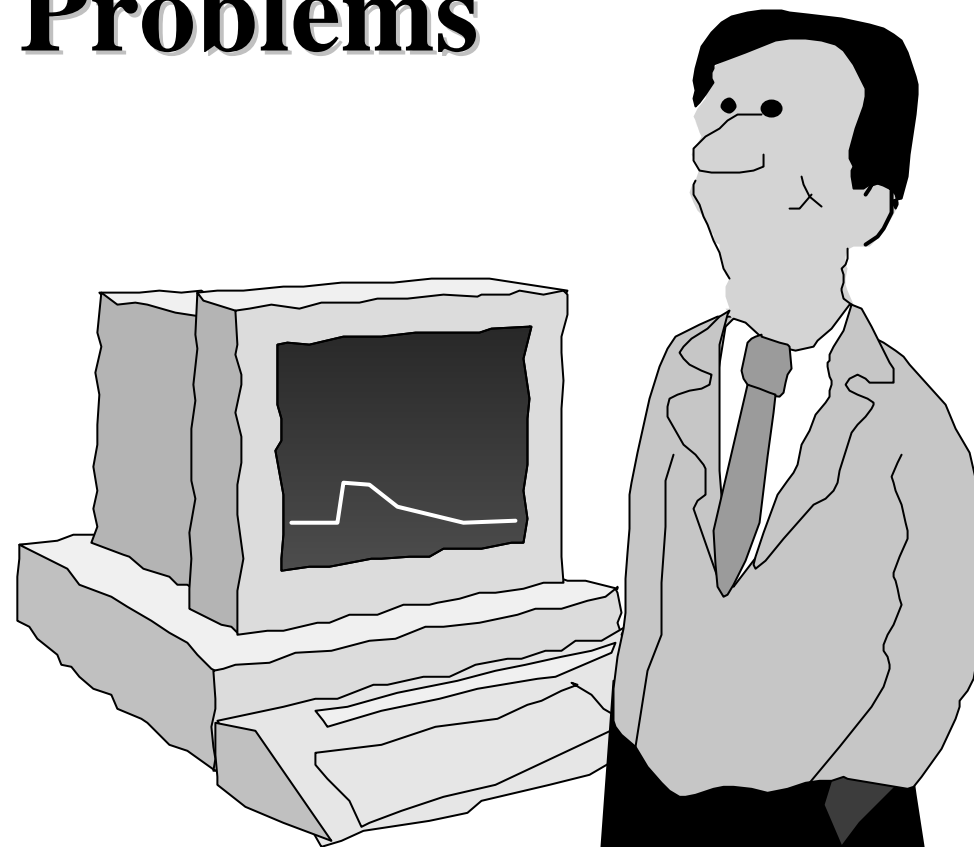
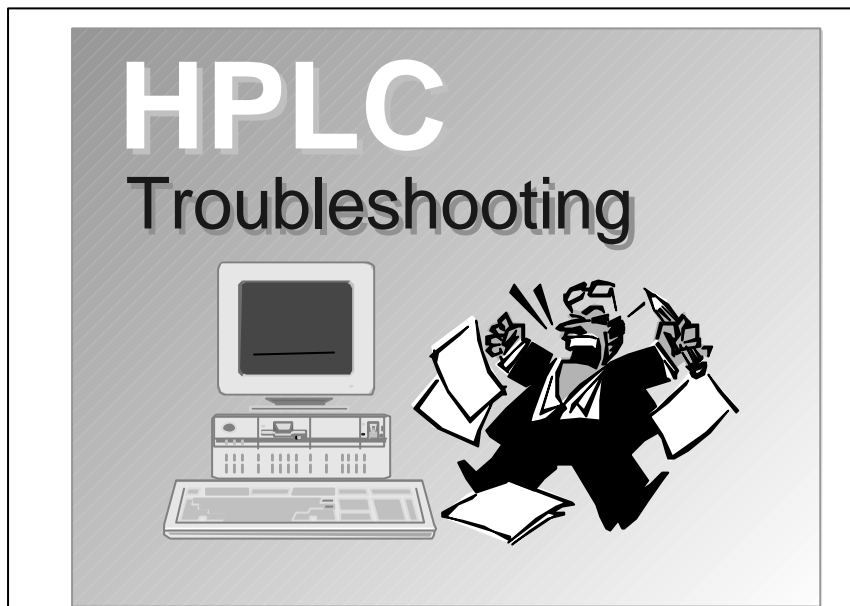


Troubleshooting Common HPLC Problems

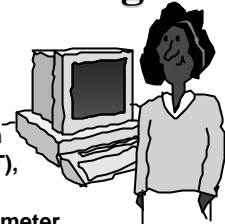


<http://www.hplc1.com/shodex/english/dd.htm>

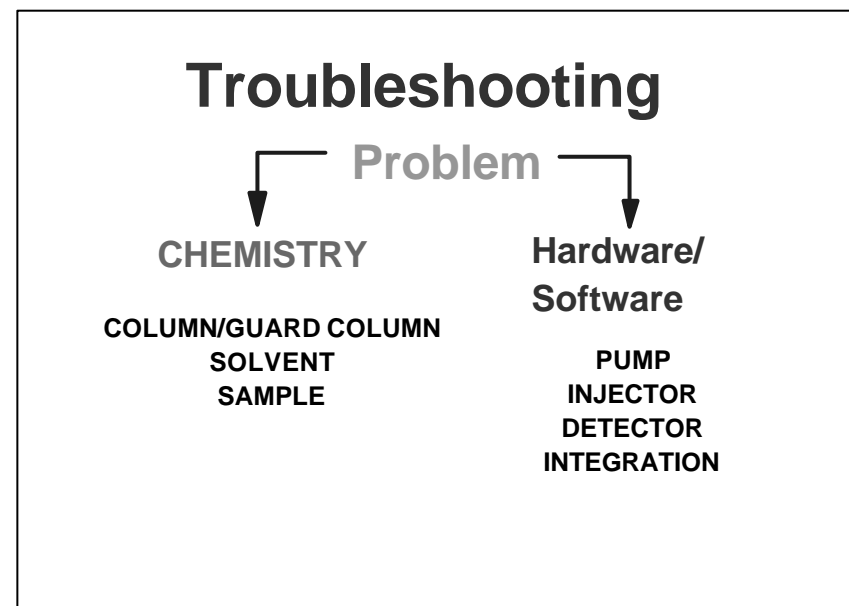
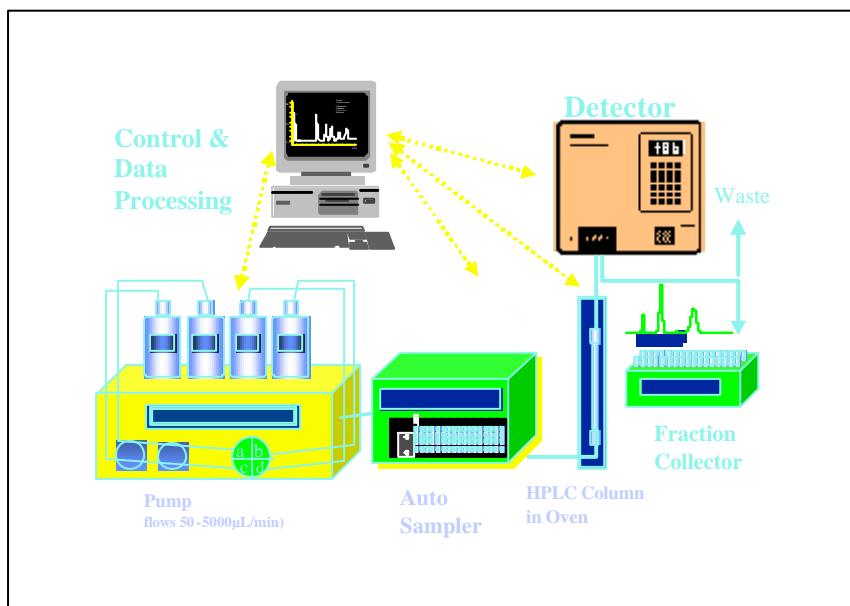


Performance Monitoring

Use Your Test Method
(Known Performance)

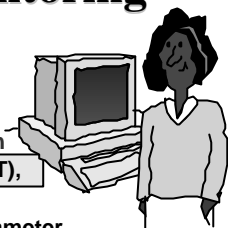


- * Monitor at least One Peak in one injection
 - Plate Count (Peak width relative to RT),
 - Peak Asymmetry,
 - Retention Time and/or Retention parameter
 - Relative Retention Time for Critical Pair of Analytes.
 - Peak Response
- * Inject Multiple Runs
 - Precision (at least 5 injections)
 - Accuracy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



Performance Monitoring

Use Your Test Method
(Known Performance)



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- Peak Response

* Inject Multiple Runs

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

Plate Count - Efficiency of the Separation

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

Performance Monitoring

Column Efficiency:

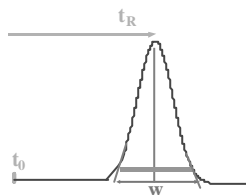
N = the number of Theoretical Plates

a = is a constant depending on the Method used

t_r = 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

Peak Width at Half Height

Peak Width at 4.4% Peak Height (σ Sigma)

Tangent

a

5.54

25.0

16.0

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

$$S_{TOT}^2 = S_{COL}^2 + S_{EC}^2$$

Band Spreading

* Column Contribution

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

* Instruments Contribution = Extra-Column

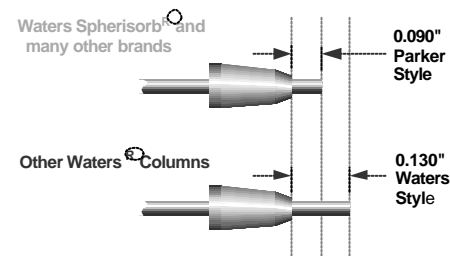
$$S_{EC}^2 = S_{TUBING}^2 + S_{CONNECTIONS}^2 + S_{INJECTORS}^2 + S_{DETECTORS}^2$$

Performance Monitoring

Extra-Column Band Spreading
(Instruments' Contribution)

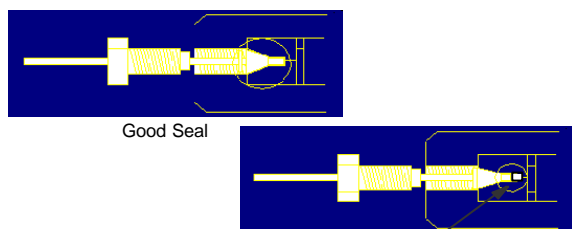
1. Injection Volume
2. Injector
3. Connection Tubing
 - a. from Injector to Column
 - b. from Column to Detector
 - c. Endfittings and Frits
4. Detector Volume

Connectors



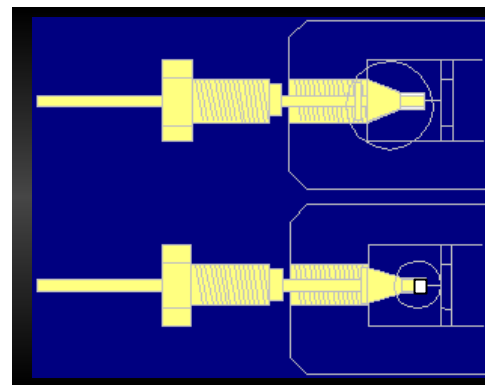
Installation and Equilibration

- ✓ 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}

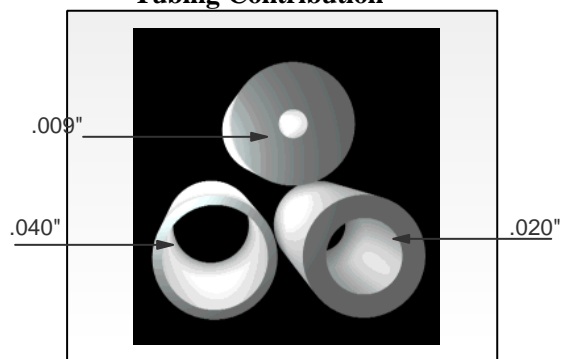
Extra-Column Band Spreading



Column Connection Contribution

Extra-Column Band Spreading

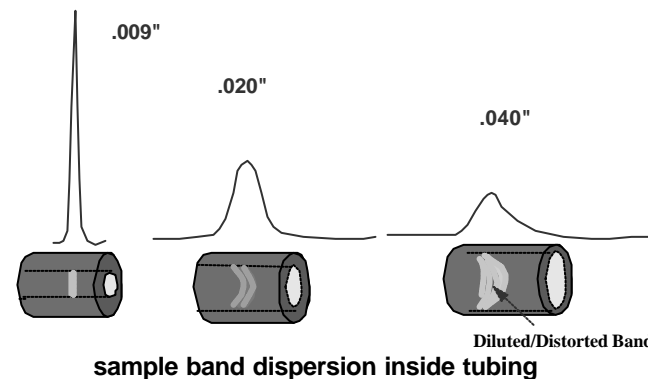
Tubing Contribution



note the differences of the inner diameter of this tubing

Performance Monitoring

Effect of Connecting Tubing on System Bandspeading



Measuring The Instruments Contribution

* Perform An Instrument Band Spreading Test

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 ml of this solution

Performance Monitoring

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

Convert to microliters using the following equation:

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

where:

1min/20cm = chart speed

1 mL/min = flow rate

1000 mL/mL = volume correction factor

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

Microbore System should be no greater than 20mL

Performance Monitoring

Impact of System Band Spread on a Plate Count:

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

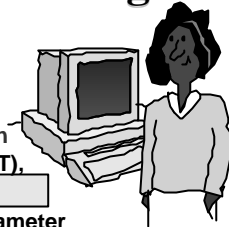
- System with 130ml Band Spread >> ~8,000 plates

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

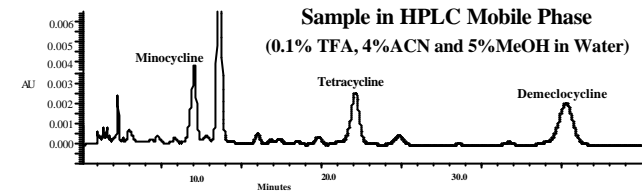
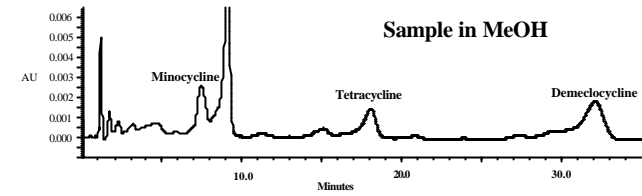
Use Your Test Method
(Known Performance)



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 - Plate Count (Peak width relative to RT),
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 - Accuracy (Use Control Samples)

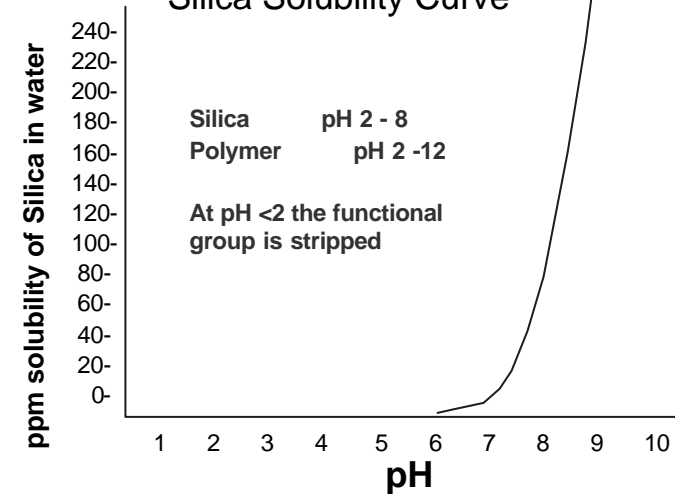
Incorrect Sample Solvent



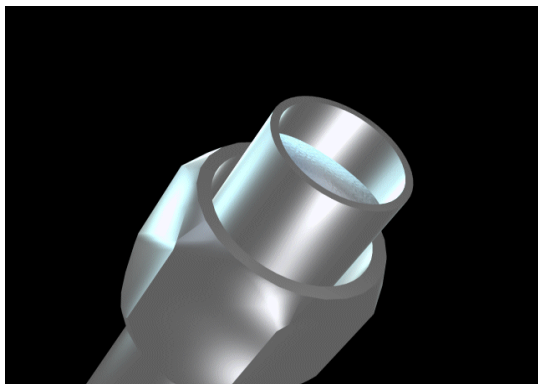
Column Use

- ✓ 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

Silica Solubility Curve

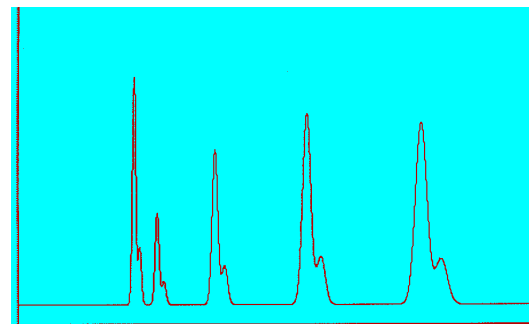


Column Collapse



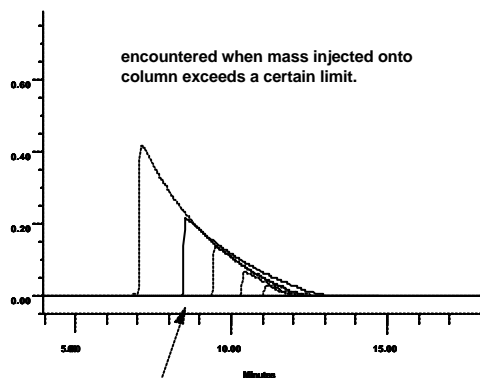
voided column

Column Collapse



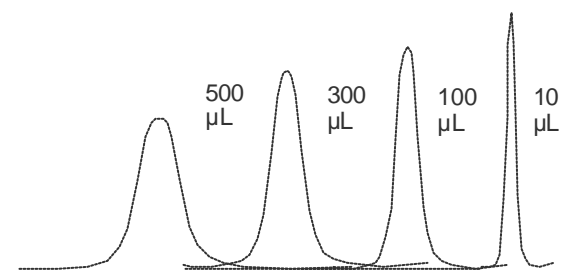
voids - high back pressure,
distorted and/or double peaks

Mass Overload



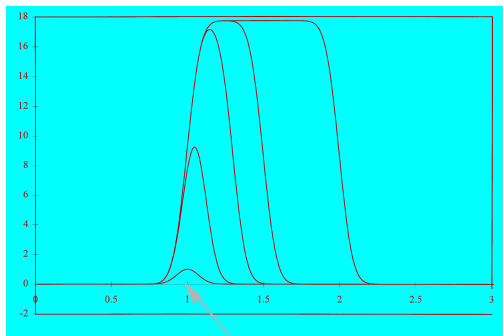
Lift-off Point Moves Earlier
Retention times are shorter

Column/Volume Overload



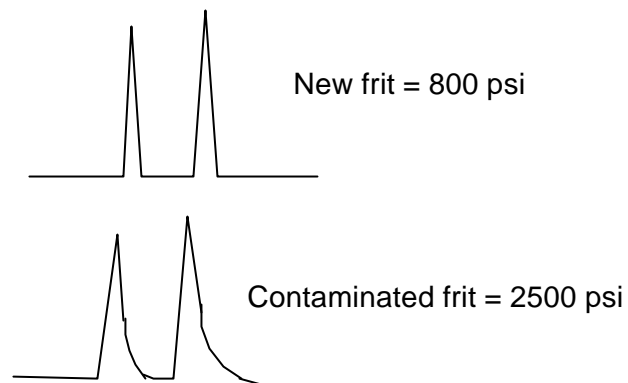
EFFECT OF INJECTION VOLUME
ON PEAK DISTORTION

Volume Overload



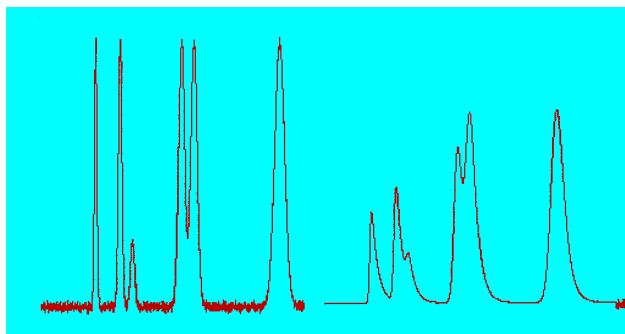
Lift-off Point Remains Constant
Retention times are longer

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)



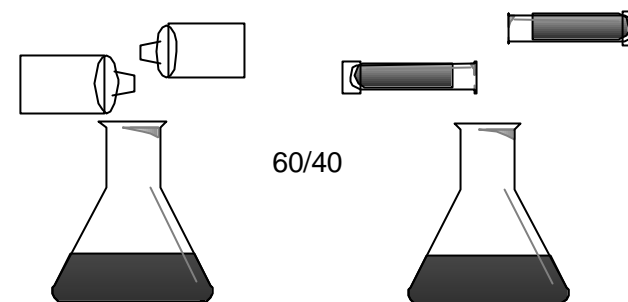
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 - Peak Response
- * Inject Multiple Runs
 - Precision (at least 5 injections)
 - Accuracy (Use Control Samples)

Retention Time Problems

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

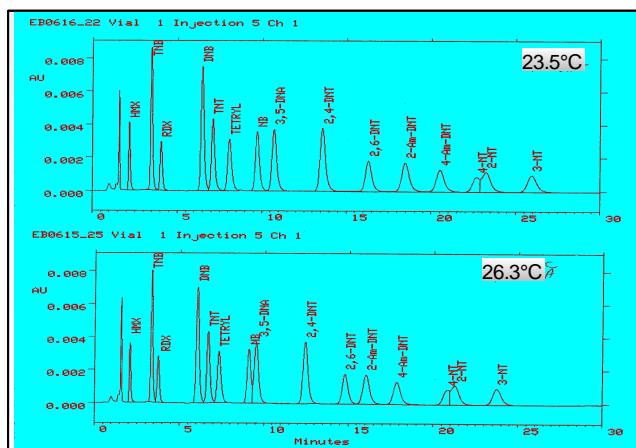
Solvent Composition

- Clearly specify HOW the Mobile Phase is to be prepared



pH Reminder: Measure pH Before the organic is added

Temperature Control

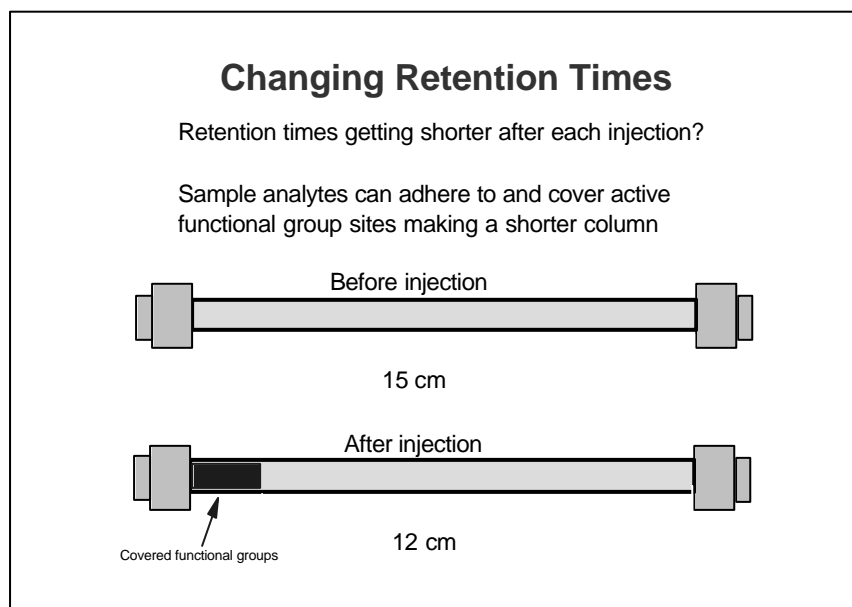
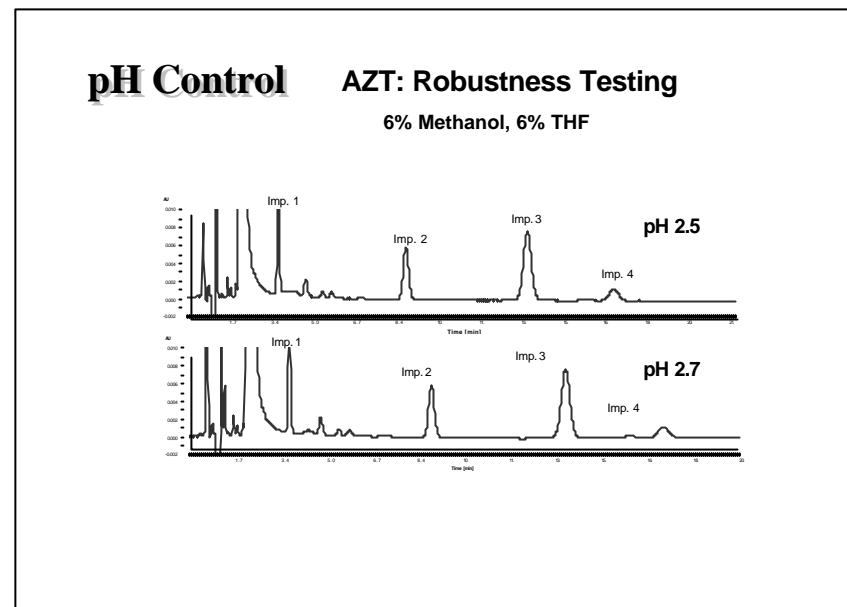
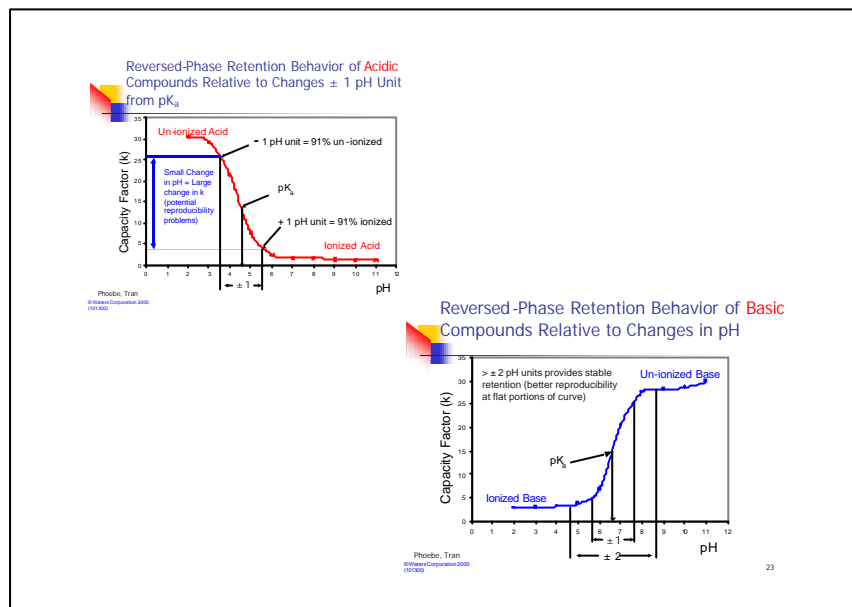


Retention Time Reproducibility

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



- 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

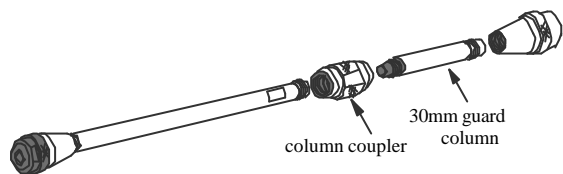
Solvent Viscosities

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

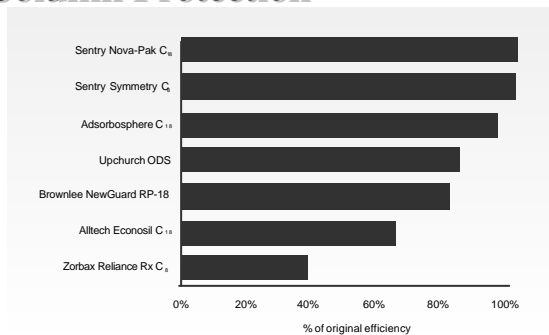


Column Protection

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

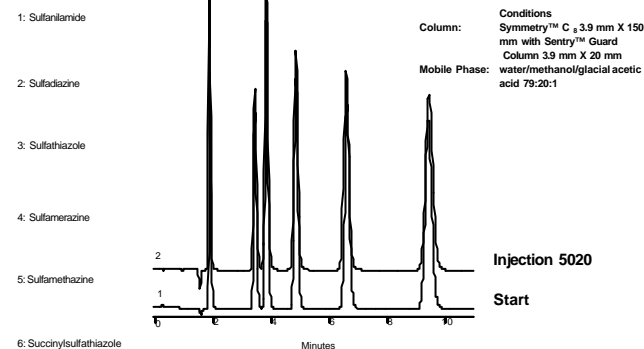
Well designed, well packed guard column will actually IMPROVE the analytical separation efficiency

Column Protection



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

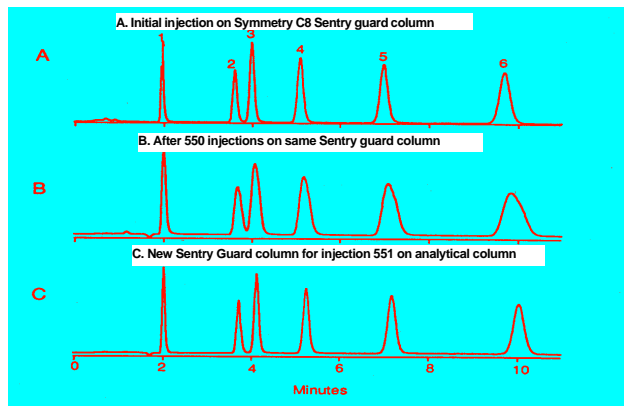
Column Protection



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
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 - Relative Retention Time for Critical Pair of Analytes.

- * 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)

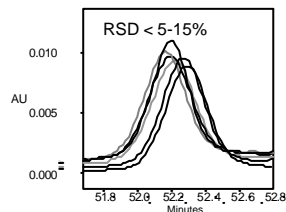
- * Multiple Injections - Same Vial -- Syringe Problem or If Only 1st Injection Low -- Septa Problem
- * Different Vials -- Evaporation -- Degradation
- * Injection Volume Test (Weight before and after injection)

- Integration Software

- * Electronic Peak Generator
- * Poor Peak Shape

- Detector

- * Cell Problem
- * Lamp Failing



Performance Monitoring

Use Your Test Method
(Known Performance)



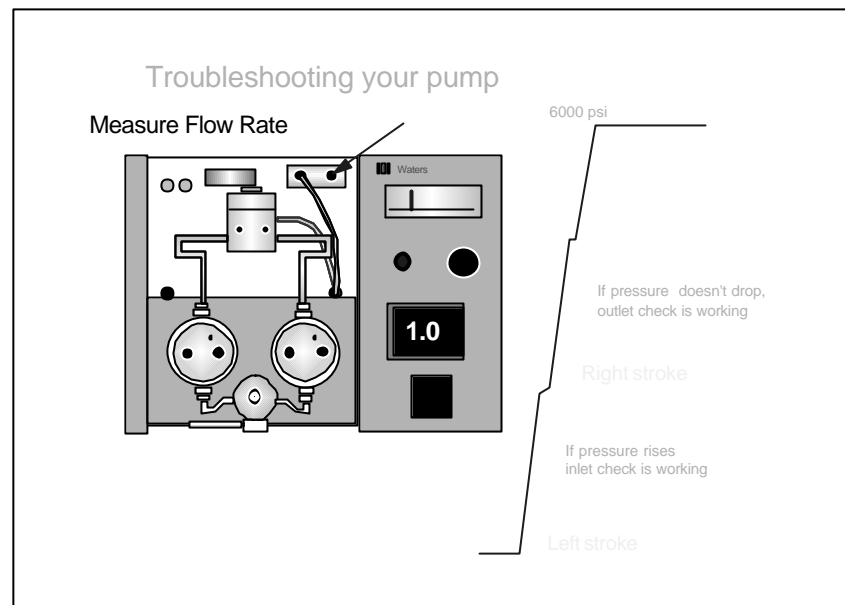
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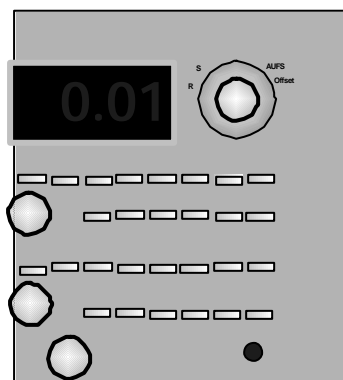
Troubleshooting your Injector

Make repetitive injections of the same volume to check reproducibility.

Make injections of varying volumes to check linearity.



Troubleshooting your UV detector

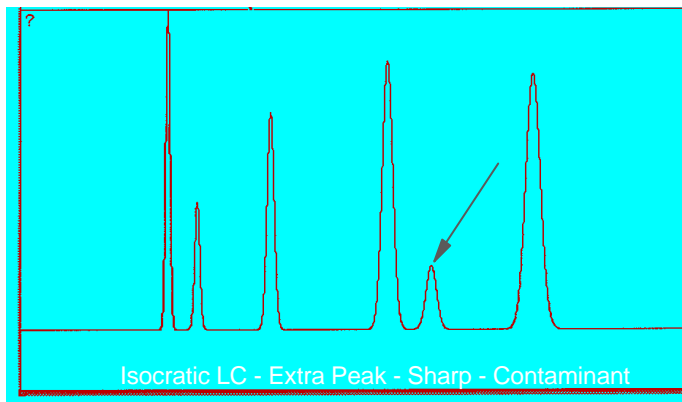


- ▶ Reference Energy
- ▶ Sample Energy
- ▶ Absorbance
- ▶ Offset

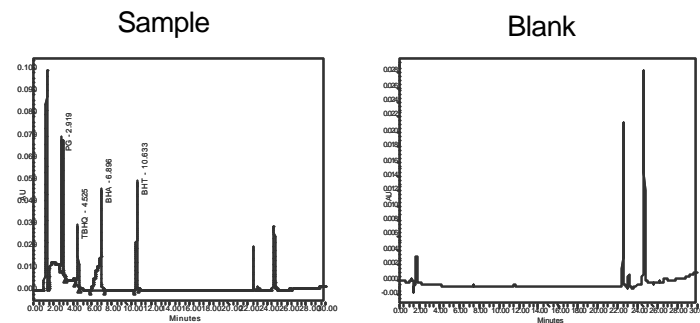
Unusual Phenomena

- ▶ Extraneous Peaks
- ▶ Problems with Baseline

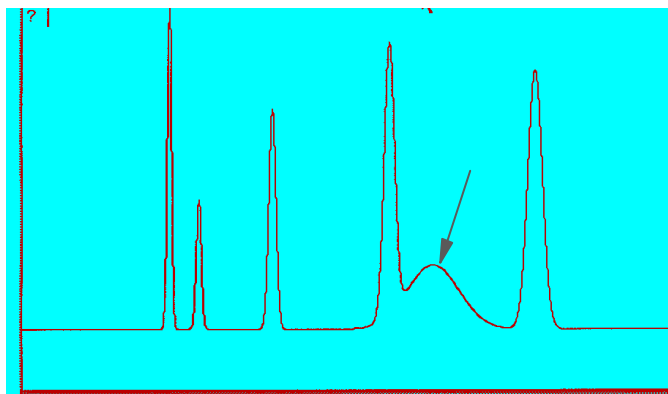
Extraneous Peaks



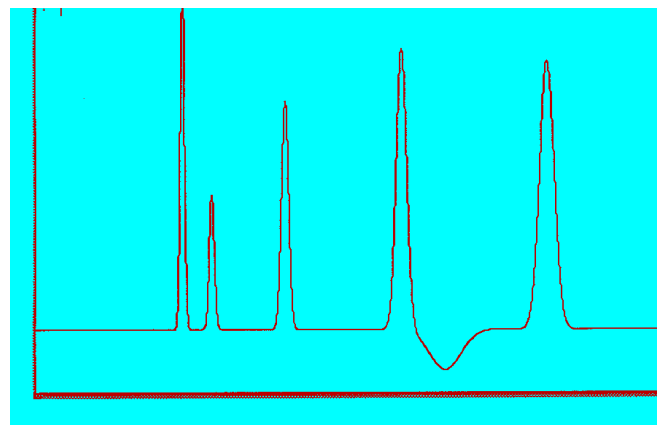
Extraneous Peaks



Extraneous Peaks

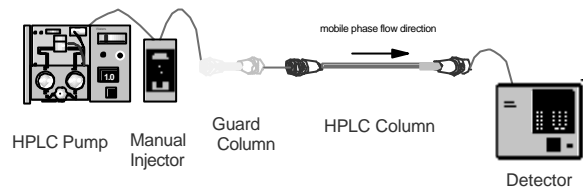


Isocratic LC - Negative Peak often occurs in Ion-Pairing -- Sample Solvent

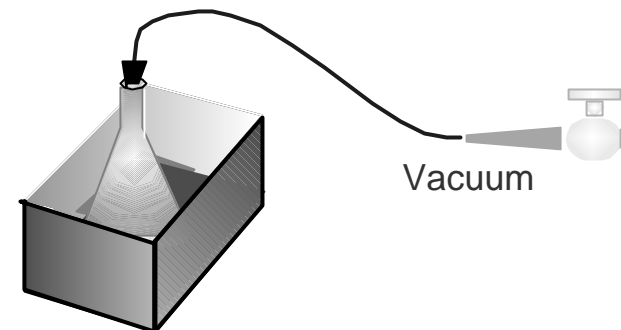


Installation and Equilibration

- ✓ 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



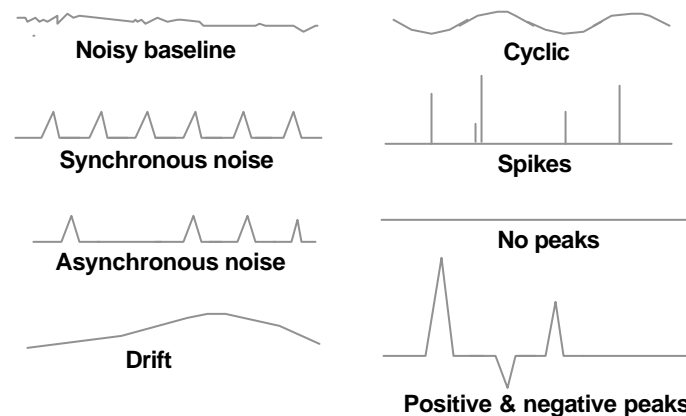
Ultrasonic bath


Time = 1 minute

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


<p>INSTRUMENTAL</p> <p>WEAK DETECTOR LAMP Replace lamp</p> <p>LEAKS Stop leaks. Replace fittings</p> <p>DETECTOR CELL DIRTY Flush with 6N nitric acid</p> <p>GAS IN MOBILE PHASE Degas solvent</p> <p>GAS BUBBLE IN DETECTOR CELL Put .009" tubing after detector (not RI!)</p> <p>ELECTRONIC NOISE Remove source. Shield cables. Clean contacts</p> <p>SENSITIVITY TOO HIGH Lower sensitivity. Adjust gain</p>	<p>CHEMICAL</p> <p>TRASH ELUTING OFF COLUMN Flush column with strong solvent</p>
---	--



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

<p>INSTRUMENTAL</p> <p>GRADIENT - SOLVENT B ABSORBS MORE THAN SOLVENT A Try a new mobile phase, use baseline subtraction</p> <p>SOLVENT CHANGING (GAS ABSORPTION, EVAPORATION) Helium sparge, enclose solvents</p> <p>SOLVENT LEAKS Tighten, replace fittings</p> <p>THERMAL EFFECTS (ESPECIALLY RI, CONDUCTIVITY, ECD) Cell temperature regulation</p> <p>BACKPRESSURE CHANGES Filter solvents and samples. Sample too viscous</p> <p>SIPHONING (RI, CONDUCTIVITY, ECD) Increase system volume</p> <p>MIXING PROBLEMS</p>	<p>CHEMICAL</p> <p>COMPOUNDS ELUTING OFF COLUMN Run strong solvent until baseline is stable</p> <p>SOLVENTS IN GRADIENT ARE NOT PURE Change the solvent batch or manufacturer. Check if the solvents are gradient grade.</p>
--	---



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

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.



NO PEAKS

<p>INSTRUMENTAL</p> <ul style="list-style-type: none"> ● Injector not making injections ● Pump not pumping ● Dead detector ● Integrator/recorder not wired correctly ● Gain setting too low ● Leaks <p>WHAT TO DO: Inject acetone solution to make a peak</p>	<p>CHEMICAL</p> <ul style="list-style-type: none"> ● Column retaining all compounds ● Bad or wrong mobile phase ● Bad or wrong standard or sample ● Wrong guard column <p>WHAT TO DO: Remove column and inject acetone solution to make a peak</p>
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NEGATIVE & POSITIVE PEAKS

<p>INSTRUMENTAL</p> <p>Air bubbles passing through cell Degas mobile phase</p> <p>You're using an RI detector May be normal since peak direction is a function of refractive index differential from mobile phase</p> <p>All peaks negative - polarity wrong Reverse leads or change detector polarity</p> <p>All peaks negative - You're using indirect UV Change polarities or reverse leads</p>	<p>CHEMICAL</p> <p>Some eluting compounds absorb less than solvent Use a different or cleaner solvent</p>
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Strange things can happen!

Radio transmitters can cause baseline noise

Contaminated helium bottles and lines can cause noise

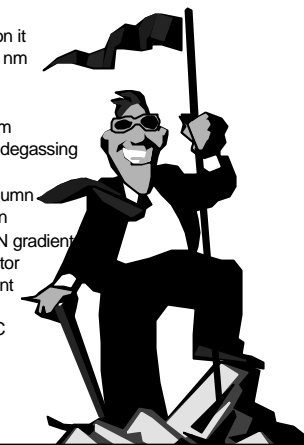
System components can get coated with impurities

Solvent vendors can misname solvent bottles

Some filters can introduce particulates

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. You're 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!



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