Troubleshooting High Performance Liquid Chromatography:

Do you have a problem with any of the following topics?

- *1.Peaks*

Broad Peak Causes and Solutions

Cause	***Solution***
Analytes eluted early due to sample overload	Dilute sample 1:10 and reinject
Detector-cell volume too large	Use smallest possible cell volume consistent with sensitivity needs; use detector with no heat exchanger in system
Injection volume too large	Decrease solvent strength of injection solvent to focus solute; inject smaller volume
Large extra column volume	Use low- or zero-dead-volume endfittings and connectors; use smallest possible diameter of connecting tubing (<0.10 in. i.d.); connect tubing with matched fittings
Mobile-phase solvent viscosity too high	Increase column temperature; change to lower viscosity solvent
Peak dispersion in injector valve	Decrease injector sample loop size; introduce air bubble in front and back of sample in loop
Poor column efficiency	Use smaller-particle-diameter packing, lower-viscosity mobile phase, higher column temperature, or lower flow rate
Retention time too long	Use gradient elution or stronger isocratic mobile phase
Sampling rate of data system too low	Increase sampling frequency
Slow detector time constant	Adjust time constant to match peak width
Some peaks broad - late elution of analytes retained from previous injection	Flush column with strong solvent at end of run; end gradient at higher solvent concentration

Ghost Peaks: Causes and Solution

Possible Cause	***Solution***
Contamination	Flush column to remove contaminatint; use HPLC-grade solvent
Elution of analytes retained from previous injection	Flush column with strong solvent at end of run; end gradient at higher solvent concentration.
Ion-pair chromatography - upset equilibrium	Prepare sample in mobile phase; reduce injection volume

Oxidation of trifluoroacetic acid in peptide mapping	Prepare trifluoroacetic acid solutions fresh daily; use antioxidant
Reversed-phase chromatography - contaminated water	Check suitability of water by running different amounts through column and measure peak height of interferences as function of enrichment time; clean water by running it through old reversed-phase column; use HPLC-grade water.
Unknown interferences in sample	Use sample cleanup or prefractionation before injection.

Negative Peaks Causes and Solutions

Cause	***Solution***
Refractive index detection - refractive index of solute less than that of mobile phase	Reverse polarity to make peak positive
UV-absorbance detection - absorbance of solute less than that of mobile phase	Use mobile phase with lower UV absorbance; if recycling solvent, stop recycling when recycled solvent affects detection

Peak Doubling Causes and Solutions

Cause	***Solution***
Blocked frit	Replace or clean frit; install 0.5-um porosity in-line filter between pump and injector to eliminate mobile-phase contaminants or between injector and column to eliminate sample contaminants
Coelution of interfering compound	Use sample cleanup or prefractionation; adjust selectivity by changing mobile or stationary phase
Coelution of interfering compound from previous injection	Flush column with strong solvent at end of ran; end gradient at higher solvent concentration
Column overloaded	Use higher-capacity stationary phase; increase column diameter; decrease sample amount
Column void or channeling	Replace column, or, if possible, open top endfitting and clean and fill void with glass beads or same column packing; repack column
Injection solvent too strong	Use weaker injection solvent or stronger mobile phase
Sample volume too large	Use injection volume equal to one-sixth of column volume when sample prepared in mobile phase for injection
Unswept injector flow path	Replace injector rotor

Peak Fronting Causes and Solutions

Cause	***Solution***
Channeling in column	Replace or repack column
Column overloaded	Use higher-capacity stationary phase; increase column diameter; decrease sample amount

Peak Tailing Causes and Solutions

Cause	***Solution***
Basic solutes - silanol interactions	Use competing base such as triethylamine; use a stronger mobile phase; use base-deactivated silica-based reversed-phase column; use polymeric column
Beginning of peak doubling	See peak doubling
Chelating solutes - trace metals in base silica	Use high purity silica-based column with low trace-metal content; add EDTA or chelating compound to mobile phase; use polymeric column
Silica-based column - degradation at high pH	Use polymeric, sterically protected, or high-coverage reversed-phase column; install silica gel saturatorcolumn between pump and injector
Silica-based column - degradation at high temperature	Reduce temperature to less than 50 C
Silica-based column - silanol interactions	Decrease mobile-phase pH to suppress silanol ionization; increase buffer concentration; derivatize solute to change polar interactions
Unswept dead volume	Minimize number of connections; ensure injector rotor seal is tight; ensure all compression fittings arecorrectly seated
Void formation at head of column	Replace column, or, if possible, open top endfitting and clean and fill in void with glass beads or samecolumn packing; rotate injection valve quickly; use injection valve with pressure bypass; avoid pressure shock

Spikes Causes and Solutions

Cause	***Solution***
Bubbles in mobile phase	Degas mobile phase; use back-pressure restrictor at detector outlet; ensure that all fittings are tight
Column stored without caps	Store column tightly capped; flush reversed-phase columns with degassed methanol

2.Leaks

Leak at column or fittings: Causes and Solution

Possible Cause	***Solution***
Catastrophic loose fitting	Tighten or replace fitting
Noncatastrophic white powder at loose fitting	Cut tubing and replace ferrule; disassemble fitting, rinse and reassemble.

Leak at Detector: Causes and Solution

Possible Cause	***Solution***
Catastrophic detector-seal failure	Replace detector seal or gaskets.

Leak at injection valve: Causes and Solution

Possible Cause	***Solution***
Catastrophic worn or scratched valve rotor	Replace valve rotor

Leak at Pump: Causes and Solution

Possible Cause	***Solution***
Catastrophic pump-seal failure	Replace pump seal; check piston for scratches and, if necessary, replace.

3.Recovery

Poor sample recovery: Causes and Solution

Possible Cause	***Solution***
Absorption or adsorption of proteins	Change HPLC mode to reduce nonspecific interactions; add protein-solubilizing agent, strong acid or base (with polymeric columns only), or detergent such as SDS to mobile phase.
Adsorption on column packing	Increase mobile phase strength to minimize adsorption; for basic compounds add competing base or use base-deactivated packing
Adsorption on tubing and other hardware components	Use inert (PEEK), glass-lined, or titanium tubing and flow-path components
Chemisorption on column packing	Ensure no reactive groups are present; use

	polymeric packing; change column type and mode
Hydrophobic interactions between stationary	Use short-chain reversed-phase packing; use 300-A pore diameter packing; use hydrophilic packing or ton-exchange media; use hydrophobic interaction chromatography
Less than 99% yield for basic compounds irreversible adsorption on active sites	Use endcapped, base-deactivated, sterically protected, high coverage, or polymeric reversed-phase
Less than 90% yield for acidic compounds - irreversible adsorption on active sites	Use endcapped or polymeric packing; acidify mobile phase

4. Sensitivity

Lack of Sensitivity: Causes and Solutions

Possible Cause	***Solution***
Autosampler flow lines blocked	Check flow and clear any blockages
Detector attenuation set too high	Reduce detector attenuation
First few sample injections - sample adsorption in injector sample loop or column	Condition loop and column with concentrated sample.
Injector sample loop underfilled	Overfill loop with sample
Not enough sample injected	Increase amount of sample inected
Peaks are outside detector's linear range	Dilute or concentrate sample to bring detector response into linear range
Sample losses during sample preparation	Use internal standard during sample prepartion; optimize sample preparation method
Sample losses on column	See problem: Recovery

5. Retention

Changing retention times: Causes and Solution

Possible Cause	***Solution***
Buffer retention times	Use buffer with concentration greater than 20 mM.
Contamination buildup	Flush column occasionally with strong solvent
Equilibration time insufficient for gradient run or changes in isocratic mobile phase	Pass at least 10 column volumes through the column for gradient regeneration or after solvent

	changes
First few injections - active sites	Condition column by injecting concentrated sample
Inconsistent on-line mobile-phase mixing	Ensure gradient system is delivering a constant composition; compare with manually prepared mobile phase; partially premix mobile phase
Selective evaporation of mobile-phase component	Cover solvent reservoirs; use less-vigorous helium purging; prepare fresh mobile phase
Varying column temperature	Thermostat or insulate column; ensure laboratory temperature is constant.

Decreasing Retention times: Causes and Solutions

Possible Cause	***Solution***
Active sites on column packing	Use mobil-phase modifier, competing base (basic compounds), or increase buffer strength; use higher coverage column packing.
Column overloaded with sample	Decrease sample amount or use larger-diameter column
Increasing flow rate	Check and reset pump flow rate.
Loss of bonded stationary phase or base silica	Use mobile-phase pH between pH 2 and pH 8
Varying column temperature	Thermostat or insulate column; ensure laboratory temperature is constant.

Increasing Retention times: Causes and Solutions

Possible Cause	***Solution***
Decreasing flow rate	Check and reset pump flow rate; check for pump cavitation; check for leaking pump seals and other leaks in system.
Changing mobile-phase composition	Cover solvent reservoirs; ensure that gradient system is delivering correct composition.
Loss of bonded stationary phase	Use mobile-phase pH between pH 2 and pH 8

Slow column equilibration time: Causes and Solutions

Possible Cause	***Solution***
Reversed phase ion pairing - long chain ion pairing reagents require longer equilibration time	Use ion-pairing reagent with shorter alkyl chain length

6. Equilibration

Slow column equilibration time: Causes and Solutions

Possible Cause	*Solution*
Reversed phase ion pairing - long chain ion pairing reagents require longer equilibration time	Use ion-pairing reagent with shorter alkyl chain length

Varying retention times: Causes and Solutions

Possible Cause	*Solution*
Gradient - insufficient column-regeneration time	Increase equilibrating time in mobile-phase A to obtain constant retention times for early peaks
Ion pairing - insufficient equilibration time	Increase equilibration time; ion pairing may require as much as 50 column volumes for mobile-phase changeover
Isocratic - insufficient equilibration time	Pass 10-15 column volumes of mobile phase through column for equilibration

7.Baseline

Disturbance at void time: Causes and Solutions

Possible Cause	*Solution*
Air bubbles in mobile phase	Degas or use back pressure restricor on detector
Positive-negative - difference in refractive index of injection solvent and mobile phase	Normal with many samples; use mobile phase as sample solvent

Drifting baseline: Causes and Solutions

Possible Cause	*Solution*
Negative direction (gradient elution) - absorbance of mobile-phase A	Use non-UV absorbing mobile phase solvents; use HPLC grade mobile phase solvents; add UV absorbing

	compound to mobile phase B.
Positive direction (gradient elution) - absorbance of mobile phase B	Use higher UV absorbance detector wavelength; use non-UV absorbing mobile phase solvents; use HPLC grade mobile phase solvents; add UV absorbing compound to modile phase A.
Positive direction - contamination buildup and elution	Flush column with strong solvent; clean up sample; use HPLC grade solvents
Wavy or undulating - temperature changes in room	Monitor and control changes in room temperature; insulate column or use column oven; cover refractive index detector and keep it out of air currents.

Noise: Causes and Solutions

Possible Cause	***Solution***
Continous - detector lamp problem or dirty flow cell	Replace UV lamp(each should last 2000 h); clean and flush flow cell
Gradient or isocratic proportioning - lack of solvent mixing	Use proper mixing device; check proportioning precision by spiking one solvent with UV absorbing compound and mointor UV absorbance detector output
Gradient or isocratic proportioning - malfunctioning proportioning valves	Clean or replace proportioning precision valves; partially remix solvents
Occasional sharp spikes - external electrical interference	Use voltage stabilizer for LC system; use independent electrical circuit
Periodic - pump pulses	Service or replace pulse damper; purge air from pump; clean or replace check valves.
Random - contamination buildup	Flush column with strong solvent; clean up sample; use HPLC grade solvent
Spikes - bubble in detector	Degas mobile phase; use back pressure restrictor at detector outlet
Spikes - column temperature higher than boiling point of solvent	Use lower column temperature

8. Pressure

Decreasing Pressure: Causes and Solutions

Possible Cause	***Solution***
Insufficient flow from pump	Loosen cap on mobile phase reservior
Leak in hydralic lines from pump to column	Tighten or replace fittings; tighten rotor in injection valve
Leaking pump check valve or seals	Replace or clean check valves; replace pump seals.
Pump cavitation	Degas solvent; check for obstruction in line from solvent reservoir to pump; replace inlet-line frit

Flucuating Pressure: Causes and Solutions

Possible Cause	***Solution***
Bubble in pump	Degas solvent; sparge solvent with helium
Leaking pump check valve or seals	Replace or clean check valves; replace pump seals

High back pressure: Causes and Solutions

Possible Cause	***Solution***
Column blocked wth irreversibly adorbed sample	Improve sample cleanup; use guard column; reverse-flush column with strong solvent to dissolve blockage
Column particle size too small (for example 3 micrometers)	Use larger particle size (for example 5 micrometer)
Microbial growth on column	Use at least 10% organic modifier in mobile phase; use fresh buffer daily; add 0.02% sodium azide to aqueous mobile phase; store column in at least 25% organic solvent without buffer
Mobile phase viscosity too high	Use lower viscosity solvents or higher temperature
Plugged frit in in-line filter or guard column	Replace frit or guard column
Plugged inlet frit	Replace endfitting or frit assembly
Polymetric columns - solvent change causes swelling of packing	Use correct solvent with column; change to proper solvent compositionl consult manufacturer's solvent-compatibility chartl use a column with a higher percentage of cross-linking
Salt precipitation (especially in reversed-phase chromatography with high concentration of organic solvent in mobile phase)	Ensure mobile phase compatibility with buffer concentration; decrease ionic strength and water-organic solvent ratio; premix mobile phase
When injector disconnected from column - blockage in injector	Clean injector or replace rotor

Increasing pressure: Causes and Solutions

Possible Cause	***Solution***
Blocked flow lines	Systematically disconnect components from detector end to column end to find blockage; replace or clean blocked component
Particulate buildup at head of column	Filter sample; use .5 micrometer in-line filter; disconnect and backflush column; replace inlet frit
Water-organic solvent systems - buffer precipitation	Ensure mobile phase compatibility with buffer concentration; decrease ionic strength or water organic solvent ratio

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