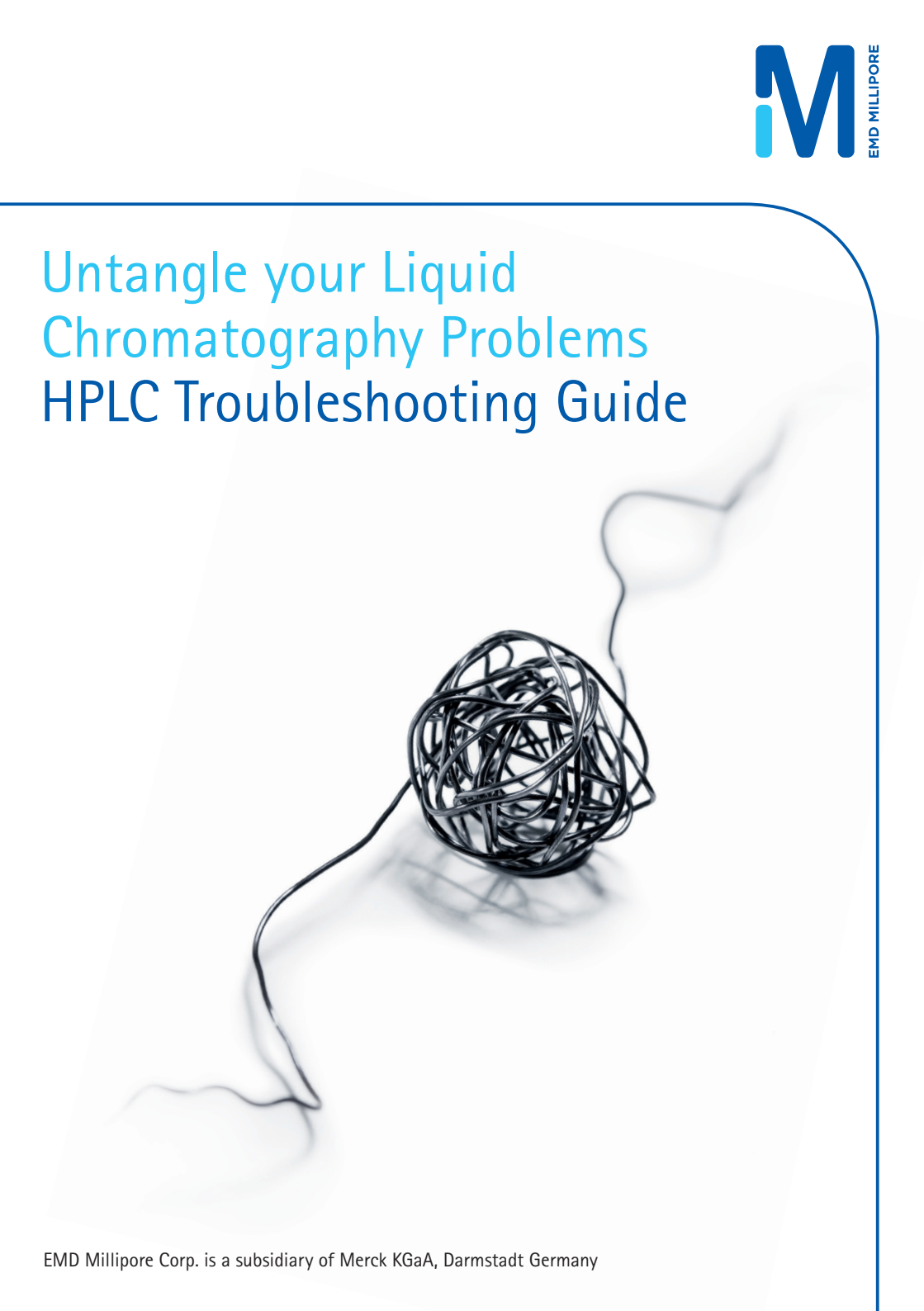
Untangle your Liquid Chromatography Problems

HPLC Troubleshooting Guide



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Chromatographers frequently have to identify and rectify problems that can be divided in different categories. In this guide, we will discuss some of the most common issues that may appear and how to solve them. Emphasis is on reversed phase separation. Often problems can be avoided by routine maintenance (e.g. planned replacement of worn out parts). Simple rules are useful for classifying deficiencies and can help in avoiding follow-up mistakes.

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Retention

Small differences in mobile phase composition may cause huge differences in retention time when the column is overloaded and this also changes with temperature. However, even if the mobile phase is buffered and the pump is working properly, the retention times may fluctuate if the pH is too close to the pK of the sample substance. The pH of the mobile phase should therefore be chosen to be at least one pH unit above or below the pK value of the analytes being separated. **Retention time drift indicates insufficient column conditioning.** With increasing column life, the retention times may shift towards less retentivity, especially if the user is working at acidic pH (≤ pH 2). Abrupt changes in retention time are usually due to errors in the system.

Problem: Changing retention times

Possible cause	Solution
Flow rate variation	• Fix system leaks • Replace pump seals • Remove bubbles • Check for cavitations
Insufficient buffer capacity	• Use buffer concentration > 20 mM and < 50 mM
Column contamination build-up	• Flush column occasionally with strong solvent or regenerate the column
Equilibration time insufficient for gradient run or changes in isocratic mobile phase	• Allow at least 10 column volumes through the column for gradient regeneration or after solvent changes. True equilibration is achieved after 30 column volumes
First few injections – active sites	• Condition column by injecting concentrated sample

Troubleshooting
Retention

Possible cause	Solution
Inconsistent on-line mobile phase mixing	• Ensure gradient system is delivering a constant composition • Compare with manually prepared mobile phase • Partially premix mobile phase. Avoid running from 100% pure solvent to 100% aqueous
Selective evaporation of mobile phase component	• Use closed solvent reservoirs • Use less-vigorous purging • Prepare fresh mobile phase • Check pump • Check frit • Avoid evaporation or degradation of mobile phase
Column temperature variation	• Thermostat or insulate column • Use column oven • Ensure constant laboratory temperature
Column aging	• Replace column • If aging is premature, it may originate from sample matrix. Perform column regeneration • Use guard column

Problem: Decreasing retention times

Possible cause	Solution
Active sites on column packing	• Use mobile phase modifier • Competing base (basic compounds), or increase buffer strength • Use higher coverage column packing
Column mass overload	• Decrease sample amount or use larger-diameter column
Increasing flow rate	• Check and reset pump flow rate
Loss of bonded stationary phase	• Use mobile phase pH that is within the specifications given for the particular column (normally between pH 2 and pH 7.5) • With Purospher® STAR pH 1.5 – 10.5 is possible
Column temperature variation	• Thermostat or insulate column • Use column oven • Ensure constant laboratory temperature
Mobile phase composition changing	• Check pump • Check frit • Avoid evaporation or degradation of mobile phase
Column fouling	• Stationary phase modified by sample. Regenerate or change the column

Troubleshooting
Retention

Problem: Increasing retention times

Possible cause	Solution
Decreasing flow rate	• Check and reset pump flow rate • Check for pump cavitations • Check for leaking pump seals and / or 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 that is within the specifications given for the particular column (normally between pH 2 and pH 7.5) • With Purospher® STAR pH 1.5 – 10.5 is possible
Mobile phase composition changing- online mixing	• Check pump • Check frit • Avoid evaporation or degradation of mobile phase
Failing or insufficient pH control for ionic compounds	• Use buffered mobile phases • Increase buffer concentration • Use buffer more suitable for required pH range
Temperature decreasing / Temperature variations in the column	• Use column thermostat
Column fouling	• Stationary phase modified by sample Regenerate or change the column

Equilibration

Problem: Slow column equilibration time

Possible cause	Solution
Reversed phase ion-pair reagents long chain ion-pair reagents require longer equilibration time	• Use ion-pair reagents with shorter alkyl chain length

Problem: Varying retention times

Possible cause	Solution
Gradient – insufficient column regeneration time	• Increase equilibration time (volume) with initial mobile phase composition (A) to achieve constant retention for early peaks
Ion-pair reagents – insufficient equilibration time	• Increase equilibration time (volume) • Ion-pair reagents 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

Peaks

If all peaks have same appearance in the chromatogram, the problem originated before the separation. If only some of the peaks or only one peak in the chromatogram elute with a distorted shape, the source is of chemical nature.

Problem: Broad peaks

Wide peaks are generated either by substantial influence on the part of the HPLC system (bad capillary connections, void volumes, too large detector cells or ill-chosen time constants) or by poor column performance.

Possible cause	Solution
Sample overload	• Dilute sample 1:10 with mobile phase and re-inject
Detector-cell volume too large	• Use smallest possible cell volume consistent with sensitivity needs • Use detector with no heat exchanger in system

Problem: Broad peaks [continued]

Possible cause	Solution
Injection volume too large	• Decrease solvent strength of injection solvent to focus solute • Decrease injection volume • Dilute sample • Rule of thumb: Inject maximum 1% of total column tube volume
Large extra column volume	• Use low- or zero-dead-volume end-fittings 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 • Use segmented injection techniques (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
Column head contaminated	• Exchange inlet frit or filter
Fouled or worn out column	• Regenerate column or replace with new column
Sampling rate of data system too low	• Increase sampling frequency

Possible cause	Solution
Slow detector time constant	• Adjust time constant to match peak width
Column temperature too low	• Increase column oven temperature
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
Guard column / pre-column or column defective or soiled	• Change guard column / pre-column or column
Sample dissolved in strong solvent	• Dissolve sample in mobile phase
Wrong buffer pH	• Test influence of eluent pH on peak shape
Buffer concentration too low	• Use concentrated buffer or add salt to increase total ionic strength of the mobile phase
Extra column effects	• Check capillary connections • Use shorter capillaries with smaller i.d. • Check for dead-volume
Leak between column and detector	• Fix leak
Large detector cell	• Use smaller cell
Sample incompatibility with system or sample precipitation	• Use inert surfaces in system parts (injector, pump) • Use simple test-tube experiment to determine solubility of sample in mobile phase to prevent on-column precipitation

Problem: Ghost peaks

Ghost peaks may be caused by unknown sample components, late eluting peaks from previous injections, impurities or mixing problems in connection with the mobile phase. The sample should therefore preferably always be dissolved in the eluent or in a solvent with weaker eluting strength. Substances with UV absorption lower than the eluent may generate negative peaks.

Possible cause	Solution
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
Unknown interferences in sample	• Use sample cleanup or pre-fractionation before injection
Column contamination	• Flush column with strong solvent after each run • Improve sample cleanup
Solvent impurities	• Use HPLC-grade solvents

Problem: Negative peaks

Possible 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

Problem: Peak doubling

If all peaks have shoulders or elute as double peaks, the cause may origin from clogged inline filters, column inlet frits, contaminated pre-columns or a void volume at the column head. In most cases, the column may be returned to its original state by cleaning or replacement of the inlet frit. A short-term solution to this problem may also be to invert the column. Destroyed bed at the column outlet contributes only marginally to peak spreading.

Possible cause	Solution
Blocked frit	• Replace or clean frit • Install 0.5 µm porosity inline filter between pump and injector to eliminate mobile phase contaminants or between injector and column to eliminate sample contaminants
Co-elution of interfering compound	• Use sample cleanup or pre-fractionation • Adjust selectivity by changing mobile or stationary phase
Co-elution of interfering compound from previous injection	• Flush column with strong solvent at end of run • End gradient at higher solvent concentration
Column overloaded	• Use higher-capacity stationary phase • Increase column diameter • Decrease sample amount
Column void or channelling	• Replace column
Injection solvent too strong	• Use weaker injection solvent or stronger mobile phase
Sample volume too large	• Use injection volume equal to 1% of the total column tube volume when sample is diluted in mobile phase • Reduce sample volume • Dilute sample • Inject sample prepared in mobile phase
Sample dissolved in strong solvent	• Dissolve sample in mobile phase or (if not possible) inject very small sample volume

Problem: Peak fronting

Possible cause	Solution
Channelling in column	• Replace or repack column
Column overloaded	• Use higher-capacity stationary phase • Increase column diameter • Decrease sample amount • Dilute sample
Pre-column defective or soiled	• Change pre-column
Sample dissolved in wrong solvent	• Dissolve sample in mobile phase or (if not possible) inject smaller sample volume
Interfering compounds in the sample	• Test column using a test- or calibrations sample • Sample clean-up advised
Sample precipitation	• Use simple test-tube experiment to determine solubility of sample in mobile phase to prevent on-column precipitation

Problem: Peak tailing

The tailing of peaks that are eluted early is caused by extra column effects. To remedy, the entire system should be checked – capillary connections, tubings and the detector cell. Secondary, non-specific interaction with the silica gel surface leads to a tailing of late eluting peaks and even to the appearance of double peaks. Addition of triethylamine or acetate to the mobile phase or selecting a suitable stationary phase will considerably improve the peak form. An inappropriately selected pH-value for the mobile phase may also lead to peak tailing. In principle, chromatography should be carried out one pH unit above or below the pK values of the sample substances.

Possible cause	Solution
Basic solutes – silanol interactions	• Use competing base such as triethylamine • Use a stronger mobile phase • Increase buffer or salt concentration (ion-pair-chromatography) • Use base-deactivated silica-based reversed-phase column • Use polymeric column • Use lower mobile phase pH

Possible cause	Solution
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 saturator column 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
Void formation at head of column	• Replace column • To prevent: Rotate injection valve quickly • Use injection valve with pressure bypass • Avoid pressure shock
Column overload	• Decrease sample size • Increase column diameter • Use higher capacity stationary phase
Blocked column frit	• Replace frit • Add in-line filter • Filter samples
Interfering compounds in the sample / Impurities	• Improve sample cleanup • Test column with test sample or calibrations sample • Use HPLC-grade solvents
Adsorption of the sample onto the column (especially basic compounds)	• Use a different stationary phase (special phase for basic compounds) • Use buffered mobile phase

Troubleshooting
Peaks

Problem: Spikes

Possible 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

Problem: No peaks

Possible cause	Solution
No flow through detector; Leak	• Check pump • Check connections and fittings in the system and column end-fittings and tighten • Check frit • Check mobile phase composition • Fix leak
Sample injection is not reproducible	• Check sample injection system
No sample injected	• Make sure the injector is working properly and sample is not precipitated
No detectability	• Make sure analytes are monitored under proper conditions

Problem: Peaks with shoulders, split peaks

Possible cause	Solution
Guard column defect or dirty	• Exchange guard column
Column head dirty	• Exchange inlet frit or filter
Dead space of column head or channels in column	• Use new analytical column
Sample dissolved in solvent which is not compatible with eluent	• Dissolve sample in eluent • Decrease injection volume

Recovery

Problem: Poor sample recovery

Possible cause	Solution
Absorption or adsorption of proteins	• Change HPLC mode to reduce non-specific interactions • Add protein-solubilising 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
Chemisorptions 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 Å pore diameter packing • Use hydrophilic packing or ion-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

Leaks

Problem: Leak at column or fittings

Possible cause	Solution
Loose fitting	• Check connections and fittings in the system and column end-fittings and tighten or replace fitting
Precipitation (white powder) at loose fitting	• Cut tubing and replace ferrule • Disassemble fitting, rinse and reassemble

Problem: Leak at detector

Possible cause	Solution
Detector-seal failure	• Replace detector seal or gaskets

Problem: Leak at injection valve

Possible cause	**Solution**
Worn or scratched valve rotor	• Replace valve rotor

Problem: Leak at pump

Possible cause	**Solution**
Pump-seal failure	• Replace pump seal • Check piston for scratches and, if necessary, replace

Selectivity

Problem: Differences in selectivity

Possible cause	Solution
Differences in mobile phase composition	• Check pump • Check frit • Avoid evaporation or degradation of mobile phase
New eluent composition is slightly different (i.e. pH is not adjusted, solvent contains contaminants)	• Make up new eluent • Accurately determine volume, salt addition and pH value
Too weak solvent / eluent not buffered	• Use buffer or ion-pair system
Sample dissolved in different solvents	• Dissolve sample in mobile phase or (if not possible) inject very small sample volume
Decreasing column life; Contamination	• Replace column • Improve sample cleanup • Check column with test mixture • Use HPLC-grade solvents
Temperature variations in the buffer	• Use column thermostat
Column to column reproducibility	• Replace column • Check with manufacturer
Column irreversibly changed	• Use new column

Baseline

Problem: Disturbance at void time

Possible cause	Solution
Air bubbles in mobile phase	• Degas or use back pressure restrictor on detector
Positive-negative – difference in refractive index of injection solvent and mobile phase	• Normal with many samples • Use mobile phase as sample solvent

Problem: Drifting baseline

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
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
Positive direction – contamination build-up 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

Problem: Noise

Possible cause	Solution
Continuous – detector lamp problem or dirty flow cell	• Replace UV lamp (each should last 2,000 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 monitor 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 • Change piston seals • Clean or replace check valves
Random – contamination build-up	• 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

Pressure

Problem with pressure is usually connected with too high back pressure, and to elucidate where the problem is originating, a good laboratory practice is to disconnect the system stepwise starting at the pump and working towards the detector.

Problem: Decreasing pressure

Possible cause	Solution
Insufficient flow to pump	• Loosen cap on mobile phase reservoir
Leak in hydraulic 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 cavitations	• Degas solvent • Check for obstruction in line from solvent reservoir to pump • Replace inlet-line frit

Problem: Fluctuating pressure

Possible cause	Solution
Bubble in pump	• Degas solvent • Purge solvent with helium
Leaking pump check valve or seals	• Replace or clean check valves • Replace pump seals

Problem: High back pressure

Possible cause	Solution
Pre / guard column blocked	• Exchange pre / guard column • Exchange column inlet frit • Back-flush column • Exchange column
Column head blocked	• Change filter of column head • Flush column • Change column

Possible cause	Solution
Capillary blocked	• Exchange capillary
Column blocked with irreversibly adsorbed sample	• Improve sample cleanup • Use guard column • Reverse-flush column with strong solvent to dissolve blockage
Column particle size too small (for example 3 µm)	• Use larger particle size (for example 5 µm)
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 end-fitting or frit assembly
Polymeric columns – solvent change causes swelling of packing	• Use correct solvent with column • Change to proper solvent composition • Consult manufacturer's solvent-compatibility chart • 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

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