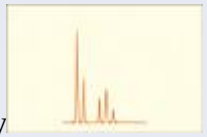
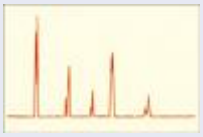
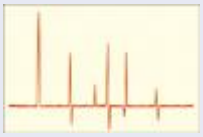
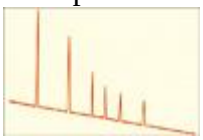
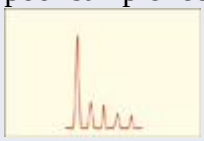
HPLC Troubleshooting

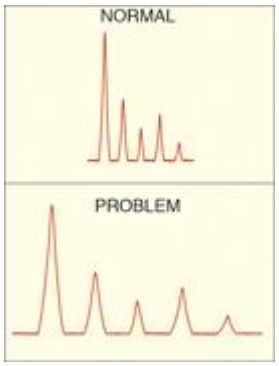
We can help you.

General instructions for column maintenance and individual rinsing procedures can be found in the accompanying column leaflet or on the corresponding website.

Peaks		Baseline	Retention time	Pressure	Miscellaneous
broad 	fronting / tailing 	drifting to higher absorption 	increasing 	no / low decreasing 	lack of selectivity 
double 	negative 	drifting to lower absorption 	decreasing 	fluctuating 	poor sample recovery 
ghost 	spikes 	undulating / noise 	fluctuating 	too high / increasing 	leaks 

Problem area: Peaks

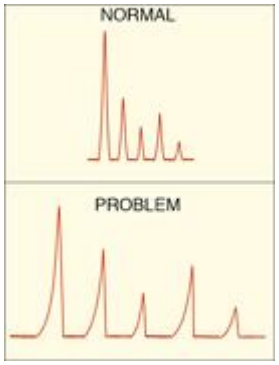
Observation: Broad peaks



Possible causes	Prevention / suggested remedy
1. Injection volume too large.	1. Inject smaller volumes or reduce solvent strength for injection to focus the sample components.
2. Poor column efficiency.	2. Use mobile phases of lower viscosity, elevated column temperature, lower flow rate or a packing with smaller particle size.
3. Extra column volume of the LC system too large.	3. Use zero dead volume fittings and connectors; use smallest possible tubing diameter (<0.25 mm) and matched size of fittings.
4. Volume of detector cell too large.	4. Use smallest possible cell volume for the sensitivity required; use a detector

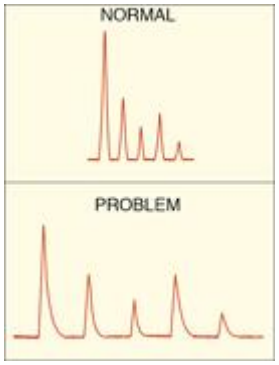
	without heat exchanger in the system.
5. Detector time constant too slow.	5. Adjust the time constant to the peak width.
6. Sampling rate of the data system is too low.	6. Increase the sampling rate.
7. only some peaks broad: late elution of analytes from a previous run.	7. Flush the column with a strong eluent after each run, or end gradient at a higher concentration.
8. Retention times too long.	8. Use gradient elution or a stronger mobile phase for isocratic elution.
9. Viscosity of mobile phase is too high.	9. Increase column temperature or use a solvent of lower viscosity.

Observation: Fronting



Possible causes	Prevention / suggested remedy
1. Column overload.	1. Decrease sample amount; increase column diameter.
2. Formation of channels in the column.	2. Buy a new column or have the column repacked.

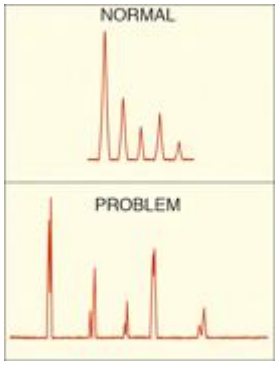
Observation: Tailing



Possible causes	Prevention / suggested remedy
1. Basic analytes: interactions with silanol groups.	1. Use silica-based base deactivated RP phases (e.g., NUCLEODUR® Gravity, HTec, Isis, Pyramid); switch to polymer-based columns (e.g., NUCLEOGEL® RP). Silica-based column: silanol interactions. Decrease the pH value of the mobile phase to suppress ionization of the silanol groups; increase the buffer concentration; derivatize the sample to avoid polar interactions.
2. Sample components which can form chelates: metal traces in the packing.	2. Only use high-purity silica-based packings (e.g., NUCLEODUR®) with their very low metal contamination; add

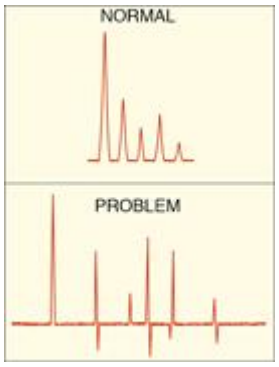
	EDTA or another chelating compound to the mobile phase; switch to polymer-based columns (e.g., NUCLEOGEL® RP).
3. Dead volume at the column head / compressed column packing.	3. Avoid pressure pulses; replace the deteriorated column, or, if possible, open the upper end fitting and fill the void with the column packing or some silanized glass fiber wadding; for preparative HPLC: with our VarioPrep columns you can compensate dead volumes with the adjustable end fitting.
4. System dead volume.	4. Minimize the number of connections; use zero-dead-volume connectors; use new capillaries to column/ to detector; check whether all fittings are tight.
5. silica-based column: degradation at high temperatures.	5. Use temperatures below 50 °C.

Observation: Double peaks



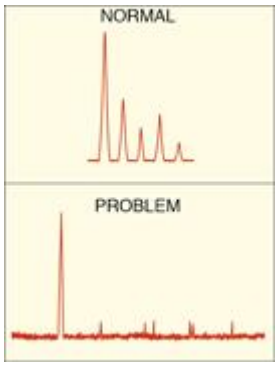
Possible causes	Prevention / suggested remedy
1. Column overload.	1. Decrease sample amount; increase column diameter.
2. Injection solvent too strong.	2. Use a weaker solvent for the sample or a stronger mobile phase.
3. Sample volume too large.	3. If the sample is dissolved in the mobile phase, the injection volume should be smaller than one-sixth of the column volume.
4. Dead volume or formation of channels in the column.	4. Replace the column or, if possible, open the upper end fitting and fill the void with the same packing; have the column repacked.
5. Simultaneous elution of an interfering substance.	5. Use sample clean-up or fractionation prior to injection (e.g., SPE with CHROMABOND® or CHROMAFIX®); improve selectivity by choice of another mobile or stationary phase.
6. Simultaneous late elution of a substance from a previous run.	6. Flush the column with a strong eluent after each run, or end gradient at a higher concentration.

Observation: Negative peaks



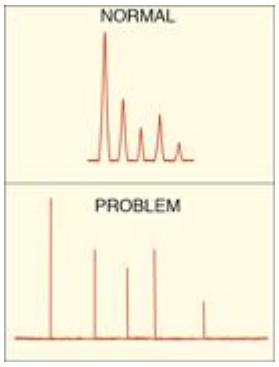
Possible causes	Prevention / suggested remedy
1. RI detector: refractive index of the analyte lower than that of the mobile phase.	1. Reverse detector polarity to obtain positive peaks.
2. UV detector: absorption of the analyte lower than absorption of the mobile phase.	2. use a mobile phase with lower UV absorption; if recycling solvent, use fresh HPLC grade eluent when the recycled mobile phase starts to affect detection.

Observation: Ghost peaks



Possible causes	Prevention / suggested remedy
1. Contamination.	1. only use HPLC grade solvents; flush the column to remove impurities.
2. Late elution of an analyte from a previous run.	2. Flush the column with a strong eluent after each run, or end gradient at a higher concentration.
3. Unknown interfering substances in the sample.	3. use sample clean-up or fractionation prior to injection (e.g., SPE with CHROMABOND® or CHROMAFIX®).
4. RP chromatography: contaminated water.	4. Check the suitability of the water by passing different amounts through the column and measure the peak height of the impurity as a function of enrichment time; use HPLC grade water or purify the water by running it through an old RP column.
5. Peptide mapping: oxidation of trifluoroacetic acid.	5. prepare fresh trifluoroacetic acid solution daily; add an antioxidant.
6. Ion pairing chromatography: disturbed equilibrium.	6. Prepare the sample in the mobile phase; reduce the injection volume.

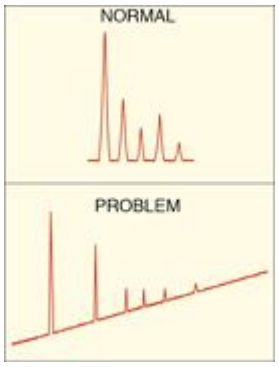
Observation: Spikes



Possible causes	Prevention / suggested remedy
1. Air bubbles in the mobile phase.	1. Degas the mobile phase; install a back pressure restrictor at the detector outlet; ensure that all fittings are tight.
2. Column was stored without end caps.	2. Always store columns tightly capped; flush RP columns with degassed methanol.

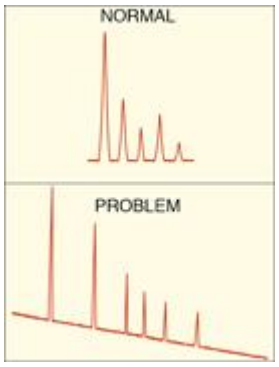
Problem area: Baseline

Observation: Baseline drifting to higher absorption



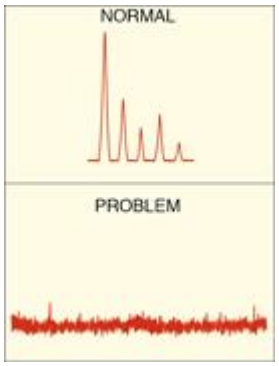
Possible causes	Prevention / suggested remedy
1. with gradient elution: strong UV absorption of the increasing mobile phase component B.	1. use a higher wavelength of the UV detector; use non-UV-absorbing HPLC grade solvents for your mobile phases; if a UV-absorbing solvent is inevitable, use a UV-absorbing additive in mobile phase A.
2. accumulation and elution of impurities.	2. use sample clean-up or fractionation prior to injection (e.g., SPE with CHROMABOND® or CHROMAFIX®); use only HPLC grade solvents; clean the contaminated column with a strong solvent.
3. Viscosity of mobile phase is too high.	3. Increase column temperature or use a solvent of lower viscosity.
4. Retention times too long.	4. Use gradient elution or a stronger mobile phase for isocratic elution.
5. Poor column efficiency.	5. Use mobile phases of lower viscosity, elevated column temperature, lower flow rate or a packing with smaller particle size.

Observation: Baseline drifting to lower absorption



Possible causes	Prevention / suggested remedy
1. with gradient elution: strong UV absorption of the decreasing mobile phase component A.	1. use non-UV-absorbing HPLC grade solvents for your mobile phases; if a UV-absorbing solvent is inevitable, use a UV-absorbing additive in mobile phase B.

Observation: Undulating baseline and noise



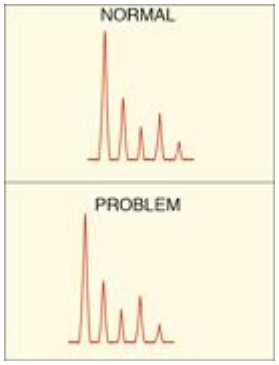
Possible causes	Prevention / suggested remedy
1. wave-like / undulating baseline: temperature changes in the room.	1. Monitor or avoid changes in room temperature; isolate the column or use a column oven; cover the RI detector to protect it from air currents.
2. Continuous noise: detector lamp problem or dirty detector cell.	2. Replace the UV lamp or clean the detector cell.
3. Periodic: pump pulses.	3. Repair or replace the pulse damper; purge any air from the pump; clean or replace the check valves.
4. Random: accumulation of impurities.	4. use sample clean-up or fractionation prior to injection (e.g., SPE with CHROMABOND® or CHROMAFIX®); use only HPLC grade solvents; backflush contaminated column with a strong solvent.
5. Insufficient solvent mixing during gradient elution or isocratic proportioning.	5. Mix by hand, or – if possible – use solvents of lower viscosity; monitor proportioning precision by spiking one solvent with a UV absorbing substance and measure the resulting detector output.
6. Malfunctioning proportioning valves by gradient elution.	6. Clean or replace the proportioning valve; use partially premixed solvents.
7. Spikes: air bubble in detector, mobile phase or pump.	7. Degas the mobile phase; install a back pressure restrictor at the detector outlet; ensure that all fittings are tight; always store columns tightly capped; flush reversed phase columns with degassed methanol.
8. Spikes: column temperature higher	8. Use lower working temperature.

than boiling point of the solvent.

9. Occasional sharp spikes: external electric interferences.	9. Use a voltage stabilizer for your LC system or use an independent electrical circuit for your chromatography equipment.
10. Disturbance at dead time: air bubbles in the mobile phase.	10. Degas the mobile phase or use premixed eluents.
11. Difference in refractive index between injection solvent and mobile phase.	11. If possible, use the mobile phase as solvent for the sample.

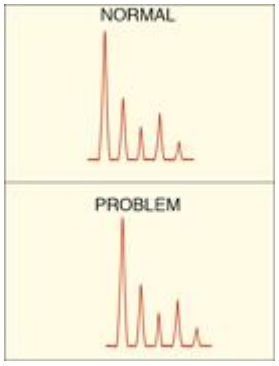
Problem area: Retention times

Observation: Decreasing retention times



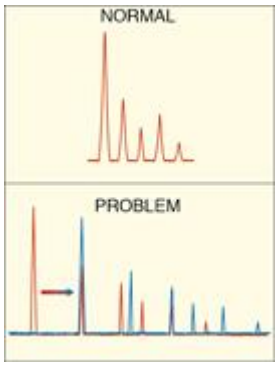
Possible causes	Prevention / suggested remedy
1. Column overloaded with sample.	1. Reduce the amount of sample or use a column with larger diameter.
2. Loss of bonded stationary phase.	2. replace column; for silica adsorbents use mobile phases between pH 2 and pH 8 or switch to phases with higher pH stability (e.g., NUCLEODUR® C_{18} Gravity)
3. Increasing flow rate.	3. Check and – if necessary – adjust the pump flow rate.

Observation: Increasing retention times



Possible causes	Prevention / suggested remedy
1. Changing mobile phase composition.	1. Cover the solvent reservoirs to avoid evaporation of volatile solvent component; ensure that the gradient system supplies the proper composition; if possible, mix the mobile phase by hand.
2. Decreasing flow rate.	2. check and – if necessary – adjust the pump flow rate; check for pump cavitation; check for leaking pump seals and other leaks in the system.

Observation: Fluctuating retention times



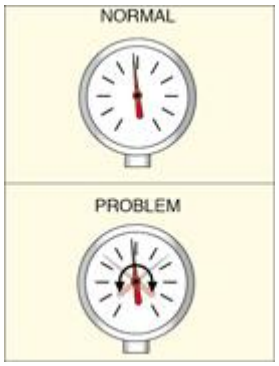
Possible causes	Prevention / suggested remedy
1. Fluctuating column temperature.	1. Ensure that the room temperature is constant; if necessary, thermostat or isolate the column.
2. leaks	2. see at "leaks"
3. Only during first few injections: active groups.	3. condition the column with concentrated sample.
4. Insufficient buffer capacity.	4. use buffer concentrations above 20 mmol/L.
5. Insufficient mixing of the mobile phase.	5. Ensure that the gradient system supplies a mobile phase with constant composition; compare with manually mixed eluents; use partially premixed mobile phases.
6. Insufficient equilibration at isocratic separation.	6. Pass 10 to 15 column volumes of mobile phase through the column for equilibration.
7. Insufficient equilibration at gradient elution.	7. Increase equilibration time with mobile phase A in order to obtain constant retention times for early peaks, also: pass at least 10 column volumes of eluent A through the column for gradient regeneration.
8. Insufficient equilibration at reversed phase ion-pairing chromatography.	8. Increase the equilibration time; in ion-pairing chromatography sometimes 50 column volumes may be required for equilibration; long-chain ion-pairing reagents require more time; if possible, use ion-pairing reagents with shorter alkyl chains.

Problem area: Pressure

Observation: No / low or decreasing pressure

Possible causes	Prevention / suggested remedy
1. Insufficient flow (interrupted, obstructed).	1. Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary; loosen the cap on the mobile phase reservoir to avoid underpressure.
2. Leak in liquid lines between pump and column.	2. Tighten all fittings; replace defective fittings; tighten the rotor in the injection valve.
3. Leaking pump, check valve or seals.	3. Clean the check valve; replace defective check valves or seals.

Observation: Fluctuating pressure



Possible causes	Prevention / suggested remedy
1. Air bubbles in the pump.	1. Degas all solvents; flush the solvent with helium.
2. Leak in liquid lines between pump and column.	2. Tighten all fittings; replace defective fittings; tighten the rotor in the injection valve.

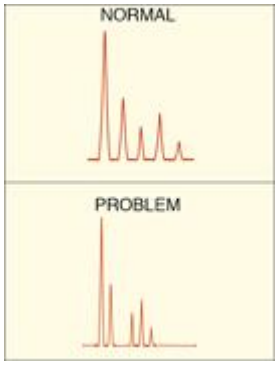
Observation: Too high / increasing pressure

Possible causes	Prevention / suggested remedy
1. Salt precipitation / precipitation of buffer components.	1. Especially in reversed phase chromatography with high proportions of organic solvents in the mobile phase; ensure that the solvent composition is compatible with the buffer concentration; reduce the ionic strength and the ratio organic-aqueous in the mobile phase; premix the mobile phase.
2. contamination at the column inlet	2. use sample clean-up or fractionation prior to injection (e.g., SPE with CHROMABOND® or CHROMAFIX®);

	use an 0.5 µm in-line filter; use guard columns; backflush column with a strong solvent in order to dissolve the impurity; replace plugged inlet frits /guard column.
3. Microbial growth in the column.	3. use a mobile phase with at least 10 % organic solvent; prepare fresh buffer daily; add 0.02 % sodium azide to aqueous mobile phases; for storage equilibrate the column with at least 25 % organic solvent and without buffer.
4. Viscosity of mobile phase too high.	4. Use a solvent of lower viscosity or increase the temperature.
5. Particle size of packing too small.	5. Use a packing with larger particle size (e. g. 3 µm instead of 1.8 µm).
6. for polymer-based columns: swelling of the adsorbent caused by eluent changes.	6. use only solvents compatible with the column; check proper eluent composition; consult instructions for use for solvent compatibility; use a column with a higher degree of cross-linking.
7. plugged frit in in-line filter / at column inlet or guard column.	7. Replace the end fitting / the frit or guard column.
8. When the injector is disconnected from the column: plugged injector.	8. Clean the injector or replace the rotor.
9. Plugged liquid lines.	9. Systematically disconnect system components from the detector end to the blockage; clean or replace the plugged component.

Problem area: Miscellaneous

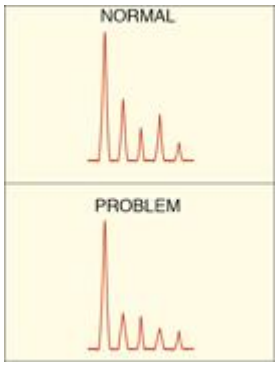
Observation: Lack of selectivity



Possible causes	Prevention / suggested remedy
1. Detector attenuation set too high.	1. Reduce detector attenuation.
2. Not enough sample injected.	2. Increase amount of sample for injection.
3. Sample loss during sample preparation.	3. use an internal standard for sample preparation and optimize your method.
4. Sample loss on column.	4. Use an internal standard and optimize your method.
5. autosampler line blocked.	5. check the flow and clear any blockages.
6. Peaks outside the linear range of the	6. Dilute or enrich the sample until the

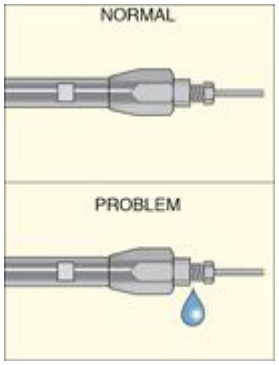
detector.	concentration is in the linear range of the detector.
7. Only during first few injections: sample absorption in sample loop of injector or column.	7. Condition sample loop and column with concentrated sample.

Observation: Poor sample recovery



Possible causes	Prevention / suggested remedy
1. adsorption on stationary phase.	1. increase mobile phase strength to minimize adsorption; for basic compounds use a base deactivated packing like NUCLEODUR® Gravity, HTec, Isis, Pyramid
2. hydrophobic interactions between stationary phase and biomolecules.	2. use short-chain reversed phase packings; as an alternative you may use hydrophilic stationary phases or ion exchangers.
3. Adsorption of proteins.	3. use another HPLC mode to reduce nonspecific interactions (e.g., gel filtration or ion exchange); use a mobile phase containing reagents which enhance solubility of the proteins, strong acids or bases (only with polymer-based columns) or detergents like SDS (sodium dodecyl sulfate).
4. Adsorption on tubing and other hardware components.	4. Use inert tubing and fittings made from, e.g., PEEK or titanium.

Observation: Leaks



Possible causes	Prevention / suggested remedy
1. Column loses stationary phase.	1. Replace column.
2. Serious leaks at column or fittings.	2. Tighten loose fittings or use new fittings.
3. Serious leak at detector.	3. Replace defective detector seals or gaskets.
4. Serious leak at the injector.	4. Replace worn or scratched valve rotors.
5. Serious leak at the pump.	5. Replace defective pump seals; check the piston for scratches and replace piston, if necessary.