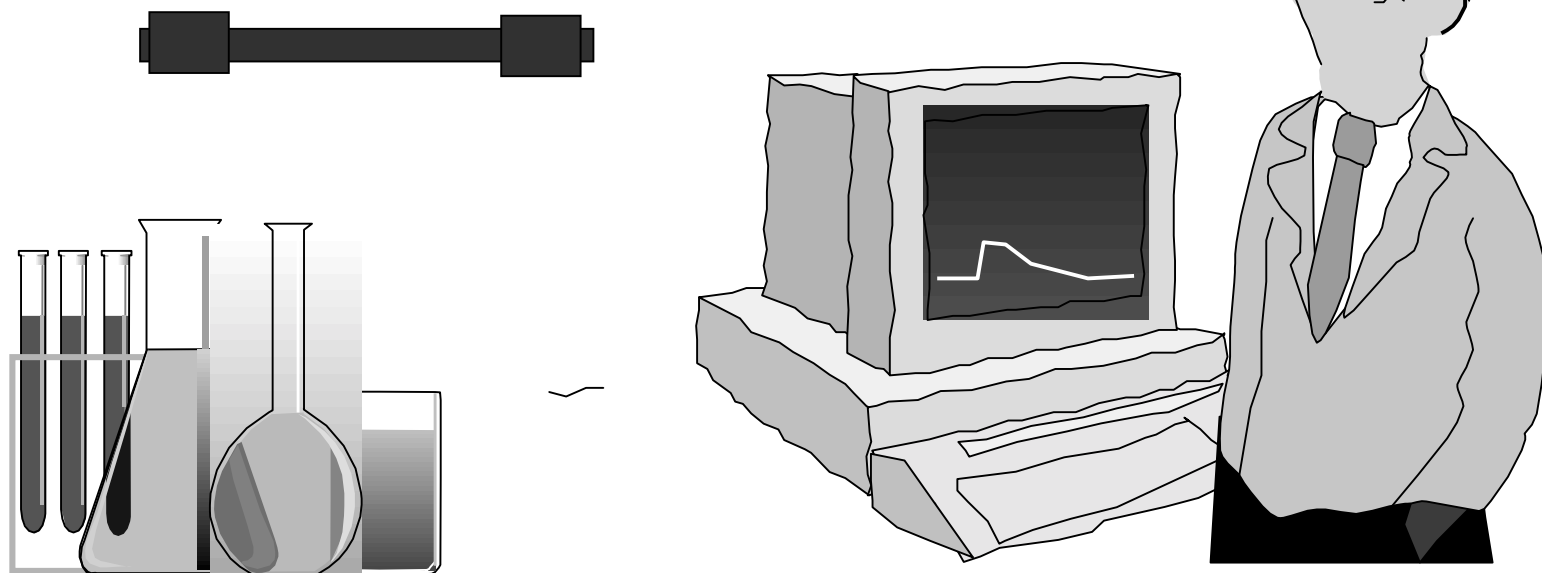


Troubleshooting Common HPLC Problems



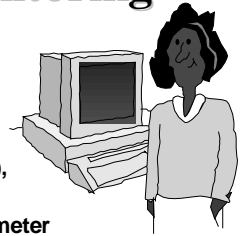
<http://www.hplc1.com/shodex/english/dd.htm>

HPLC Troubleshooting



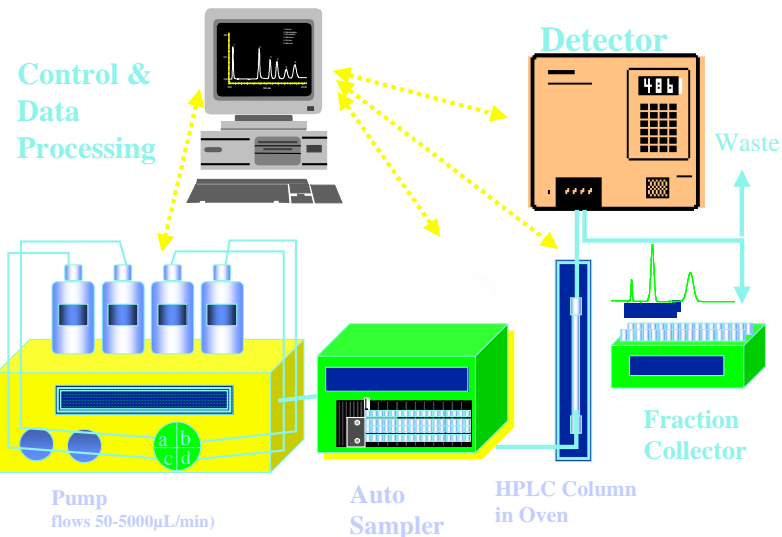
Performance Monitoring

Use Your Test Method
(Known Performance)

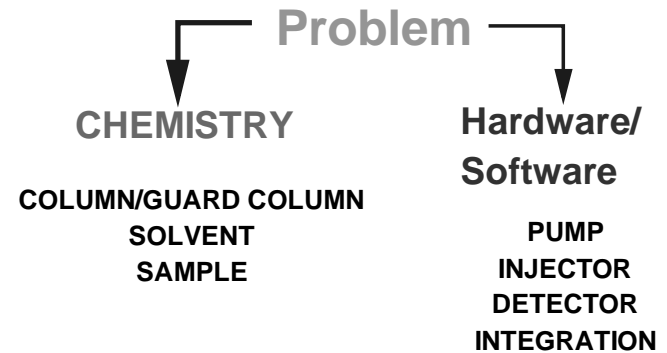


- * Monitor at least One Peak in one injection
 - Plate Count (Peak width relative to RT),
 - Peak Asymmetry,
 - Retention Time and/or Retention parameter
 - Relative Retention Time for Critical Pair of Analytes.
 - Peak Response

- * Inject Multiple Runs
 - Precision (at least 5 injections)
 - Accuracy (Use Control Samples)

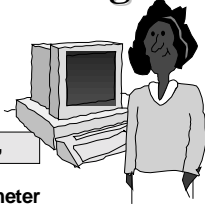


Troubleshooting



Performance Monitoring

Use Your Test Method
(Known Performance)



* Monitor at least One Peak in one injection

- Plate Count (Peak width relative to RT),
- Peak Asymmetry,
- Retention Time and/or Retention parameter
- Relative Retention Time for Critical Pair of Analytes.
- Peak Response

* Inject Multiple Runs

- Precision (at least 5 injections)
- Accuracy (Use Control Samples)

Performance Monitoring

Column Efficiency:

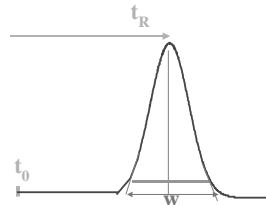
N = the number of Theoretical Plates

a = is a constant depending on the Method used

t = retention time of peak

W = the peak width (time units) at a given peak height

$$N = a \left(\frac{t_r}{W} \right)^2$$



METHOD	a
Peak Width at Half Height	5.54
Peak Width at 4.4% Peak Height (5 sigma)	25.0
Tangent	16.0

Plate Count - Efficiency of the Separation

* A "Plate Count" Actually Is a Determination Of Both The Column AND Instruments' Performance

Performance Monitoring

Band Spreading

* Band Spreading Impacts Chromatographic Performance -- The Greater The Band Spreading, The Poorer The Performance (ie; Resolution)

* Band Spreading Contains Both An Instrument AND A Column Contribution

Extra-Column Band Spreading

The Observed Bandwidth (TOT)

* Sum of the Bandspreading Contributions

- Column (COL)
- Extra-Column (EC) Instrument components

$$\sigma_{TOT}^2 = \sigma_{COL}^2 + \sigma_{EC}^2$$

Band Spreading

* Column Contribution

σ_{COLUMN}^2 = optimized by choosing the correct column and conditions

* Instruments Contribution = Extra-Column

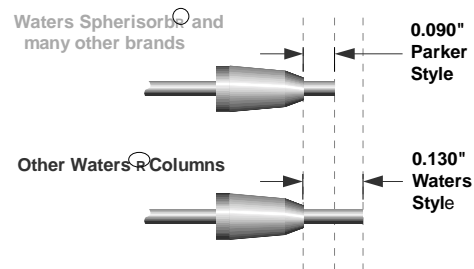
$$\sigma_{EC}^2 = \sigma_{TUBING}^2 + \sigma_{CONNECTIONS}^2 + \sigma_{INJECTORS}^2 + \sigma_{DETECTORS}^2$$

Performance Monitoring

Extra-Column Band Spreading
(Instruments' Contribution)

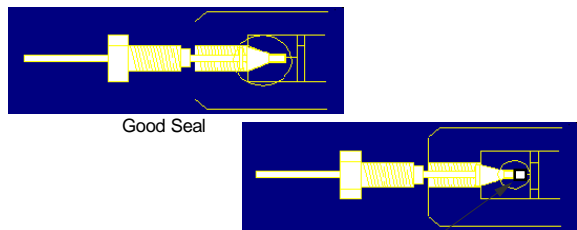
1. Injection Volume
2. Injector
3. Connection Tubing
 - a. from Injector to Column
 - b. from Column to Detector
 - c. Endfittings and Frits
4. Detector Volume

Connectors



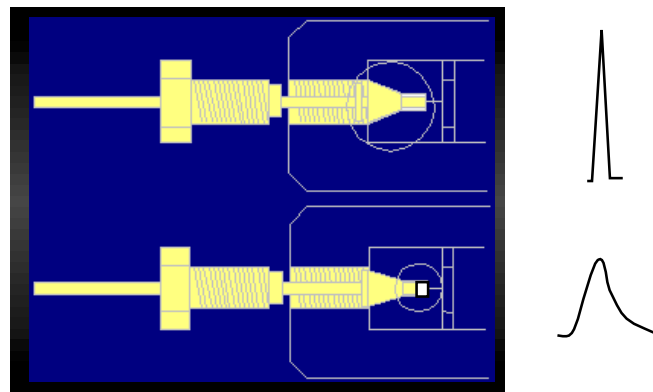
Installation and Equilibration

- ✓ Make sure column inlet connected correctly
- ✓ Make sure nut and ferrule are seated



NOTE: column inlet connector not seated properly
 {PEEK Connectors Easier to Use --
 THF makes PEEK brittle}

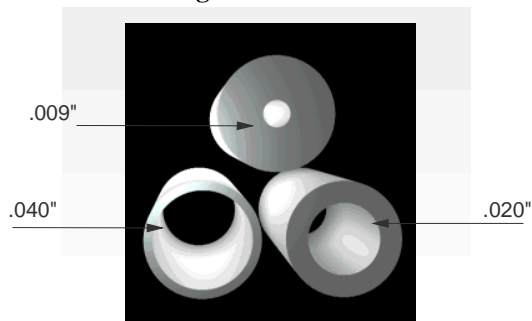
Extra-Column Band Spreading



Column Connection Contribution

Extra-Column Band Spreading

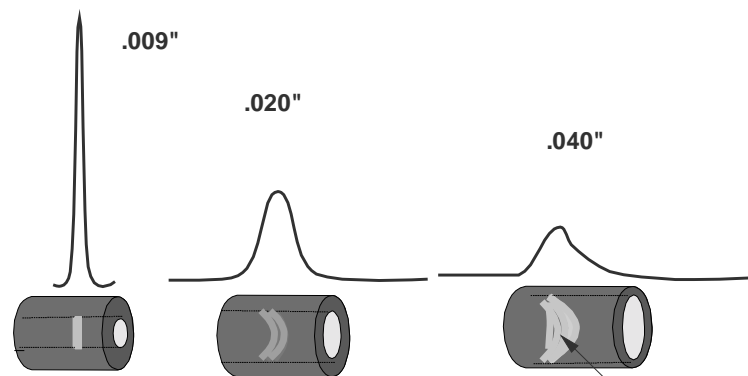
Tubing Contribution



note the differences of the inner diameter of this tubing

Performance Monitoring

Effect of Connecting Tubing on System Bandspreading



sample band dispersion inside tubing

Measuring The Instruments Contribution

* Perform An Instrument Band Spreading Test

Performance Monitoring

Using 5 sigma efficiency method, measure the peak width at 4.4% of peak height

Convert to microliters using the following equation:

$$\left(\frac{2\text{cm}}{\text{PW}} \right) \left(\frac{1\text{min}}{20\text{cm}} \right) \left(\frac{1\text{mL}}{\text{min.}} \right) \left(\frac{1000\mu\text{L}}{\text{mL}} \right) = 100 (\mu\text{L})$$

where:

1min/20cm = chart speed

1 mL/min = flow rate

1000 $\mu\text{L}/\text{mL}$ = volume correction factor

Typical LC System should be 100 μL +/- 30 μL

Microbore System should be no greater than 20 μL

Performance Monitoring

To perform a measurement:

- disconnect column from system
- connect injector directly to detector

Parameter	Setting
Flow Rate	1.0 mL/min
Chart Speed	20 cm/min
Detector Sensitivity	0.5 - 1.0 AUFS
Time Constant	0.2 seconds or less

dilute test mixture 1 to 10 in mobile phase
inject 2 to 5 μl of this solution

Performance Monitoring

Impact of System Band Spread on a Plate Count:

- System with 70 μl Band Spread >> 10,000 plates

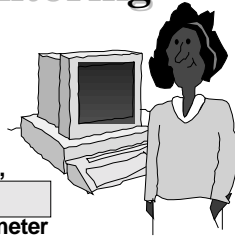
- System with 130 μl Band Spread >> ~8,000 plates

On the Same Column!

Assumption: <40% loss in resolution at $k' = 5$ and $N = 10,000$ and <20% loss in resolution at the preferred value

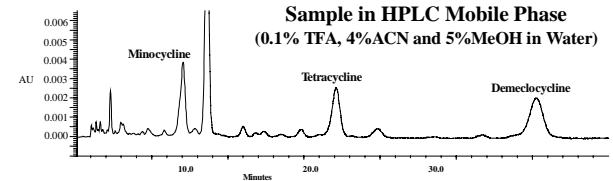
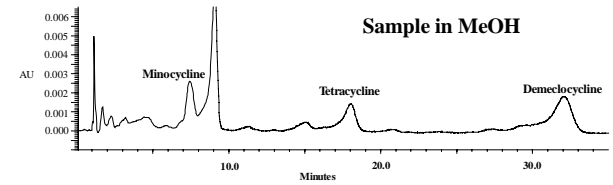
Performance Monitoring

Use Your Test Method
(Known Performance)



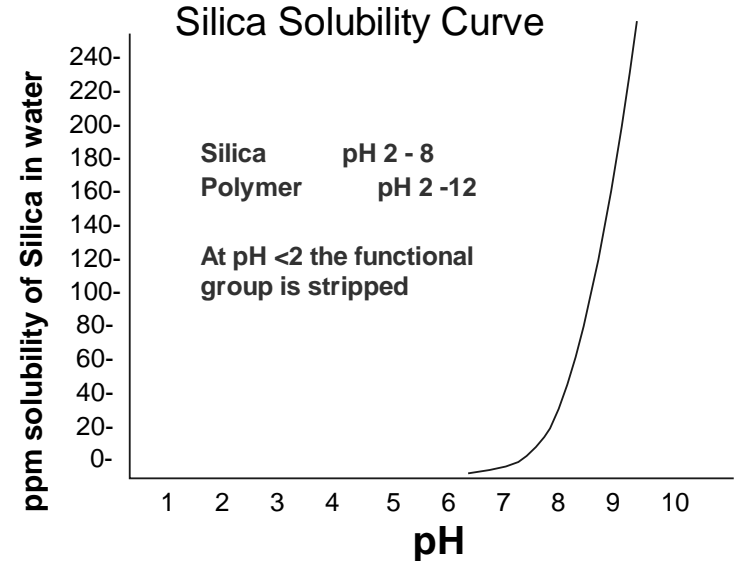
- * Monitor at least One Peak in one injection
 - Plate Count (Peak width relative to RT),
 - Peak Asymmetry,
 - Retention Time and/or Retention parameter
 - Relative Retention Time for Critical Pair of Analytes.
 - Peak Response
- * Inject Multiple Runs
 - Precision (at least 5 injections)
 - Accuracy (Use Control Samples)

Incorrect Sample Solvent

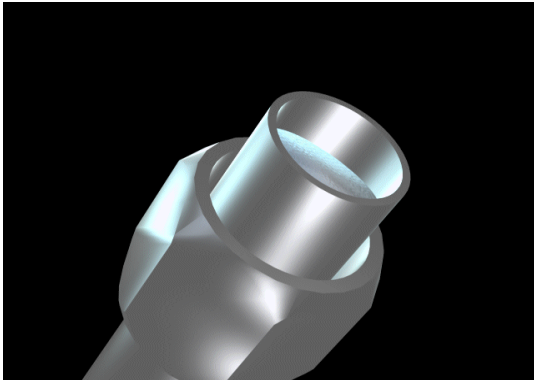


Column Use

- ✓ Silicas hydrolyze at high pH
- ✓ Instability of bonded phase at low pH
- ✓ Elevated temperatures decrease column lifetime
- ✓ C18 approximately 1000 times more stable than CN

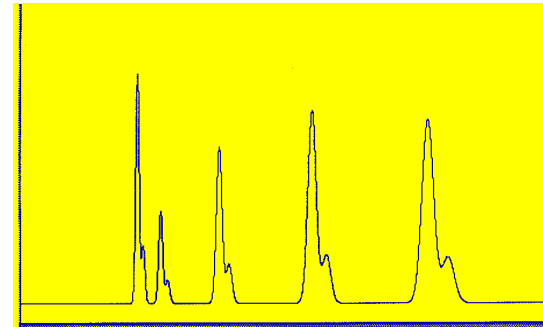


Column Collapse



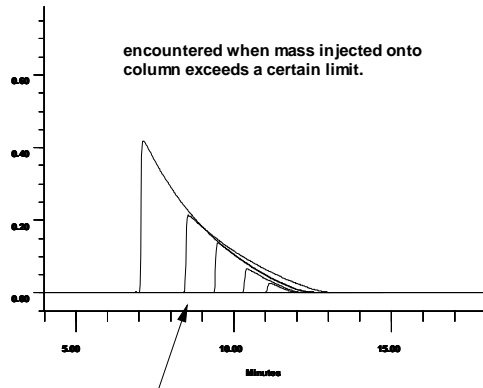
voided column

Column Collapse



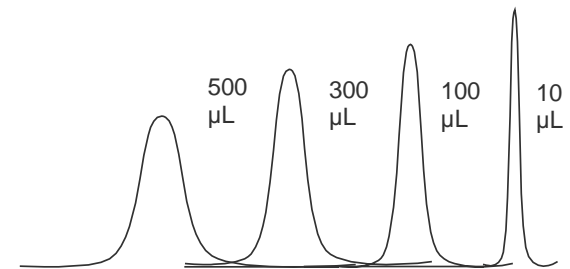
voids - high back pressure,
distorted and/or double peaks

Mass Overload



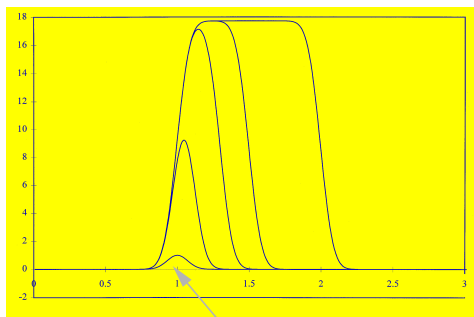
Lift-off Point Moves Earlier
Retention times are shorter

Column/Volume Overload



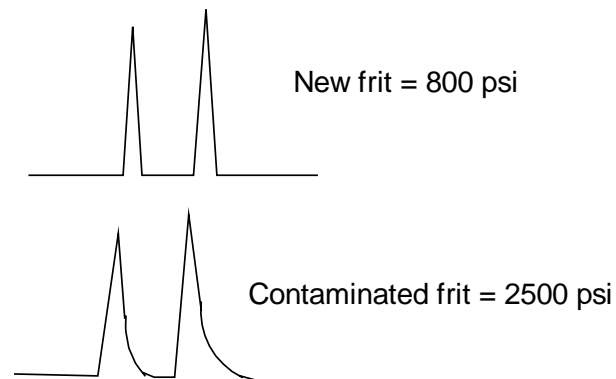
EFFECT OF INJECTION VOLUME
ON PEAK DISTORTION

Volume Overload



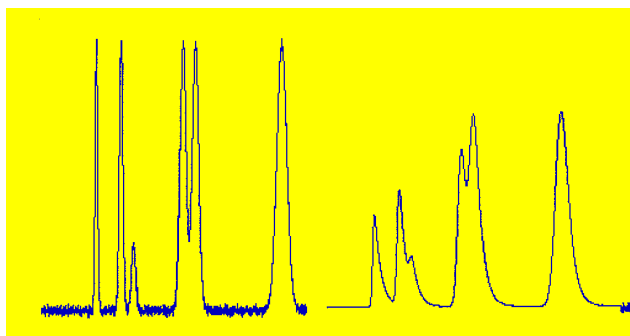
Lift-off Point Remains Constant
Retention times are longer

Contaminated In-Line Filter



Extra Column Effects

Isocratic LC - Time Constant Differences
(Detector setting)



left is 0.1 secs right is 10 secs
note the noisy baseline on left chromatogram

Performance Monitoring

Use Your Test Method
(Known Performance)



- * Monitor at least One Peak in one injection
 - Plate Count (Peak width relative to RT),
 - Peak Asymmetry,
 - Retention Time and/or Retention parameter
 - Relative Retention Time for Critical Pair of Analytes.
 - Peak Response

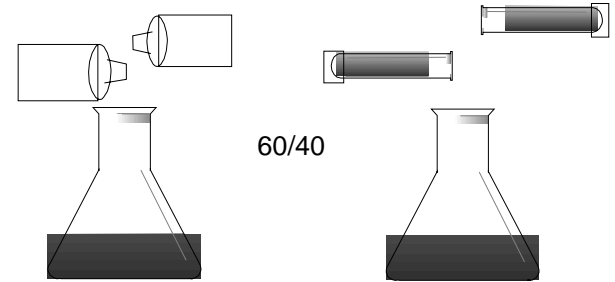
- * Inject Multiple Runs
 - Precision (at least 5 injections)
 - Accuracy (Use Control Samples)

Retention Time Problems

- Reproducibility
 - ▶ Solvent Composition
 - ▶ Temperature
 - ▶ pH-Control
 - ▶ Ion Pairing
- Drifting Retention
 - ▶ Equilibration
 - ▶ Stationary Phase Stability
 - ▶ Column Contamination
 - ▶ Hydrophobic Collapse

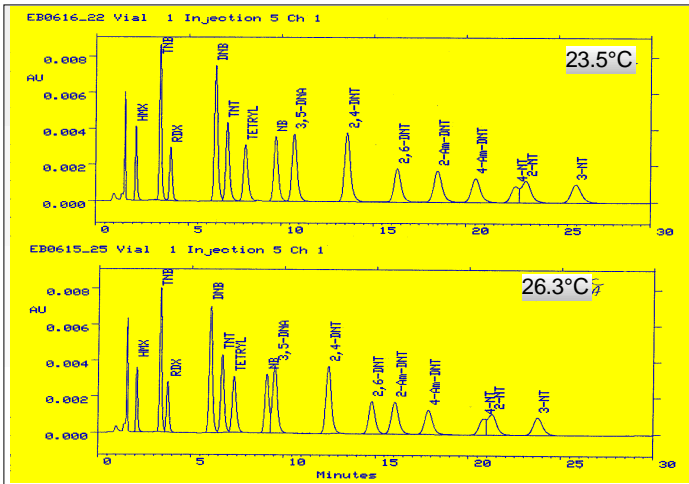
Solvent Composition

■ Clearly specify HOW the Mobile Phase is to be prepared



pH Reminder: Measure pH Before the organic is added

Temperature Control



Retention Time Reproducibility

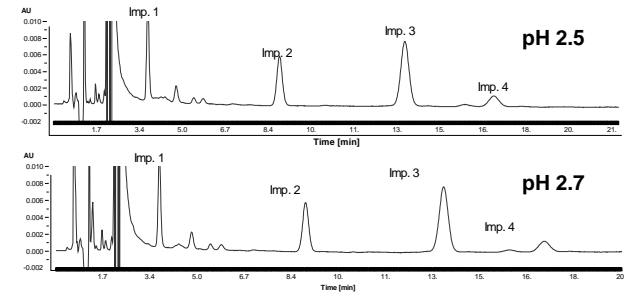
Non-Column Influences:

pH

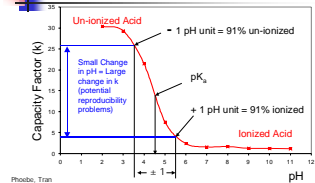
- Neutrals: No Influence
- Acids: Reduced Retention with Increasing pH
- Bases: Increased Retention with Increasing pH
- 10% Change in Retention per 0.1 pH Units

pH Control AZT: Robustness Testing

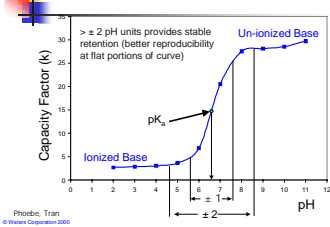
6% Methanol, 6% THF



Reversed-Phase Retention Behavior of Acidic Compounds Relative to Changes ± 1 pH Unit from pK_a



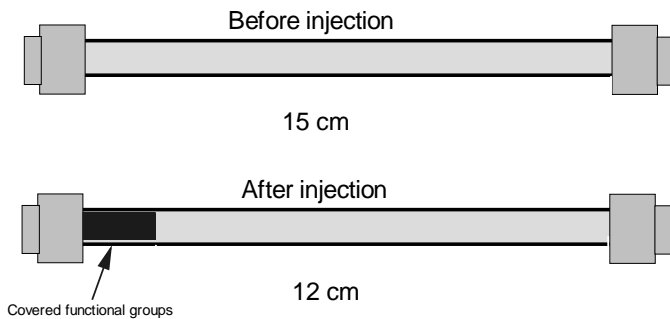
Reversed-Phase Retention Behavior of Basic Compounds Relative to Changes in pH



Changing Retention Times

Retention times getting shorter after each injection?

Sample analytes can adhere to and cover active functional group sites making a shorter column



COLUMN REGENERATION

REVERSE PHASE

1. Wash with unbuffered mobile phase
2. Wash with 100% water
3. Wash with methanol (or ACN)
4. Wash with THF or IPA
5. Wash with methylene chloride
6. Wash with N-Heptane
7. Wash with methylene chloride
8. Wash with methanol (or ACN)
9. Wash with water
10. Return to solvent

Installation and Equilibration

- ✓ Purge column with 10 column volumes of mobile phase to be used in analysis
(\gg 4.6x150mm = 25mL)
- ✓ Reversed-Phase (C18 etc.) columns equilibrate quicker than Normal Phase columns
→ (magnitude of ten)
- ✓ Normal phase columns (silica or alumina) may take several DAYS at flow rates of 1.0 ml/min

Installation and Equilibration

Internal Diameter (mm)	Length (mm)	Column Volume (mL)
2.0	150	.47
2.0	300	.94
3.9	50	.6
3.9	75	.9
3.9	100	1.2
3.9	150	1.8
3.9	300	3.6
4.6	150	2.5
4.6	250	4.2
5	100	2.0
8	100	5.0
7.8	300	4.3
19	150	43
25	100	49
30	300	212
40	100	125
47	300	520
50	300	589



Solvent Viscosities

Solvent	Viscosity [cP] at 20° C
Acetone	0.32
Acetonitrile	0.37
Cyclohexanone	0.98
Di-isopropylether	0.37
Diethyl ether	0.23
Dimethyl acetamide	2.1
Dimethyl formamide	0.92
Dimethyl sulfoxide	2.2
Dioxane	1.54
Ethanol	1.2
Ethyl acetate	0.45
Hexafluoroisopropanol	1.0
iso-Propanol	2.5
Isooctane	0.5
Methanol	0.6

Remember: Some mixtures are more viscous than either pure solvent -- 50/50 MeOH/H₂O is almost 2x

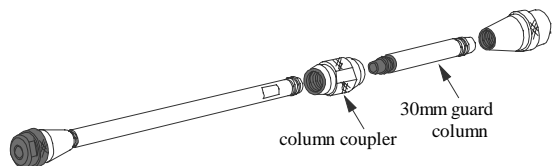
Solvent Viscosities

Solvent	Viscosity [cP] at 20° C
Methyl acetate	0.37
Methylene chloride	0.44
Methylethyl ketone	0.4
n-Heptane	0.42
n-Hexane	0.33
N-Methyl pyrrolidone	1.67 (25° C)
n-Pentane	0.235
n-Propanol	2.3
o-Dichlorobenzene	1.41
Tetrahydrofuran	0.46
Toluene	0.59
1,2,4-Trichlorobenzene	1.89 (25° C)
Water	1.0
m-Xylene	0.62
o-Xylene	0.81

Remember: Some mixtures are more viscous than either pure solvent -- 50/50 MeOH/H₂O is almost 2x

Column Protection

Major cause of column deterioration is contamination.
Use of guard columns may increase column life-time
to > 10,000 analyses

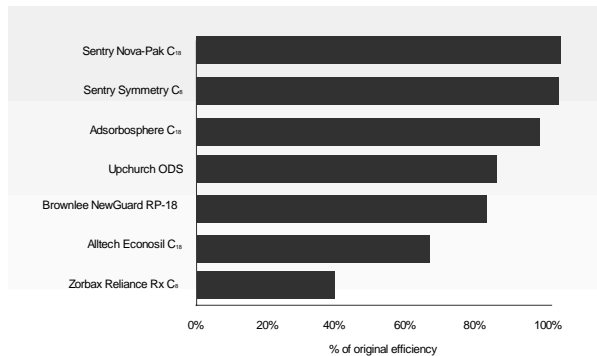


Column Protection

1. Guard column should be regarded as a cost-effective sacrifice to extend analytical column life-time
2. Should contain IDENTICAL packing material as the analytical column
e.g. using a different C18, with different retention properties could actually destroy the separation

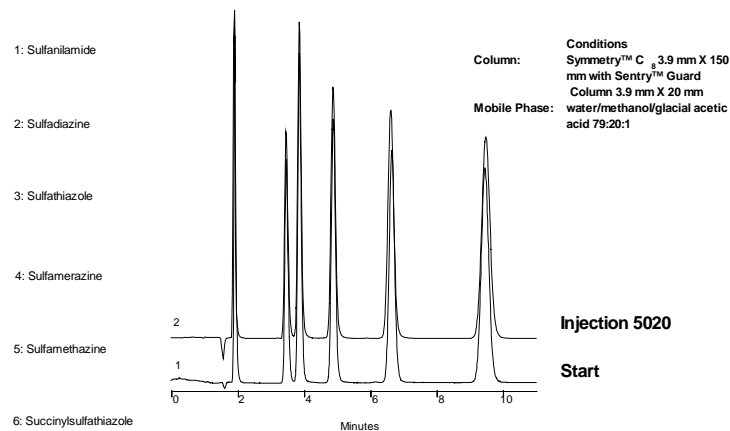
Well designed, well packed guard column will actually IMPROVE the analytical separation efficiency

Column Protection



Effect of guard column on HPLC column efficiencies
Analytical column Nova-Pak C₁₈ (150 x 3.9mm or 4.6mm) except Zorbax Rx C₁₈ (150 x 4.6mm)
Sample was 0.5µL injection acenaphthene (2.9 mg/mL) and acetone (34 µL/mL) in ACN/Water

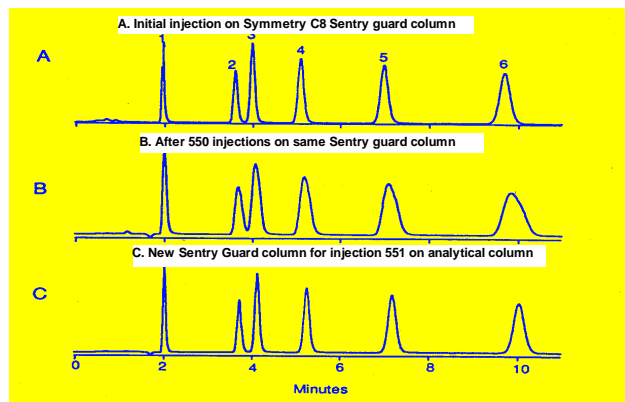
Column Protection



Chromatogram of Life-time Test

* Guard Column Changed Every 500 Injections

Column Protection



Extension of column lifetime with Guard Column using a mixture of sulfa drugs as the sample

Performance Monitoring

Use Your Test Method
(Known Performance)



- * Monitor at least One Peak in one injection
 - Plate Count (Peak width relative to RT),
 - Peak Asymmetry,
 - Retention Time and/or Retention parameter
 - Relative Retention Time for Critical Pair of Analytes.
 - Peak Response

- * Inject Multiple Runs
 - Precision (at least 5 injections)
 - Accuracy (Use Control Samples)

Variable Reported Concentrations Problems with Peak Response

■ Linearity Test of Concentrations

- Check Injector (Use Standards)

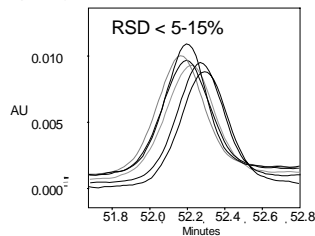
- * Multiple Injections - Same Vial -- Syringe Problem
or If Only 1st Injection Low -- Septa Problem
- * Different Vials -- Evaporation -- Degradation
- * Injection Volume Test (Weight before and after injection)

- Integration Software

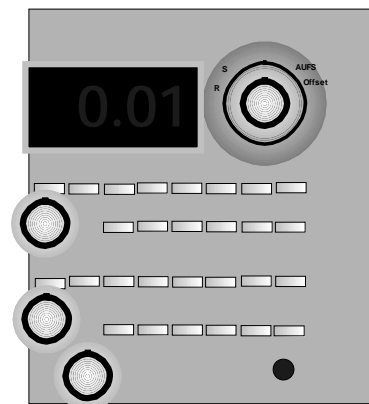
- * Electronic Peak Generator
- * Poor Peak Shape

- Detector

- * Cell Problem
- * Lamp Failing



Troubleshooting your UV detector

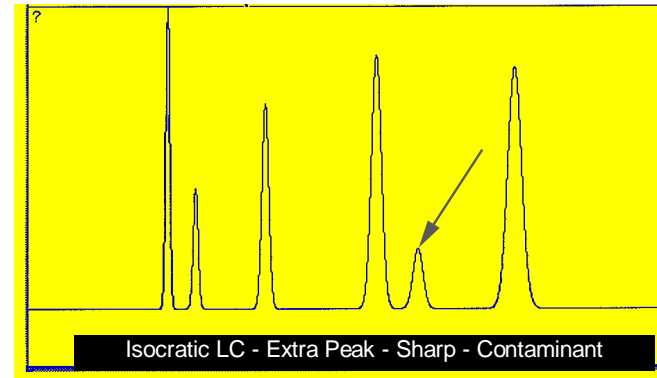


- ▶ Reference Energy
- ▶ Sample Energy
- ▶ Absorbance
- ▶ Offset

Unusual Phenomena

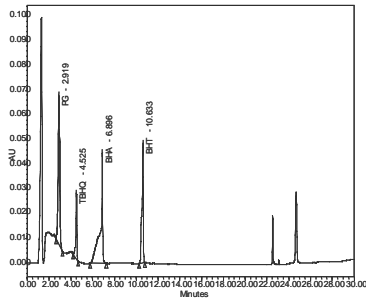
- ▶ Extraneous Peaks
- ▶ Problems with Baseline

Extraneous Peaks

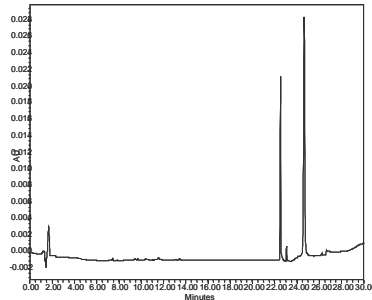


Extraneous Peaks

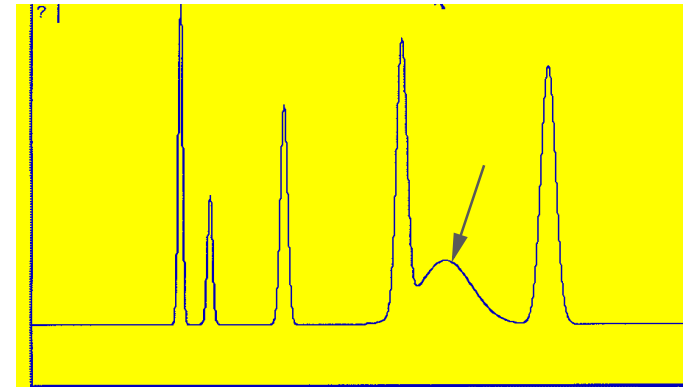
Sample



Blank

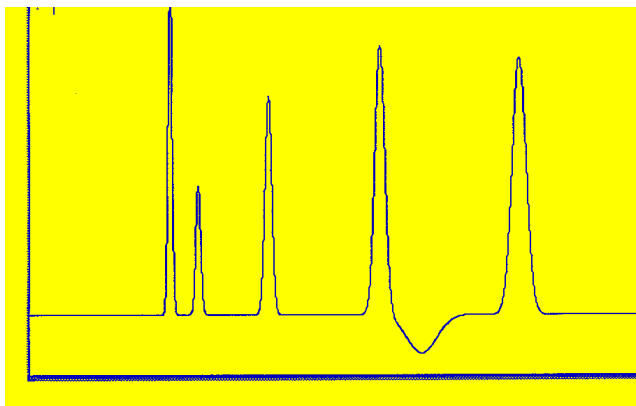


Extraneous Peaks



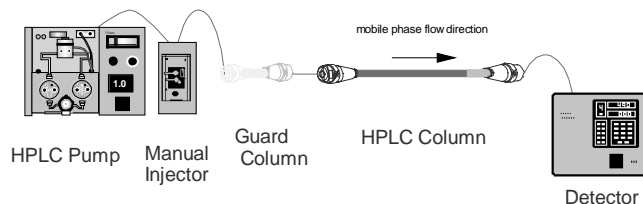
Isocratic LC - Broad -Peak from Previous Injection or Injector Contamination

Isocratic LC - Negative Peak
often occurs in Ion-Pairing -- Sample Solvent

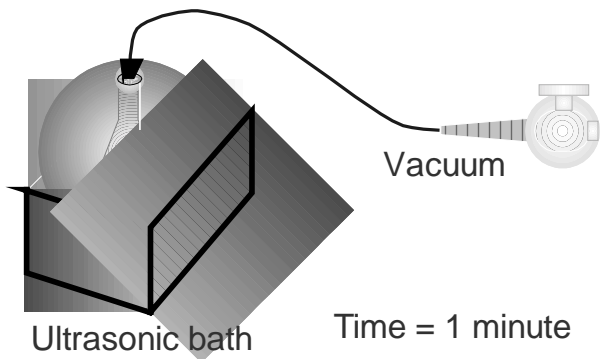


Installation and Equilibration

- ✓ Connect Column Inlet
 - ✓ Purge Column at Low Flow Rate To Waste -- Then Connect to Detector
- (begin flow of analytical columns at 0.1 ml/min
increase by 0.2 ml/min increments every 30 seconds until
final analytical flow rate is reached)



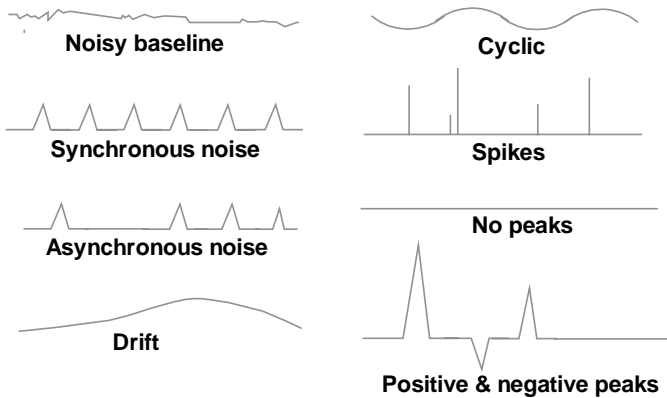
Degas Solvents



Solvent Degassing Precautions

1. Degas solvents prior to adding modifiers
2. Helium sparge is good, as long as solvent doesn't change due to volatility of solvents and/or additives
3. Solvents should be degassed daily

BASELINE TROUBLESHOOTING



NOISY BASELINE

INSTRUMENTAL

CHEMICAL

WEAK DETECTOR LAMP

Replace lamp

LEAKS

Stop leaks. Replace fittings

DETECTOR CELL DIRTY

Flush with 6N nitric acid

GAS IN MOBILE PHASE

Degas solvent

GAS BUBBLE IN DETECTOR CELL

Put .009" tubing after detector (not RII!)

ELECTRONIC NOISE

Remove source. Shield cables. Clean contacts

SENSITIVITY TOO HIGH

Lower sensitivity. Adjust gain

TRASH ELUTING OFF COLUMN

Flush column with strong solvent

SYNCHRONOUS NOISE

ALMOST ALWAYS CAUSED BY THE PUMP

Air in pump head - Prime pump and degas solvent

Check valve problem - Rebuild or replace

Broken plunger - Replace (blame it on someone else)

Mixing problem - Increase system volume

Electrical noise - Change circuits, remove source



ASYNCHRONOUS NOISE

BUBBLES

Degas mobile phase

GAS CAUGHT IN DETECTOR

Degas mobile phase. Put backpressure on cell.

LEAKS

Fix leaks, replace fittings

MIXING PROBLEMS

Increase system volume

PLUGGED LINES

Remove plug, flush system

ELECTRICAL PROBLEMS

Remove source, change circuits

BASELINE DRIFT

INSTRUMENTAL

GRADIENT - SOLVENT B ABSORBS MORE THAN SOLVENT A

Try a new mobile phase, use baseline subtraction

SOLVENT CHANGING (GAS ABSORPTION, EVAPORATION)

Helium sparge, enclose solvents

SOLVENT LEAKS

Tighten, replace fittings

THERMAL EFFECTS (ESPECIALLY RI, CONDUCTIVITY, ECD)

Cell temperature regulation

BACKPRESSURE CHANGES

Filter solvents and samples. Sample too viscous

SIPHONING (RI, CONDUCTIVITY, ECD)

Increase system volume

MIXING PROBLEMS

CHEMICAL

COMPOUNDS ELUTING OFF COLUMN

Run strong solvent until baseline is stable

SOLVENTS IN GRADIENT ARE NOT PURE

Change the solvent batch or manufacturer.

Check if the solvents are gradient grade.

CYCLIC BASELINE

TEMPERATURE FLUCTUATIONS

Thermally insulate. Move away from ventilation.

Increase cell temperature.

MIXING PROBLEMS

Increase system volume

GAS IN MOBILE PHASE

Degas solvents

ELECTRICAL PROBLEMS

Change circuits, remove source

ERRATIC PUMP

Repair pump

PLUG

Remove obstruction, flush system



SPIKES

NO PEAKS

BUBBLES

Degas solvent

POOR ELECTRICAL CONNECTION, LOOSE WIRING

Clean and tighten detector leads, check wiring, replace spade lugs.

LAMP RELAY TRYING TO FIRE A DEAD LAMP

Replace lamp

ELECTRICAL NOISE

Change circuits, remove source

Common sources include switching valves, compressors, muffle furnaces, fraction collectors, power conditioners, lighting, poor power source.

INSTRUMENTAL

- Injector not making injections
- Pump not pumping
- Dead detector
- Integrator/recorder not wired correctly
- Gain setting too low
- Leaks

WHAT TO DO:

Inject acetone solution to make a peak

CHEMICAL

- Column retaining all compounds
- Bad or wrong mobile phase
- Bad or wrong standard or sample
- Wrong guard column

WHAT TO DO:

Remove column and inject acetone solution to make a peak



NEGATIVE & POSITIVE PEAKS

INSTRUMENTAL

Air bubbles passing through cell

Degas mobile phase

You're using an RI detector

May be normal since peak direction is a function of

refractive index differential from mobile phase

All peaks negative - polarity wrong

Reverse leads or change detector polarity

All peaks negative - You're using indirect UV

Change polarities or reverse leads

CHEMICAL

Some eluting compounds absorb less than solvent

Use a different or cleaner solvent

Basic assumptions

1. The HPLC is plugged in and turned on
2. Solvent is in the reservoir
3. The pumps are primed and in good working order
4. The HPLC is plumbed and wired correctly
5. The detector has a good lamp in it
6. The solvent bottle doesn't have a vacuum on it
7. You're not using acetone for solvent at 195 nm
8. You're not injecting rocks
9. You're not doing a water to hexane gradient
10. You're not trying to detect sugars at 254 nm
11. You're not mixing MEOH and water without degassing
12. You're not sparging with nitrogen or air
13. You're not running water through a silica column
14. Solvent pH is not 13 on a silica base column
15. You're not running a 1M NaCl to 100% ACN gradient
16. You're not doing gradients with an RI detector
17. Your RI is not under the air conditioner vent
18. No buffer stalagmites on your pump heads
19. HCl vapors are not blowing onto your HPLC
20. You're having a wonderful time!



Strange things can happen!

Radio transmitters can cause baseline noise

Contaminated helium bottles and lines can cause noise

System components can get coated with impurities

Solvent vendors can misname solvent bottles

Some filters can introduce particulates

Things not to do:

- * Plug the outlet of your RI detector
- * Flush your system with methanol after running buffer
- * Inject samples that may precipitate in the eluent
- * Run long durations with HCl on your stainless steel HPLC
- * Filter organic solvents through aqueous filters
- * Spill buffers onto HPLC electronics
- * Try to change the column frits while it still has pressure in it
- * Store THF on the shelf, uncapped, for weeks
- * Pump cyclohexane above 2000 psi
- * Tightly seal your mobile phase container
- * Cut tubing with a wire cutter