



# CHROMATOGRAPHY

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## HPLC Troubleshooting Tips

### Leaks

Leaks are the easiest problems to locate and correct. A drop in system pressure usually indicates a leak. Leaks at fitting unions can often be fixed by tightening the fitting. However, a fitting may need to be disassembled and cleaned prior to tightening to remove particulates or buffer residues. If this does not correct the leak, it is best to replace the fitting. Leaks at other locations such as detector cells, pump heads and injection valves may require rebuilding or replacing seals. Check the instrument manual or contact the manufacturer for service information.

### Pressure Problems

Pressure problems are observed as a change in the system pressure. Standard operating pressure for a given assay or system condition should be recorded and used as a reference in order to determine when a problem with system pressure exists. High pressure may result from changes in system components (column length, diameter or particle size), an inadvertent change in flow rate, change in mobile phase composition, or particulate buildup within the system. Be sure to check the column specifications, mobile phase composition and flow rate before trying other corrective measures. Particulates typically originate from samples, mobile phase, or wear of system components such as pump seals. The fastest way to locate a problem causing high pressure is to start with the components that contribute most to system pressure. These include first any inline filter or guard column, and then the analytical column. Replace each component in sequence with one known to be in good working order. If an inline filter or guard column is causing the problem, replace it. If the analytical column is blocked, try back-flushing the column or replacing the inlet frit. Replace the analytical column if these procedures do not correct the problem. When pressure problems persist, begin at the detector and loosen connecting fittings one at a time, working upstream until the pressure drops dramatically. This can help to isolate the fitting or component causing the problem. Check tubing ends to make sure they are clean and free of filings. Metal fragments from an improperly finished tubing cut can create blockage and cause increased pressure. Precipitation of buffers within the system can cause pressure increases. This problem is common in buffered aqueous mobile phases when the organic concentration exceeds 50%. If buffer precipitation is suspected, remove the HPLC column and begin flushing the system with a buffer-free 5% organic aqueous mobile phase at a low flow rate. After system pressure stabilizes, reinstall the column and rinse it thoroughly at a low flow rate with the same flushing solvent. Re-equilibrate the system with the standard mobile phase and begin analysis. Premixing and filtering the mobile phase may help prevent buffer precipitation. In general, however, conditions that may lead to buffer precipitation should be avoided because in many cases, if it occurs, buffer precipitation can irreversibly damage an expensive HPLC column. Low pressure often indicates the presence of a leak, a reduction in the flow rate, or a problem with a system component. First, check for leaks throughout the system and ensure the solvent delivery flow rate setting is correct. Verify proper flow rate and pump operation with an in-line electronic flowmeter, or by filling a graduated cylinder from the detector or column outlet with a measured

volume of mobile phase over a measured time interval. (Note: Measuring flow in the system with the column disconnected may cause erroneously low readings. Most high-pressure pumps require flow restriction at the outlet for the check valves to operate properly.) If the measured flow rate is correct, check the mobile phase composition. The wrong organic content or the wrong organic solvent, for example acetonitrile instead of methanol, can cause reduced pressure. Column problems are more likely to increase pressure than to cause a decrease, but as a last resort, check the column by removing the inlet frit and observing any loss of packing. If this has occurred, replace the column. If the measured flow is too low, be sure the pump is not being starved for mobile phase. Check reservoir inlet frits and any pump inlet filters for clogging. Also ensure that mobile phase reservoirs are not sealed tightly or below the level of the pump inlet. Check for air bubbles in the pump inlet lines, and if found reprime the pump. Outgassing of mobile phase can produce air bubbles in the pump which result in variable flow and pressure, especially in the case of low-pressure-mixing gradient systems. Be sure all solvents have been degassed properly and recently by sonication, vacuum filtration, helium sparging (preferably continuous at very low flow rate), or by using an inline vacuum degasser. Flush and prime the pump with degassed solvent according to recommended procedure. If this fails to correct the problem, replace the pump check valves. Wear or particulate contamination can cause improper check-valve operation. Either the inlet or outlet check valves can cause the problem. Change the pump seals if seal leakage is detected or if the pressure continues to cycle or remains erratic. (Note: Seal leakage may be hidden in pumps with seal-washing head designs.)

## Retention-Time Problems

Before beginning troubleshooting procedures related to retention time changes, compare the retention times of several runs to verify that the problem is reproducible and determine if there is a pattern to the variation.

**Variable retention time** may result from changes in mobile phase flow rate, mobile phase composition, column stationary phase, and column temperature. If the retention time for the column void volume,  $t_0$ , changes and there have been no changes in the column configuration, the problem is related to flow rate. System leaks and air bubbles in the pump cause  $t_0$  to increase. To troubleshoot, follow the recommendations in the previous paragraph for low-flow problems. Increased flow rates cause  $t_0$  to decrease. Verify proper flow rate selection on the pump controller and check the pump flow rate accuracy as described in the "Low Pressure" section.

**A shift of all peaks** in one direction without a change in  $t_0$  represents a change in the mobile phase. Such changes are often caused by the reduction of at least one liquid mobile phase component due to evaporation or improper mixing procedures. When mobile phase is suspect, mix a new batch and determine if this corrects the problem. Degassing solvents using helium sparging may lead to the evaporation of volatile solvents, thus affecting the mobile phase concentration. Solvent degassing using an Inline HPLC Solvent Degasser (see listing in Instrumentation Section of this catalog) is the recommended alternative to helium sparging.

**When retention time changes for selected peaks**, the most common source of problems includes mobile phase pH and buffer concentration. Depending on the  $pK_a$ , the retention time of ionizable compounds can shift up to 10% when the mobile phase pH changes by as little as 0.1 pH unit. Remember to measure the pH of the aqueous component only, as the addition of organic solvents will alter pH readings. Buffer concentrations should be typically 20-50 mM. Lower levels may provide irreproducible results and higher levels may reduce column life.

**Thorough column equilibration** with the starting mobile phase before sample injection is essential for reproducible retention times. Determine adequate column equilibration by allowing longer equilibration times to see if the problem improves. Allow at least 10 to 15 column volumes of solvent to pass through the column before injecting a sample. When using on-line solvent

mixing instrumentation, compare isocratic results with a mobile phase that has been mixed manually. In gradient separations, exchange solvent inlet lines and make appropriate program adjustments. If the system is operating correctly, the results should remain the same in both configurations. Retention time drift may be observed during the first few samples of a given analysis. If retention times move and then stabilize after several sample injections, deactivation of the column may be taking place through sample loading onto the column. This may not be a problem if sample supply is not limited and the resultant chromatography is acceptable. As an alternative, a different type of column chemistry, changes in sample preparation procedures, or mobile phase modifiers may help eliminate this phenomenon. If mobile phase modifiers such as ion-pairing reagents are being used, drifting retention times may also be the result of incomplete column equilibration.

**HPLC column problems** such as contaminant buildup or general column deterioration can manifest as retention time drift. A reduction in column efficiency, or plate number (N), may result in broad peaks with poor retention. Column contamination that contributes to retention time drift is sometimes accompanied by changes in relative peak spacing and usually takes place over a large number of samples or over a long period of time. If a defective analytical column is suspected, replace or remove the guard column and run several more samples. If this does not correct the problem, or if a guard column is not being used, flush the analytical column with strong solvent to remove any contaminants. If the problem persists, replace the analytical column. Generally, a guard column should always be used to protect an analytical column. Periodic cleaning of the analytical column using strong solvents helps extend useful column lifetime and reduces long term contamination.

**HPLC column temperature problems** may show up as shifts in retention times that cycle with changes in ambient laboratory temperature. Retention times can change as much as 2% with each 1°C change in ambient temperature, depending on the analysis. It is difficult and expensive to control the ambient air temperature through control of the laboratory air conditioning or heating system. As a cost effective alternative, column thermostating can be used to either maintain the column at ambient temperature, or slightly elevate the column temperature above ambient. This helps to maintain reproducible retention times without affecting the chromatography. For a listing of thermostated HPLC column heaters and chillers, see the Instrumentation Section of this catalog.

**The sample and injection solvent** can also negatively impact retention times. If the sample is injected in a large volume of strong solvent, poor peak shape and irreproducible retention times may result for early eluting peaks. To avoid this problem, samples should be injected using the starting mobile phase solvent whenever possible.

**Insufficient sample preparation** can cause rapid contamination buildup on the guard and analytical columns, and this can reduce column life as well as affect retention times. Using an appropriate sample preparation technique such as solid phase extraction (SPE) may alleviate this problem.

## Baseline Noise

Baseline problems are indicated by a change in the appearance of the baseline. This change may occur even when no sample has been injected. Baseline noise can result from problems with the HPLC instrumentation, external environmental sources, or from the separation process itself. Locating the origin of the problem begins with determining which of these three factors is involved. The first step is to set the flow rate to zero and see if the problem disappears. If the problem is related to the chromatographic process, the baseline should be straight. If the problem is related to the instrument or environmental sources, the noise will continue.

**If the baseline noise is random or spiking and stops when the flow is turned off**, the problem

is most likely caused by bubbles in the detector cell or a contaminated HPLC column. Bubbles in the detector cell can often be removed by using a back-pressure regulator on the outlet of the flow cell. A slight positive pressure at the detector outlet causes gases to remain in solution until the mobile phase has cleared the detector cell. Often a short coil of small-diameter tubing at the detector outlet will suffice. Increasing flow rate also helps to purge bubbles from the detector cell by increasing the system pressure and forcing them back into solution. In either case, be careful not to exceed the maximum allowable pressure of the flow cell. Thoroughly degassing mobile phase solvents will also help prevent gas bubbles from forming.

**Cyclical baseline noise** can result from small variations in mobile phase composition generated by solvent mixing in a gradient system. This is most likely to occur when the percentage of one solvent in the programmed mixture is low, 5% or less. Observe whether the period of the baseline noise corresponds to the period of the piston cycle in the low-flow pump of a high-pressure-mixing system, or to cycling of the mixing valve to the low-percentage solvent in a low-pressure mixing system. If the noise is due to solvent mixing, it can best be eliminated by premixing the mobile phase for an isocratic separation or by revising the mobile-phase components and program to avoid gradient mixing ratios of less than 5%. Other methods for reducing compositional noise include using mobile phase components that are more transparent at the wavelength being monitored and installing a larger-volume mixing chamber which will act as a more effective filter to damp out low-frequency variations in mobile phase composition.

**A wavy baseline** is sometimes the result of contaminants or very late eluting components from the guard or analytical column. This may also result from contaminants in the mobile phase itself. Flush the column with a strong solvent to remove any contaminants, and always use pure HPLC grade water and solvents in the mobile phase.

**If the baseline noise continues under zero-flow conditions** the problem may be related to a defective detector lamp or an environmental problem. Rapid noise spikes may indicate the detector lamp needs replacement. Most UV lamps have a lifetime of about 1000 hours of use and should be replaced after this period. Baseline noise can also be caused by external environmental factors. If the noise appears at regular intervals, check for other equipment and devices that may be on the same electrical circuit and disconnect them or reroute them to other available circuits. Also, make sure the cases of all components of the HPLC system are connected to a common ground.

## Baseline Drift

Baseline drift generally results from interferences in the mobile phase, contaminant buildup on the column, or changes in temperature. **If the drift is repeatable between chromatographic runs**, then the problem is probably related to the mobile phase. In gradient chromatography, a changing baseline during the course of a run is common. This is especially true when using UV detection at low wavelengths because of differences in absorbance of the different solvents used. Attempt to select solvents where the absorbances are balanced as closely as possible.

**If the baseline continues drifting randomly**, then the problem may be coming from contamination building up on the column. If this is suspected, try flushing the column with a strong solvent. If the problem continues, replace the column and review the chromatographic and sample preparation procedures.

**If the drift is cyclic between operating sessions**, then the problem may be related to changes in ambient air temperature which affect the chromatographic separation. Changes in laboratory air conditioning and heating often affect baseline drift and the separation itself. The most effective way to correct these problems is to thermostatically control column temperature. Drift can also result from the detector itself. Be sure the detector is warmed up sufficiently before performing chromatography. Check the detector manufacturer's specification for drift, and be sure the lamp is not nearing the end of its expected lifetime.

## Peak Problems

Peak shape problems are difficult to assess and correct. Because peak shape problems may not adversely affect the resultant data, determine if the analytical results are truly compromised before troubleshooting.

**it peaks or peaks with shoulders** may be caused by a blocked frit, defective guard column, or column void. Begin by replacing the first frit downstream from the injector, as this is the most likely place for particles to accumulate. It is recommended that a 0.2 - 0.5  $\mu\text{m}$  in-line filter be placed immediately after the injector to trap mobile phase particulates. If this does not correct the problem, replace the guard column. If problems persist, it may be time to replace the column. If only one or two peaks in the chromatogram show splitting, this may be the result of a non-resolved or interfering compound. The mobile phase composition should be altered in an attempt to resolve the mystery peak. Sample preparation procedures should also be reviewed to determine if the interference can be removed prior to analysis. **Broad peaks or fronting**, often accompanied by shifts to shorter retention times indicate sample overload. Dilute the sample by a factor of 10 to 20 and reinject, making sure that the system settings have been changed accordingly. Sharper peaks throughout the chromatogram confirm a sample overloading problem. Adjust the method and sample preparation procedure to avoid similar problems in the future.

**If early eluting peaks are affected more than later peaks**, the problem may be related to extra-column effects or injection solvent problems. Extra-column effects are caused by excessive system dead volume. Inspect the system tubing to ensure that it is the proper internal diameter. Examine the flow cell to confirm that it is the proper volume, and check the data handling system for the proper sampling-rate setting. If the problem persists, check the injection solvent strength and sample size. A strong injection solvent can cause the sample to move quickly through the first portion of the column while it re-equilibrates with the mobile phase, thereby broadening or even splitting peaks. It is best to inject the sample in the starting mobile phase to avoid this type of problem. Band spreading often results from column degradation or contamination. If the column provides low efficiency, try cleaning it with a strong solvent before replacing the column. If the column is functioning properly, the problem may be related to a partially plugged in-line filter, an injector or detector problem, or incorrectly installed tubing. These problems will likely also produce other symptoms such as too-high pressure, leaks, or flow variation.

**Tailing peaks** are usually the result of secondary retention interactions between basic functional groups in the analytes (amino groups for example) and unbonded exposed silanol groups on the column packing material. Peak tailing caused by this type of interaction can be minimized by using mobile phase modifiers such as triethylamine (TEA) at low concentrations of 20 to 30 mM. Using these types of mobile phase modifiers will irreversibly alter the column and render it unusable for other assays not requiring similar modifiers. Ion-pairing reagents, sample derivatization and strong buffers can also be employed to reduce peak tailing. As an alternative, new ultrapure base-deactivated columns are now available. These columns do not require mobile phase modifiers in order to provide good peak shapes for basic compounds. See the descriptions of MS, EVEREST™, DENALI™ and GENESIS™ HPLC columns in the reversed-phase column sections of this catalog.

**Ghost peaks** are spurious peaks of unknown origin. One possible cause of ghost peaks is a difference between the amount of dissolved air in the sample and the mobile phase. This problem can be corrected by degassing both the mobile phase and sample by using an in-line vacuum degasser. When using UV detection, ghost peaks are more prevalent and typically larger at lower wavelengths. It is also useful to exchange acetonitrile (ACN) for methanol in the mobile phase since oxygen has lower solubility in ACN. Ghost peaks can also result from refractive-index effects, column contamination, mobile phase contamination, contaminated autosampler blank or

wash solution, or microbial growth in a mobile-phase component or solvent filter. Grace Vydac Application Note #9705, available on request or in electronic form in the Publications section of the Grace Vydac website ([www.gracevydac.com](http://www.gracevydac.com)) discusses these possibilities.

## **Sensitivity Problems**

Sensitivity problems relate to a change in peak heights while all other system settings and method parameters remain unchanged. When all peaks within a run change proportionately, an injection problem is likely. If only a few peak heights change, then there may be a problem with the sample preparation procedure. If all peak heights change when running standards, check the injection procedures. When using an autosampler, inspect the needle assembly and injection flow circuit for possible blockage or leaks and ensure that sufficient sample is available in the sample vial. With either manual or automatic injections, be sure to inject less than 50% of the total loop volume when performing partial loop injections. While performing flushed loop injections, be sure to overflow the loop with sample using 2- 3 times the loop volume to ensure reproducible results. With manual injection valves, check for leaks or blockage in the injection loop, rotor seal, and needle seal. Rebuild the injection valve and replace these components if problems are found. If only a few peak heights change, check sample preparation and sample storage procedures. Sample losses can occur during the extraction, evaporation, reconstitution, and transfer steps of the sample preparation process. Sample concentration can also change due to sample evaporation or degradation when samples are not properly capped and stored. The use of an internal standard prior to sample preparation may help to isolate problem steps. If problems occur for only large or small peaks, check the linear range of the detector or data system. Try injecting various sample concentrations and note the changes in these peaks. Make the appropriate changes to the method if this corrects the problem. Also, make sure the column is not overloaded. If sensitivity changes only for the first few injections, column conditioning may be occurring. Sample carryover from the injection loop can also appear as changes in sensitivity. Ensure that the loop is properly washed and that sample is not adsorbing to the sample loop or connecting tubing by making several blank injections and checking for carryover.