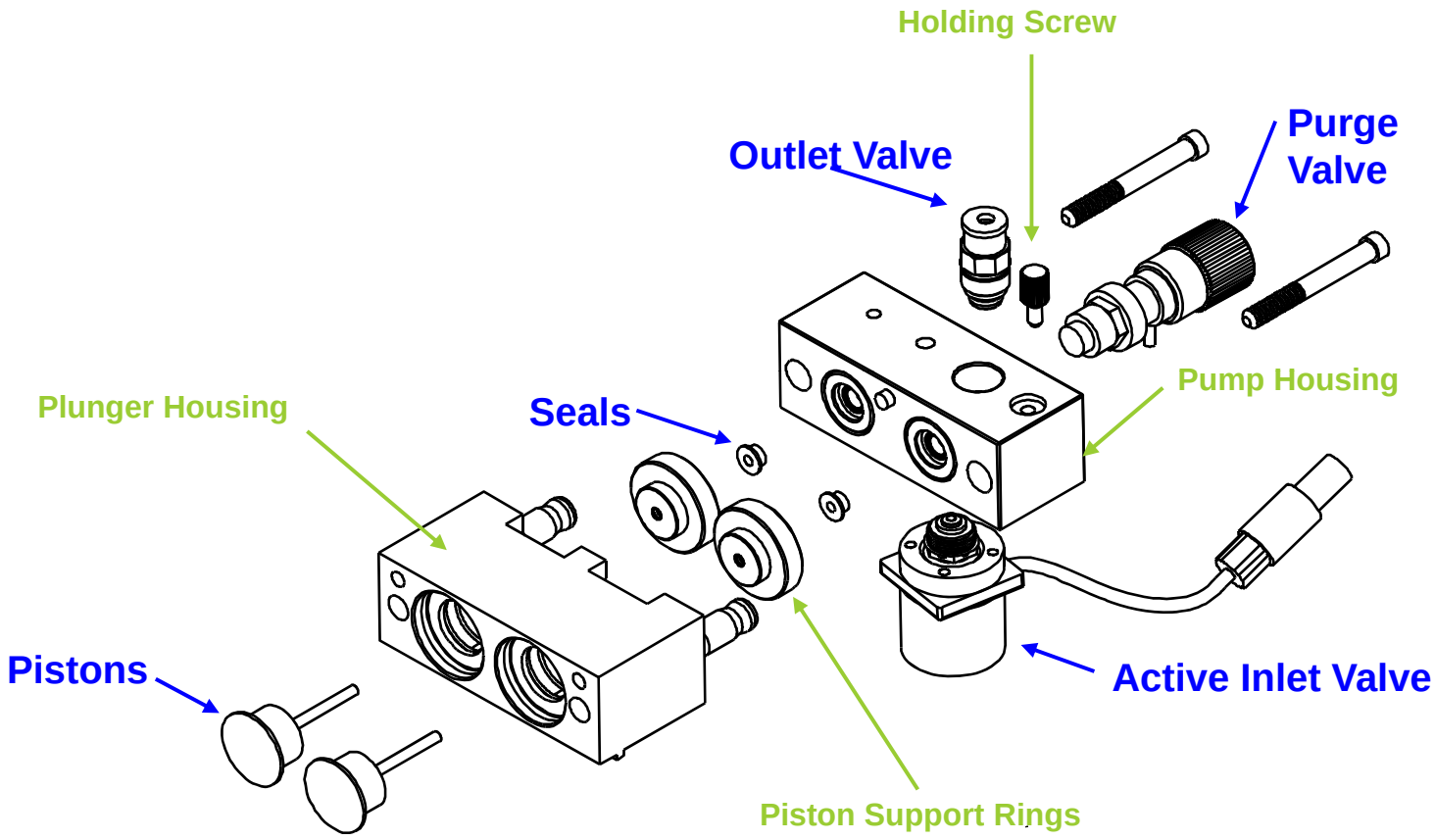
- Solvent inlet frit plugged
- Leak in a capillary connection or other part (pump seals)
- Wrong solvent or flow rate
- AIV (Active inlet valve) defective
- Multichannel Gradient valve incorrectly proportioning
- Ball valve defective
- Column defective (stationaryphase)

Pressure Measurement

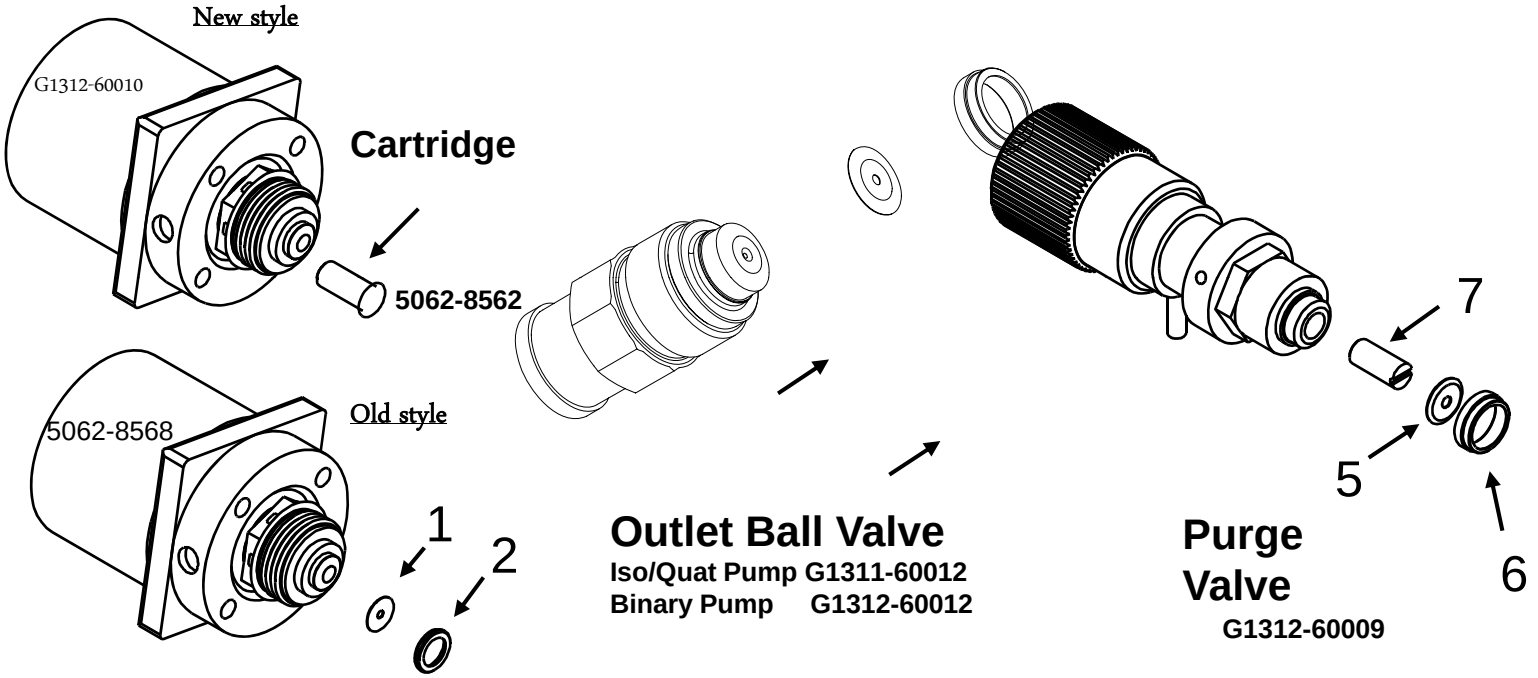


1100 and 1200 Pumps
Exploded View





Pump Check Valves



Active Inlet Valve
(common to all)

1. Gold Washer	5001-3707	5. Gold Seal	5001-3707
2. Plastic cap	01018-21207	6. Cap(4pk)	5062-2485
3. Gold Seal	5001-3707	7. PTFE (5pk)	01018-22707
4. Cap(4pk)	5062-2485		

Column Cleaning:

Flush with stronger solvents than your mobile phase. Make sure detector is taken out of flow path.

Reversed-Phase Solvent Choices
in Order of Increasing Strength

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

1. Mobile phase without buffer salts (water/organic)
2. 100% Organic (MeOH or ACN)
3. Is pressure back in normal range?
4. If not, discard column or consider more drastic conditions:
   75% Acetonitrile:25% Isopropanol, then
5. 100% Isopropanol
6. 100% Methylene Chloride*
7. 100% Hexane*

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

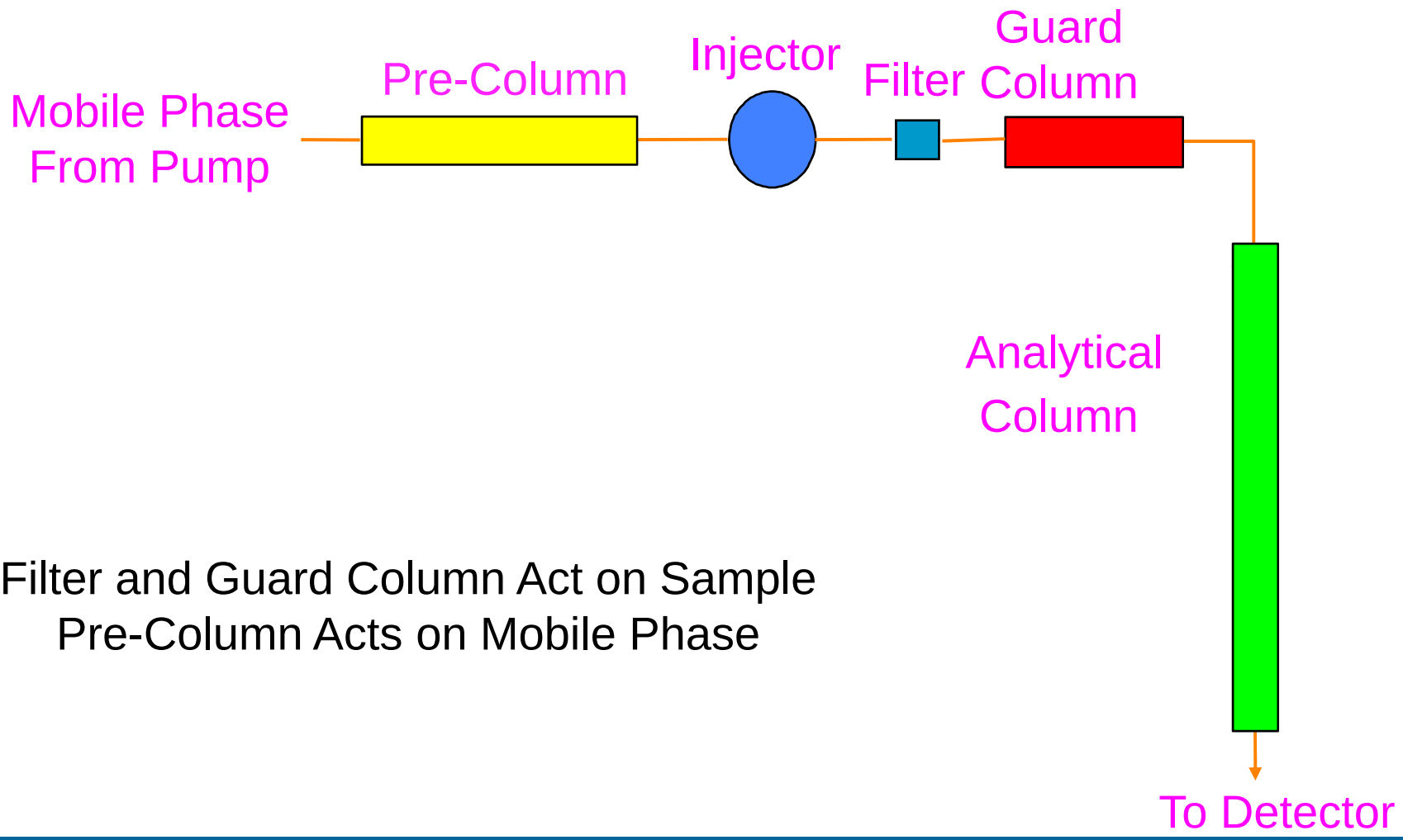
Column Cleaning

Normal Phase Solvent Choices

in Order of Increasing Strength

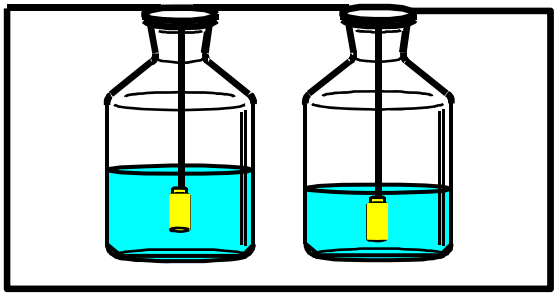
- Use at least 50 mL of each solvent
- 50% Methanol : 50% Chloroform
- 100% Ethyl Acetate

Preventing Back Pressure Problems: In-Line Devices



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

Preventing Column Back Pressure Problems:



1. Filter mobile phase:
   - filter non-HPLC grade solvents
   - filter buffer solutions
   - Install an in-line filter between auto-sampler and column (removes pump seal debris, ALS rotor debris, and sample particulates). Use 2 um frit for 3.5 um columns, use 0.5 um frit for 1.8 um columns.
2. Filter all samples and standards
3. Perform sample clean-up (i.e. SPE, LLE) on dirty samples.
4. Appropriate column flushing – flush buffers from entire system at end of day with water/organic mobile phase.

2. Peak Shape Issues in HPLC

- **Split peaks**
- **Peak tailing**
- **Broad peaks**
- **Poor efficiency (low N)**
- **Inconsistent Response**

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

Split Peaks

Can be caused by:

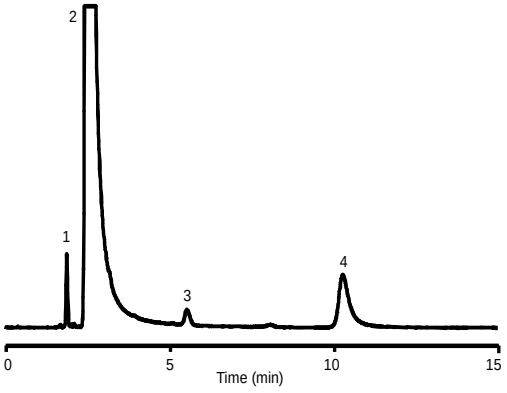
- Column contamination
- Partially plugged frit
- Column void (gap in packing bed)
- Injection solvent effects

Split Peaks

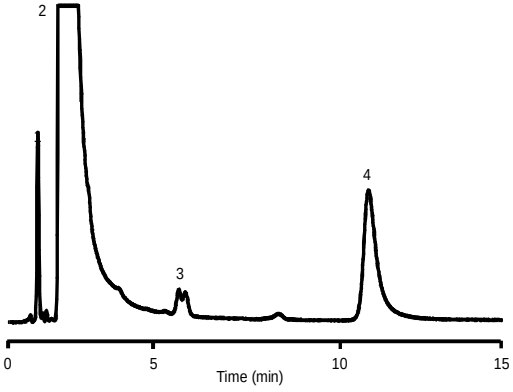
Column Contamination

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

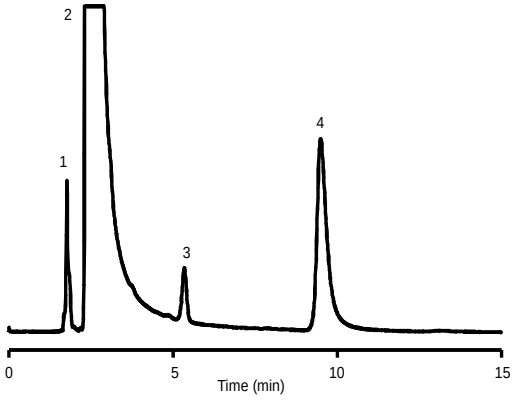
Injection 1



Injection 30



Injection 1 After Column Wash with 100% ACN



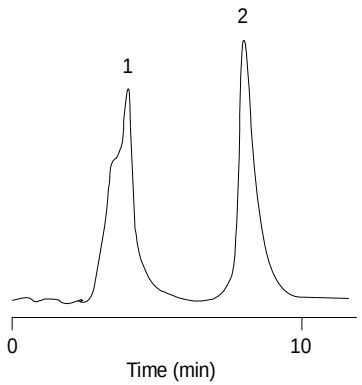
- **Column washing eliminates the peak splitting, which resulted from a contaminant on the column.**

Split Peaks

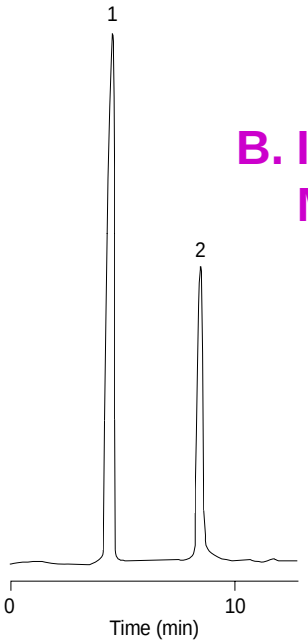
Injection Solvent Effects

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

A. Injection Solvent 100% Acetonitrile

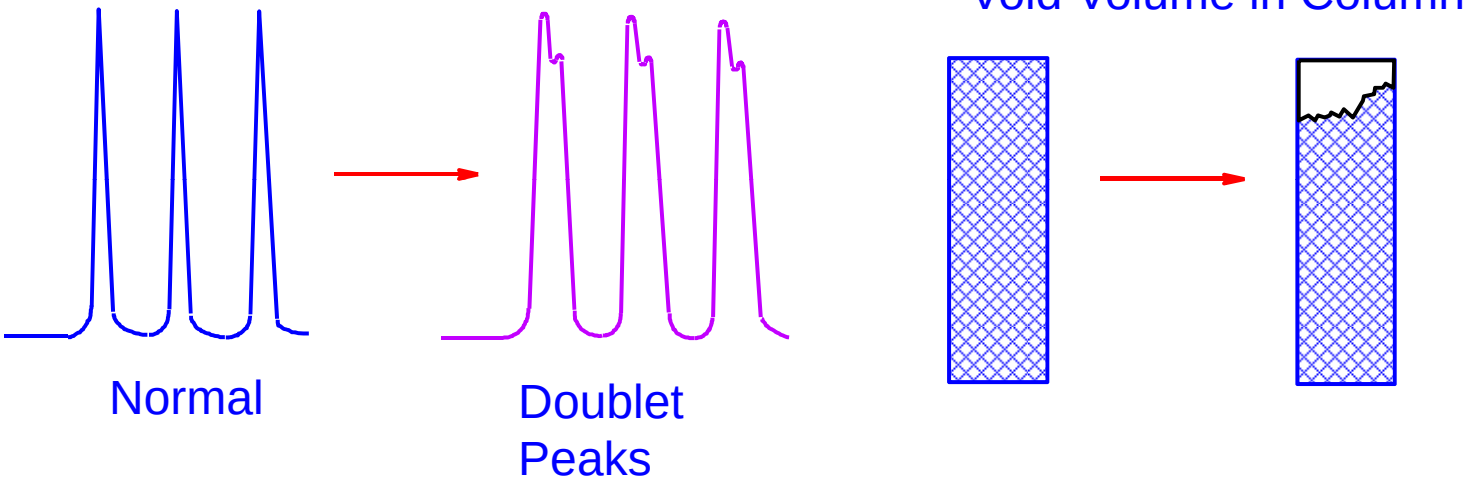


B. Injection Solvent Mobile Phase



- Injecting in a solvent stronger than the mobile phase can cause peak shape problems, such as peak splitting or broadening.
- Note: earlier peaks (low k) most affected

Peak Shape Problems - Doublets



- Void Volume in Column
- Partially Blocked Frit
- Only One-Peak a Doublet- Coeluting Components
- Early (low k) peaks most affected

Determining the Cause of Split Peaks

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

2. Mobile phase pH > 7 - likely column void due to silica dissolution (unless specialty column used, Zorbax Extend-C18 stable to pH 11)

3. Injection solvent stronger than mobile phase - likely split *and* broad peaks, shape dependent on injection volume and k value.

Peak Tailing, Broadening and Loss of Efficiency (N, plates)

May be caused by:

1. Column “secondary interactions”
2. Column packing voids
3. Column contamination
4. Column aging
5. Column loading
6. Extra-column effects

Peak Tailing

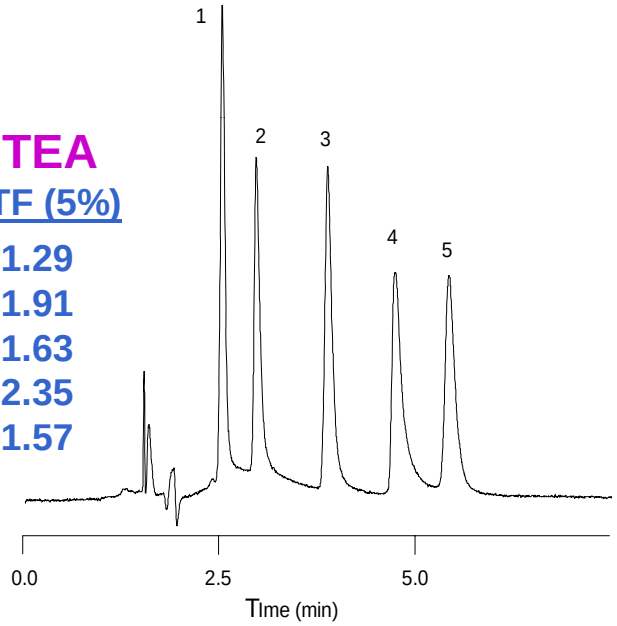
Column “Secondary Interactions”

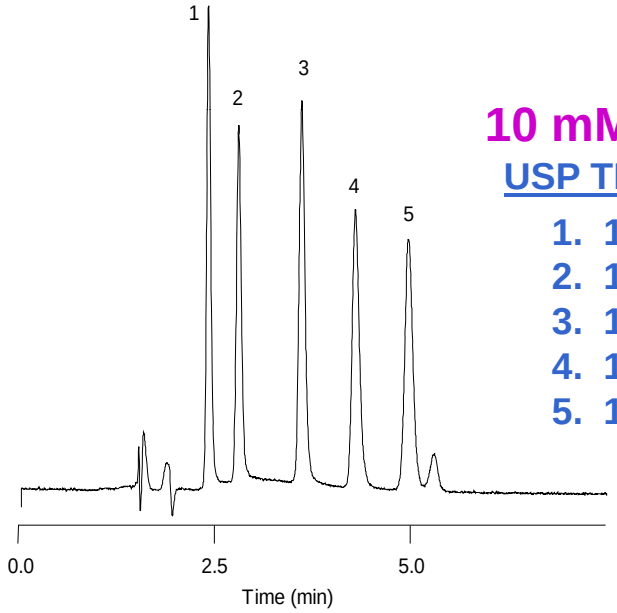
Column: Alkyl-C8, 4.6 x 150 mm, 5mm Mobile Phase: 85% 25 mM Na_2HPO_4 pH 7.0 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

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

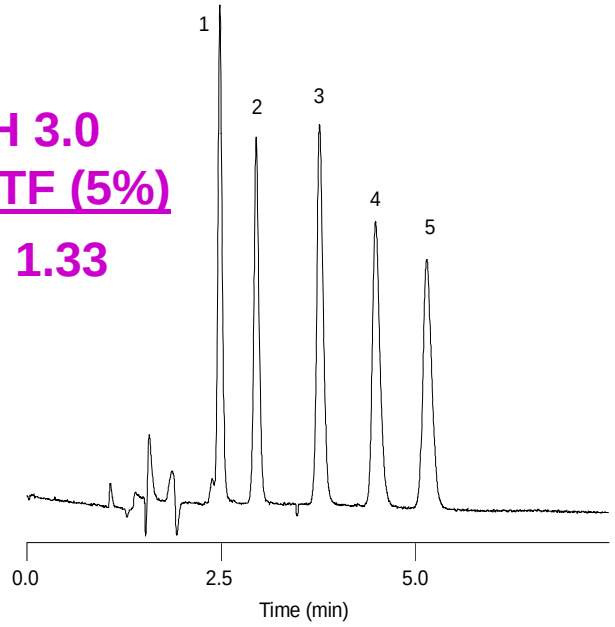
- Peak tailing of amine analytes eliminated with mobile phase modifier (TEA, triethylamine) at pH 7

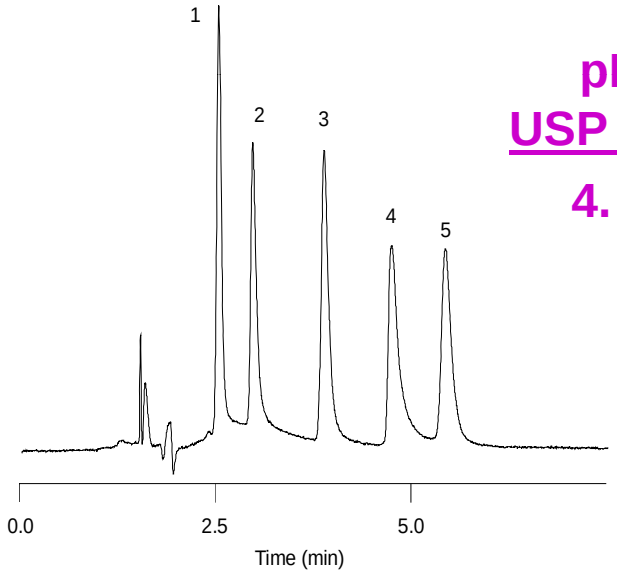
Peak Tailing

Column “Secondary Interactions”

Column: Alkyl-C8, 4.6 x 150 mm, 5mm 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. No TEA modifier required.

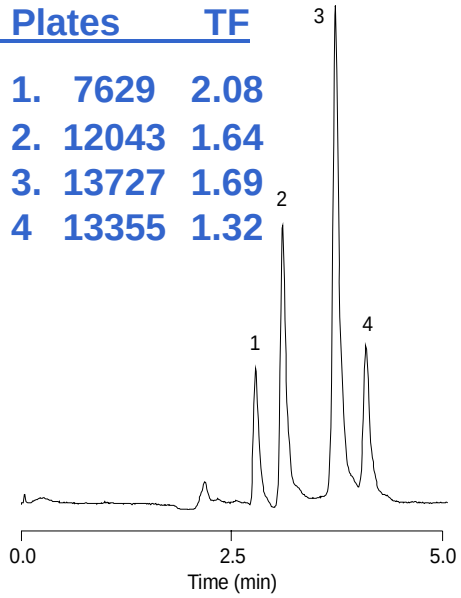
Peak Tailing

Column Contamination

Column: StableBond SB-C8, 4.6 x 250 mm, 5mm 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

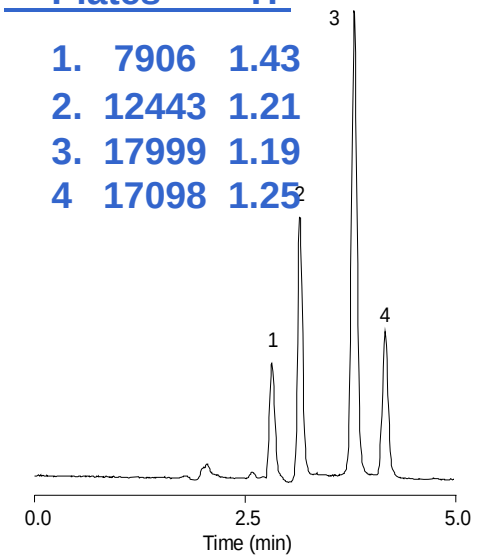
QC test forward direction

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



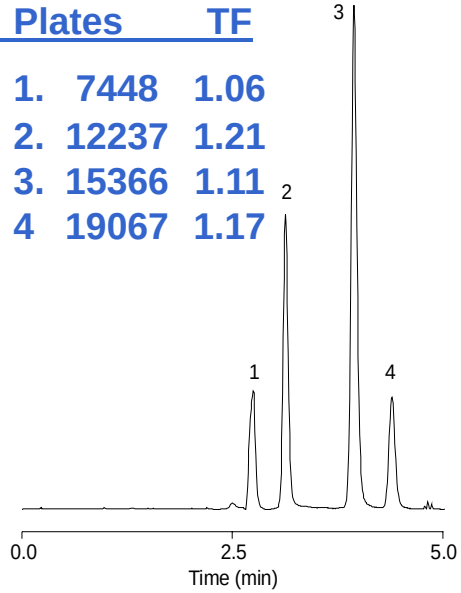
QC test reverse direction

	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

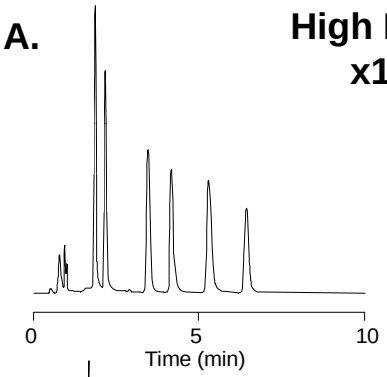
Columns: 4.6 x 150 mm, 5mm Mobile Phase: 40% 25 mM Na_2HPO_4 pH 7.0 : 60% ACN Flow Rate: 1.5 mL/min
Temperature: 40°C Sample: 1. Desipramine 2. Nortriptyline 3. Doxepin 4. Imipramine 5. Amitriptyline 6. Trimipramine

Tailing

Eclipse XDB-C8
USP TF (5%)

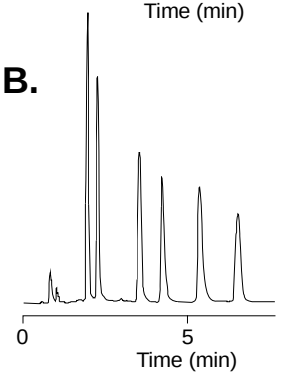
	A	B
1.	1.60	1.70
2.	2.00	1.90
3.	1.56	1.56
4.	2.13	1.70
5.	2.15	1.86
6.	1.25	1.25

A.



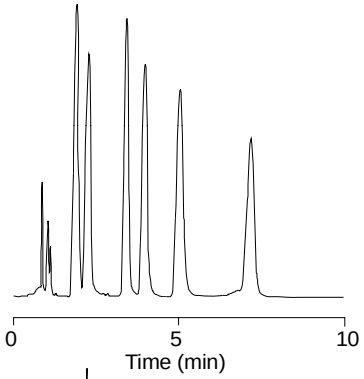
High Load x10

B.

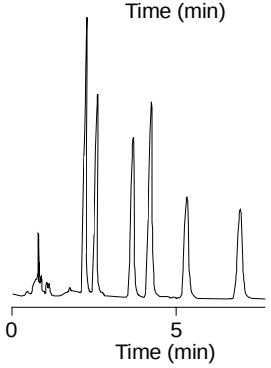


Low Load

C.



D.



Broadening

Competitive C8
Plates

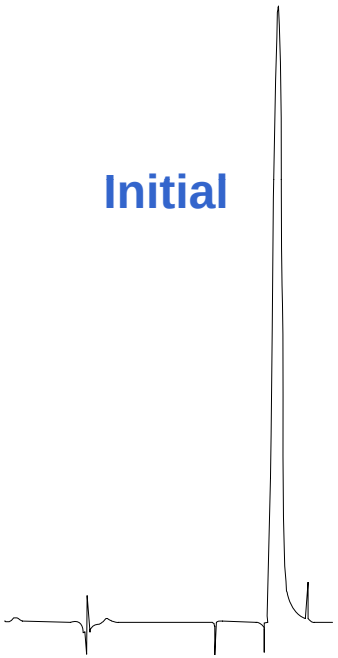
	C	D
1.	850	5941
2.	815	7842
3.	2776	6231
4.	2539	8359
5.	2735	10022
6.	5189	10725

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.

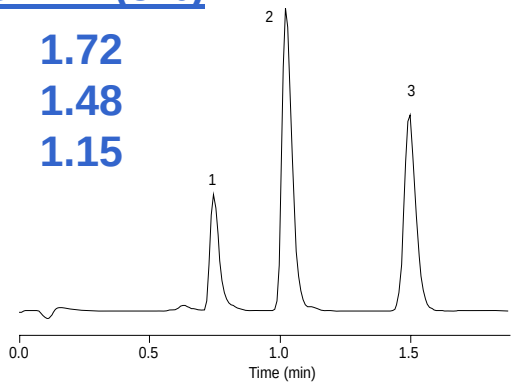
Peak Tailing

Injector Seal Failure

Column: Bonus-RP, 4.6 x 75 mm, 3.5 **mm** 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

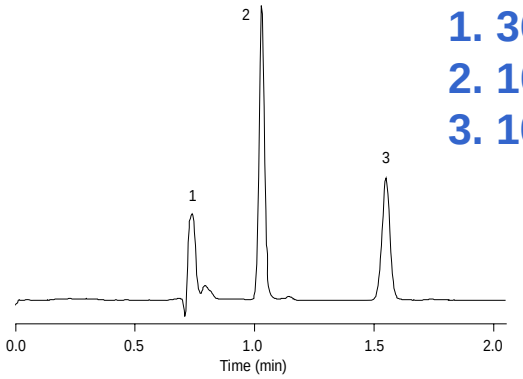
Before

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

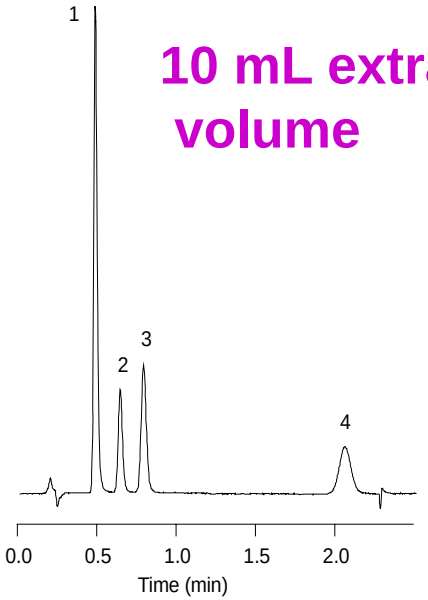


- **Overdue instrument maintenance can sometimes cause peak shape problems.**

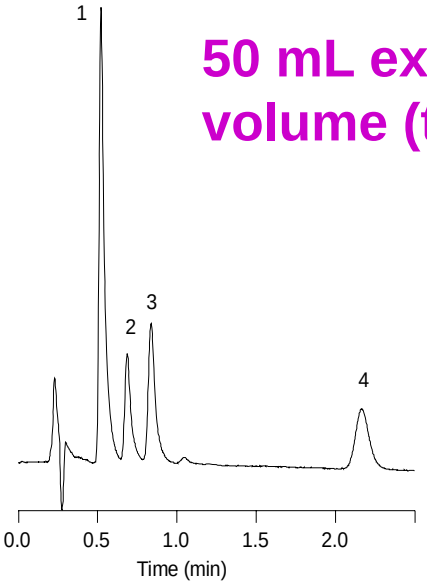
Peak Tailing

Extra-Column Volume

Column: StableBond SB-C18, 4.6 x 30 mm, 3.5 mm 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)

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 – vol inj and concentration
- Eliminate extra-column effects – tubing, fittings, Uv cell
- Flush column and check for aging/void

Reproducibility

Peak retention time precision:

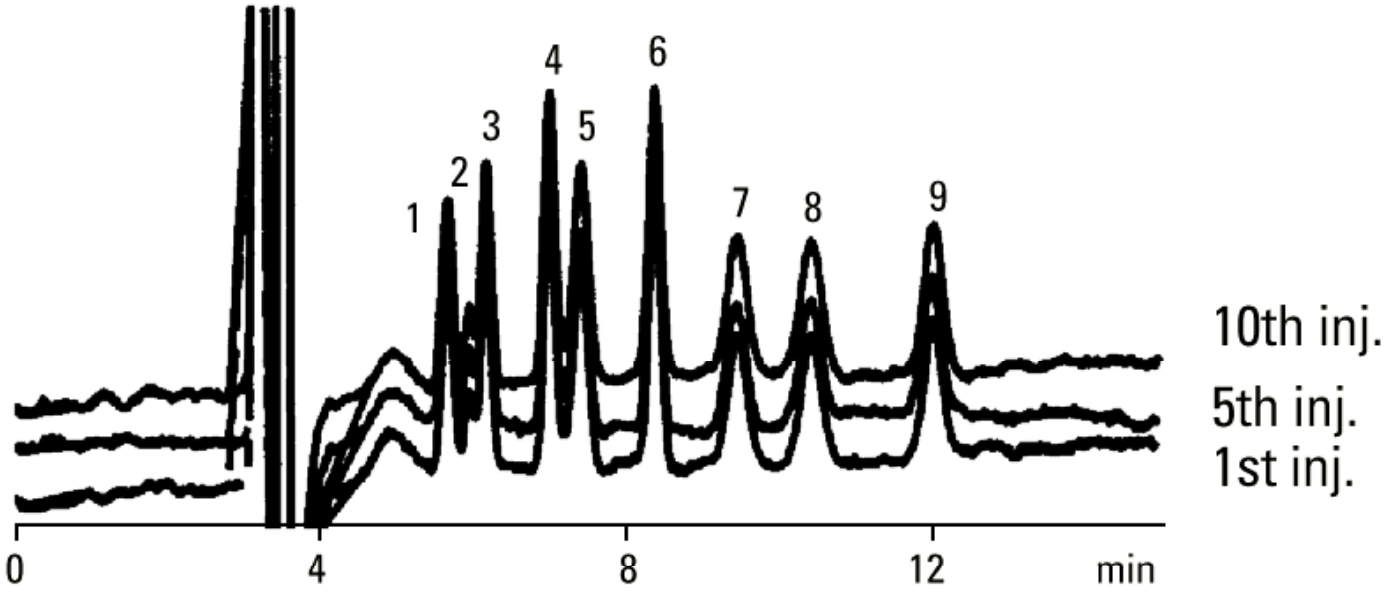
⇒ with oven: ≤ 0.3%

⇒ without oven: ≤ 0.7%

Peak area precision: ≤1.5%

Typically,

- Area and Peak Height problems together point to the autosampler system
- Area and Retention Time problems together point to the pump



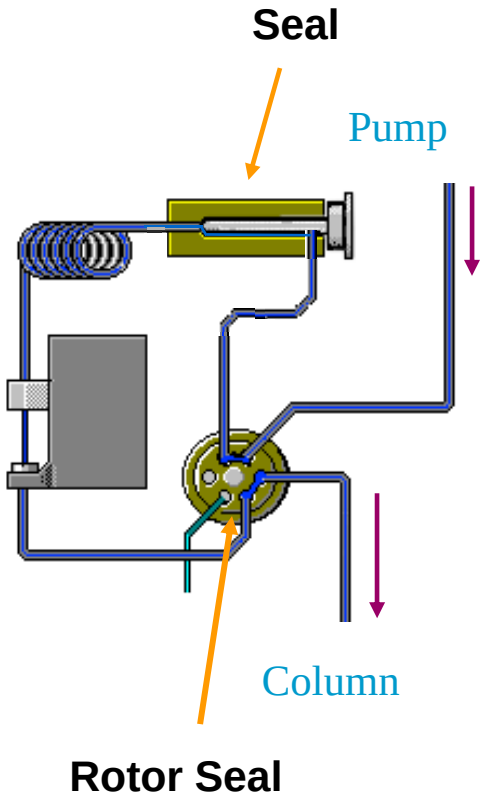
Problems with Reproducibility – Peak Areas

Peak Areas not Reproducible

With peak height
- Rotor seal cross-port leak or injection valve not tight
- Piston seal of metering unit leaking
- Needle partially blocked

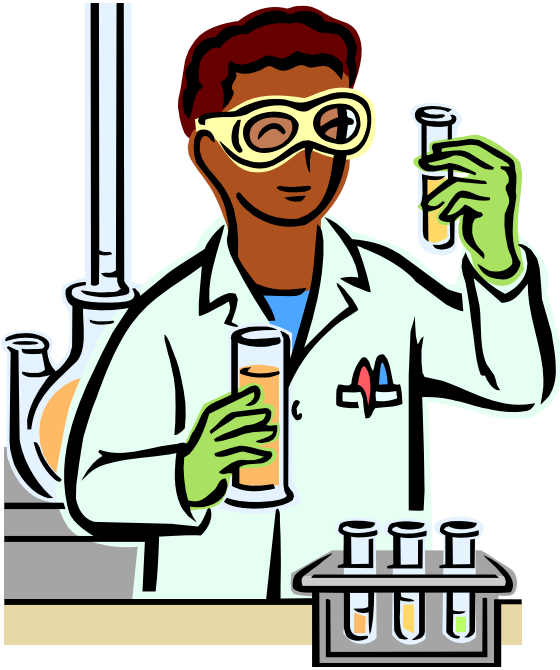
With retention time
- Variable pump flow rate

Other
- Capillary from injector to detector not tight
- Detector equilibration problems



3. Retention Issues

- **Retention time changes (t_r)**
- **Retention factor changes (k')**
- **Selectivity changes (a)**

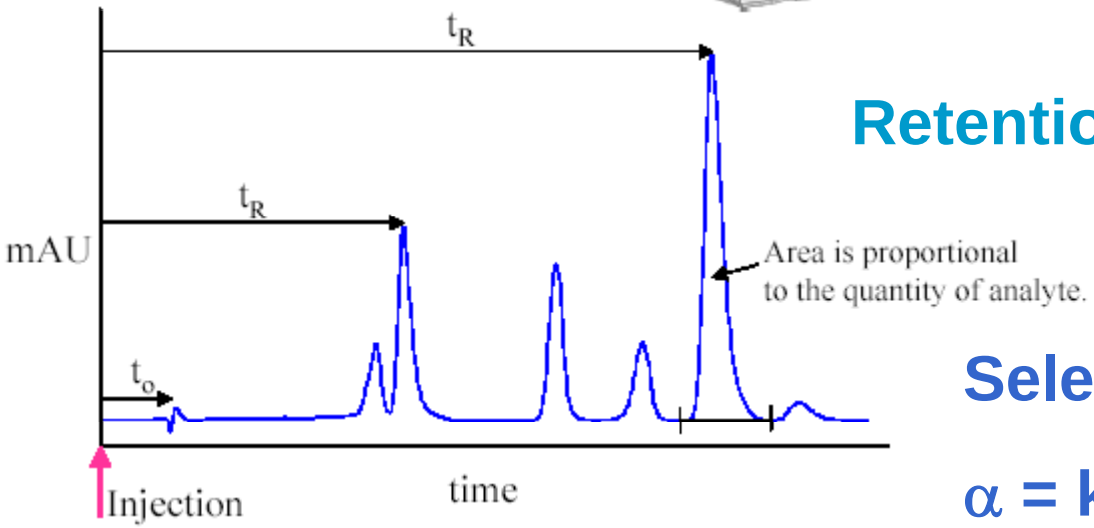


Retention time t_R, Retention factor k', and Selectivity factor α

The Chromatogram

t_o - elution time of unretained peak

t_R - retention time - determines sample identity



Retention factor k' = $(t_R-t_0)/t_0$

Selectivity factor α

$\alpha = k_2/k_1$

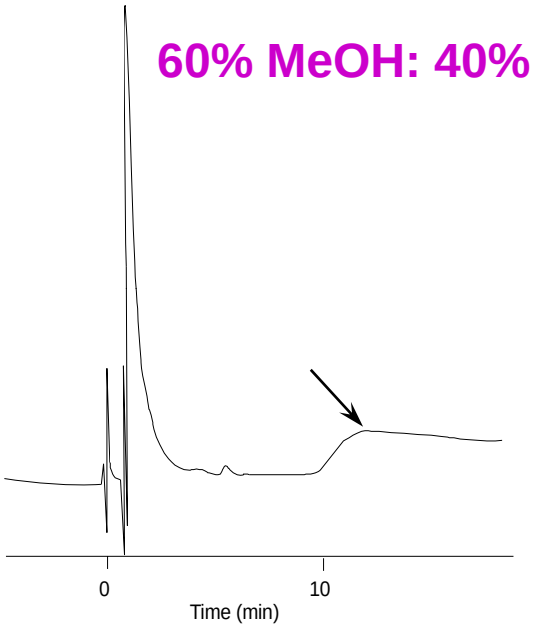
Changes in Retention (k) - Same Column, Over Time

May be caused by:

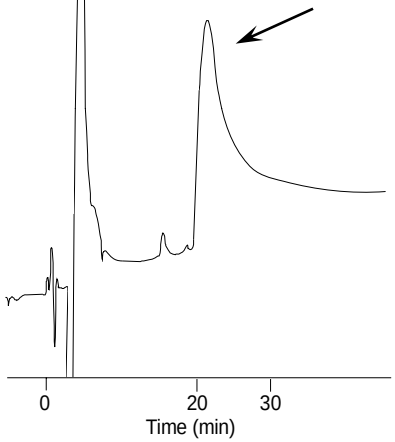
1. Column aging
2. Column contamination
3. Insufficient column equilibration
4. Poor column/mobile phase combination
5. Change in mobile phase
6. Change in flow rate
7. Change in column temperature
8. Other instrument issues

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.

Separation Conditions That Cause Changes in Retention*

Flow Rate	**± 1%**	**± 1% t_r**
Temp	**± 1° C**	**± 1 to 2% t_r**
%Organic	**± 1%**	**± 5 to 10% t_r**
pH	**± 0.01%**	**± 0 to 1% t_r**

***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', a, 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

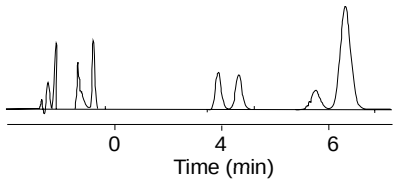
1. Different column histories (aging)
2. Insufficient/inconsistent equilibration
3. Poor column/mobile phase combination
4. Change in mobile phase
5. Change in flow rate
6. Other instrument issues
7. Slight changes in column bed volume (t_r only)

Example Change in Retention/Selectivity

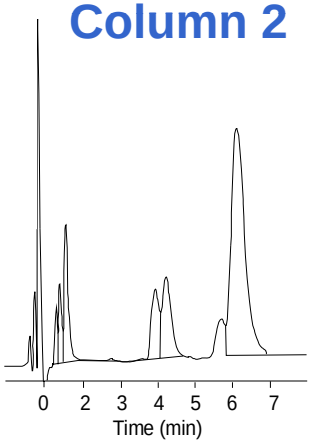
Column-to-Column

Mobile Phase Variation

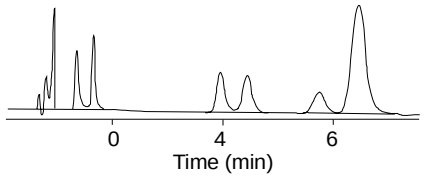
Column 1



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."

Minimize Change in Retention/Selectivity
Lot-to-Lot

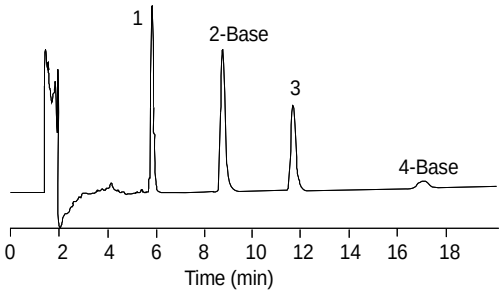
Evaluate:

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

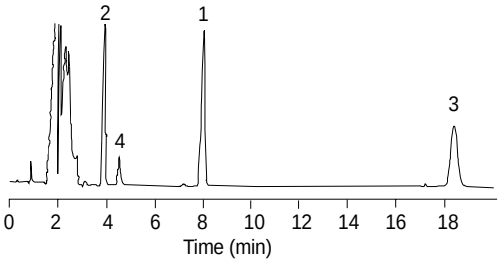
*All causes of column-to-column change should be considered first, especially when only one column from a lot has been tested.

Lot-to-Lot Selectivity Change - pH

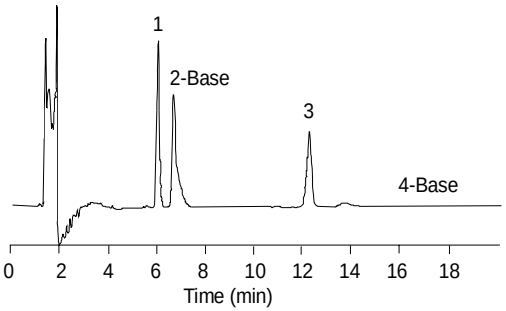
pH 4.5 - Lot 1



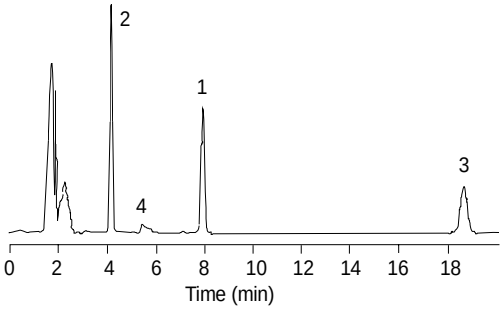
pH 3.0 - Lot 1



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**
- **Evaluate several pH levels to establish most robust choice of pH**

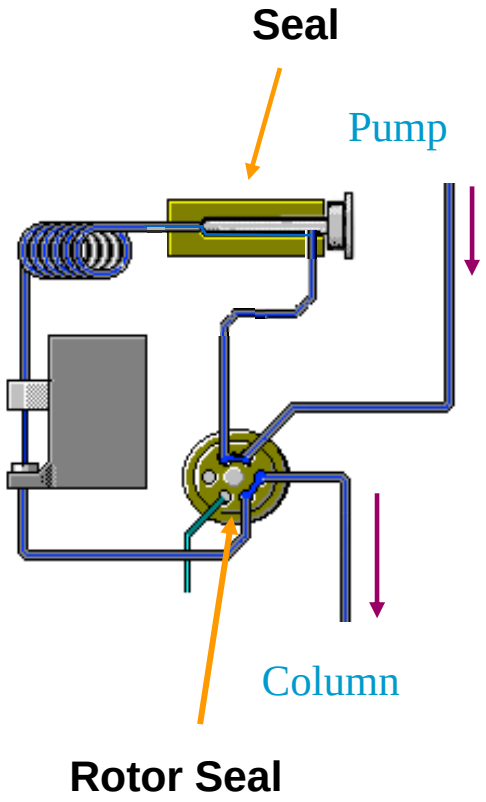
Problems with Reproducibility – Peak Areas

Peak Areas not Reproducible

With peak height
- Rotor seal cross-port leak or injection valve not tight
- Piston seal of metering unit leaking
- Needle partially blocked

With retention time
- Variable pump flow rate

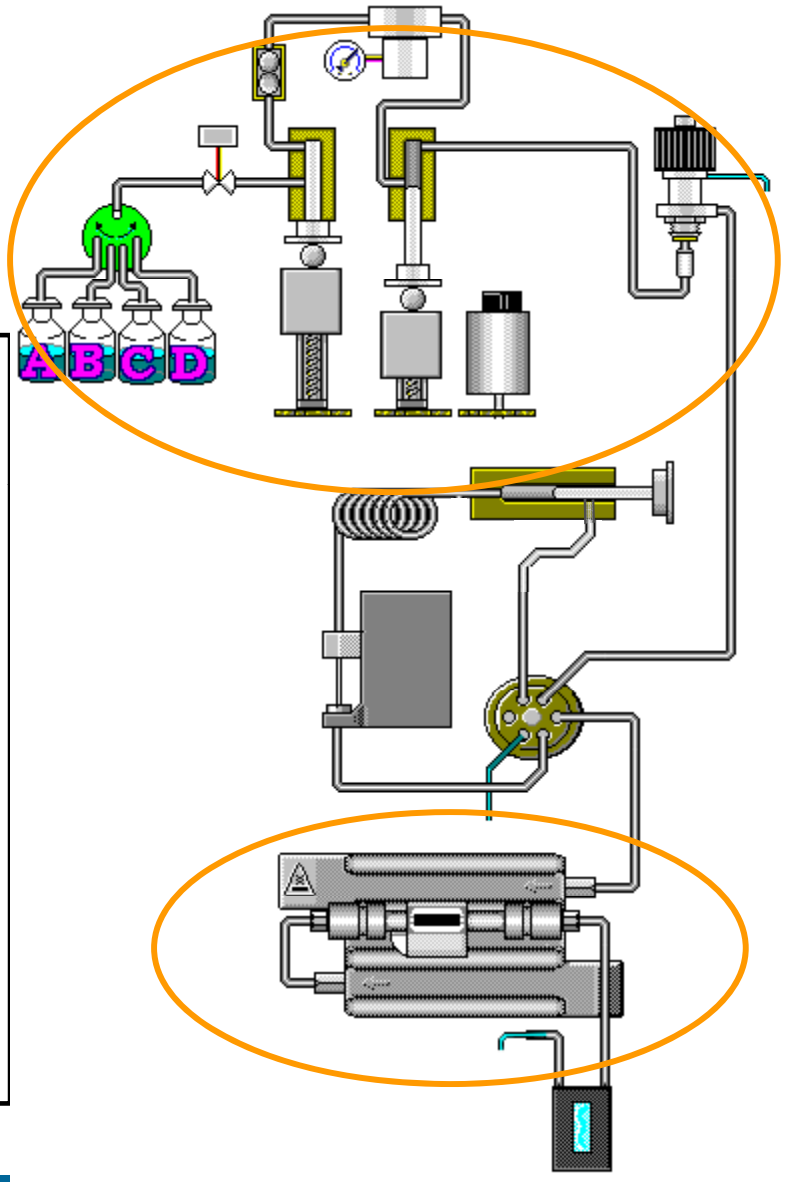
Other
- Capillary from injector to detector not tight
- Detector equilibration problems



Problems with Reproducibiliy – Retention Time

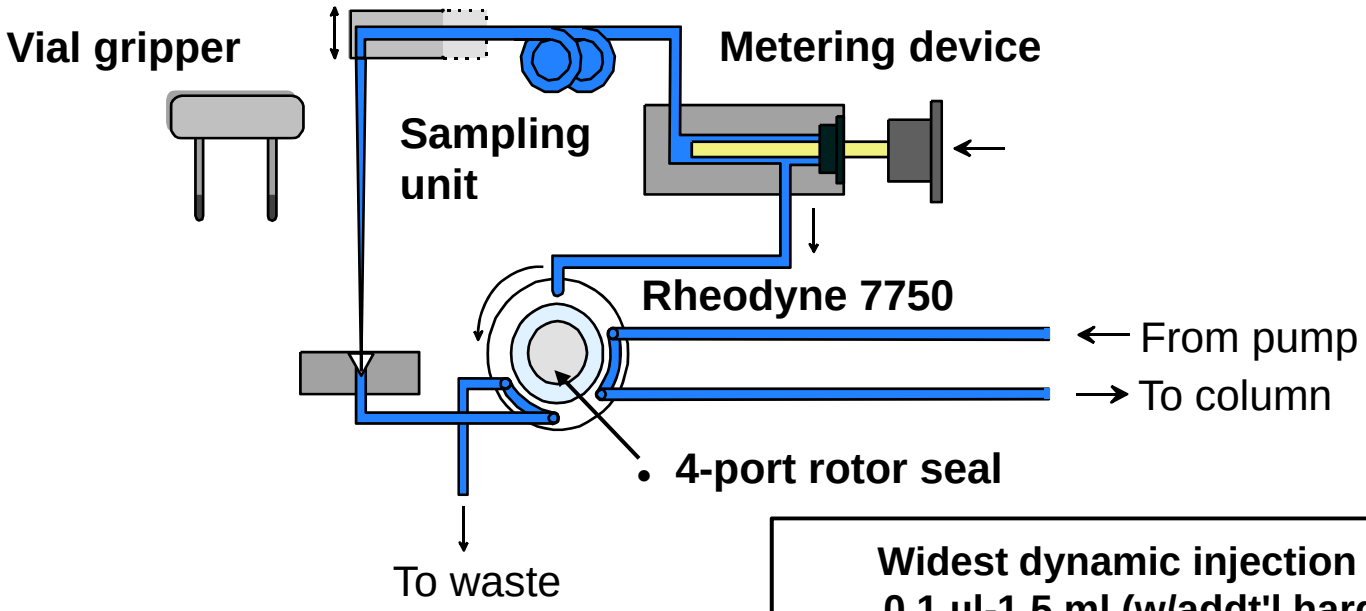
Retention Times not Reproducible

- **Pump Problems**
  - –Mobile phase composition problems
  - –Valves AIV, ball valve defective
  - –Flow rate problems
- **Column Oven Problems**
  - –Temperature fluctuations
- **Other**
  - –Column equilibration
  - –Column deterioration



Autosampler
Principle of Operation

Standard loop volume300µl
Total delay volume 300µl + Vinj
Minimal (bypass) delay volume 6.2ul



Widest dynamic injection range:
0.1 µl-1.5 ml (w/addt'l hardware)

Evaluate Retention Changes

Lot-to-Lot

1. Eliminate causes of column-to-column selectivity change
2. Re-evaluate method ruggedness - modify method
3. Determine pH sensitivity - modify method
4. Classify selectivity changes
5. Contact manufacturer for assistance*

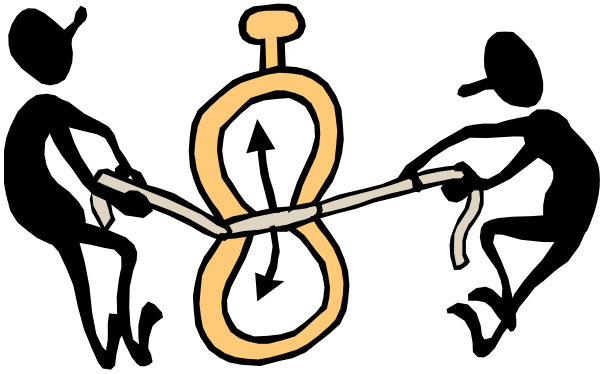
Agilent Column Support: 800-227-9770, option 4, option 2 (LC columns)

Conclusions:

HPLC column problems are evident as:

1. High pressure
2. Undesirable peak shape
3. Changes in retention/selectivity

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



The End – Thank You!

Agilent LC Column Tech Support: 800-227-9770 #4, #2 Email: Edward_kim@agilent.com