KNAUER

Application Note

► HPLC Troubleshooting Guide

Category	Troubleshooting
Matrix	-
Method	HPLC, UHPLC
Keywords	Troubleshooting, HPLC and UHPLC problems, column care and use
Analytes	-
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Summary

In HPLC or UHPLC numerous problem can arise. In comparison to former days, technology and instrumentation have been improved but typical problems still occur. Especially for inexperienced HPLC users but also for advanced learners, help in isolating, identifying and correcting typical problems is needed.

Every HPLC system consists of the same important components, no matter if it's a modular system or a specialized all-in-one unit. Problems can arise in each component and can affect the overall system performance. With this troubleshooting guide, we provide help for solving typical and frequently found problems in HPLC and UHPLC. Easy-to-use tables describe probable causes and solutions. To complement this troubleshooting guide, we have added column usage and column care guidelines for silica-based (Eurospher, Eurospher II, Eurosil Bioselect) and polymer-based materials (Eurokat) and especially for BlueOrchid silica-based UHPLC columns.

Contents

Summary of HPLC problems

In an HPLC or UHPLC system, problems can arise from many sources. The best way is to first define the problem and then to isolate the source. In table 1 we offer a tool for determining which components may be causing the trouble. A following process of elimination will enable to pinpoint the specific cause and to correct the problem.

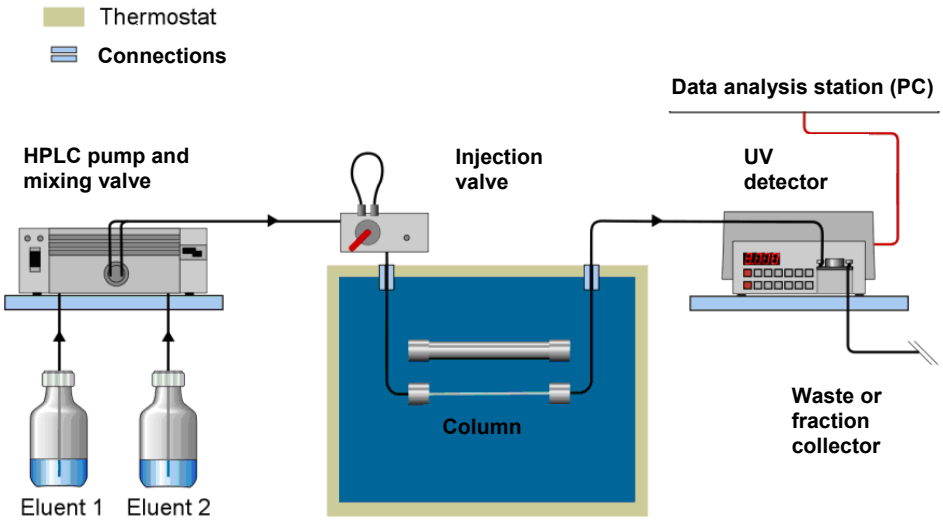


Figure 1: Components of a KNAUER HPLC system equipped with LPG

Mobile phase problems

Problems that often occur in HPLC are low sensitivity and drift, noise or spikes in the chromatogram. These phenomena can often be attributed to problems with the mobile phase. Contaminants in the eluent are especially troublesome in gradient elution. The baseline may rise, and spurious peaks can appear as the level of the contaminated component increases. Water is the most common source of contamination in reversed phase analyses. You should only use high purity deionised (DI) water when formulating mobile phases. However, several common deionizers introduce organic contaminants into the water. To remove these contaminants, pass the deionised water through activated charcoal or a preparative C18 column. Use only *HPLC grade* solvents, salts, ion pair reagents, and base and acid modifiers. Cleaning lower quality solvents is time consuming and trace levels of contaminants often remain and can cause problems when you use a high sensitivity ultraviolet or fluorescence detector. Because many aqueous buffers promote the growth of algae or bacteria, you should discard cloudy buffers and prepare them freshly. Prevent micro organism growth by adding about 100 ppm of sodium azide to aqueous buffers. Alternatively, these buffers may also be mixed with 10 to 20 % or more of an organic solvent such as methanol, ethanol or acetonitrile. To prevent bubbles in the system, degas the mobile phase before use. We recommend using a constantly working degasser unit. Filtering the mobile phase through a 0.2 or 0.45 µm filter using a vacuum filtration apparatus eliminates dissolved gas (see table 1). This will also remove particles that could produce noisy baselines or plug the column. Use ion pair reagents carefully. The optimum chain length and concentration of the reagent must be determined for each analysis. In general, increasing the concentration or chain length increases retention times. We recommend using concentrations of 0.2 to 10 mM. High concentrations (>50%) of acetonitrile and some other organic solvents can precipitate ion pair reagents. Also, some salts of ion-pair reagents are insoluble in water and will precipitate. This can be avoided by using sodium-containing buffers in the presence of long chain sulfonic acids (e.g. sodium dodecyl sulfate), instead of potassium-containing buffers. Volatile basic and acidic modifiers, such as triethylamine (TEA) and trifluoroacetic acid (TFA) are useful when you wish to recover a compound for further analysis. These modifiers also let you avoid problems associated with ion pair reagents. They can be added to the buffer at concentrations of 0.1 to 1.0% TEA and 0.05 to 0.15% TFA. Increasing the concentration may improve peak shape for certain compounds, but can alter retention times.

Pump problems

The HPLC pump must deliver a constant flow of solvent to the column over a wide range of conditions. KNAUER HPLC pumps incorporate a dual piston design. Pumping system problems are usually easy to spot and correct. Some of the more common symptoms are erratic retention times, noisy baselines, or spikes in the chromatogram. Leaks at pump fittings or seals will result in poor chromatography. A sure sign of a leak is a build up of salts at a pump connection. Buffer salts should be flushed from the system daily with fresh DI water. Run the HPLC system constantly at low flow rates (e.g. 0.1 ml/min) to avoid crystallization effects. To isolate and repair specific problems related to your HPLC system, use the troubleshooting and maintenance sections of the operation manual. Pump seals require periodic replacement. You should perform regular maintenance rather than waiting for a problem to occur. Other locations where problems can occur are the check valves in the pump head. You see it for example when the pump is not able to produce a constant flow/pressure. If this happens, clean the check valves with isopropanol for example. If this does not work, dismantle the check valves and clean them in an ultrasonic bath using isopropanol for example. Then refit the check valves in the pump head. Be sure that the valves are inserted in the right direction. If this procedure is not successful, replace check valves.

Highly concentrated salts and caustic mobile phases can reduce pump seal efficiency. In some cases, prolonged use of ion pair reagents has a lubricating effect on the pump pistons that may produce small leaks at the seal. Some seals do not perform well with certain solvents. Before using a pump under adverse conditions, read the instrument manufacturer's specifications. To replace seals, refer to the maintenance section of the pump manual.

Injector/injection problems

The injector rapidly introduces the sample into the system with minimal disruption of the solvent flow. HPLC systems currently use variable loop, fixed loop, and syringe-type injectors. Mechanical problems involving the injector (e.g., leaks, plugged capillary tubing, worn seals) are easy to spot and correct. Use a column filter unit to prevent plugging of the column frit due to physical degradation of the injector seal. Variable peak heights, split peaks and broad peaks can be caused by incompletely filled sample loops, incompatibility of the injection solvent with the mobile phase, or poor sample solubility. Whenever possible, dissolve and inject samples in the mobile phase. Otherwise, be sure the injection solvent is of lower eluting strength than the mobile phase. Be aware that some autosamplers use separate syringe wash solutions. Make sure that the wash solution is compatible with and weaker than the mobile phase. This is especially important when switching between reversed phase and normal phase analyses.

Column protection

Although not an integral part of most equipment, mobile phase inlet filters, pre-injector and pre-column filters, saturator columns, and guard columns greatly reduce problems associated with complex separations. We recommend that all samples are filtered through 0.45 µm or 0.2 µm syringe filters. The use of integrated precolumns is also strongly recommended. Filters and precolumns prevent particles and strongly retained compounds from accumulating on the analytical column. Silica particles in a saturator column dissolve in high pH mobile phases, protecting the silica based packing in the analytical column. The useful lifetime of these disposable products depends on mobile phase composition, sample purity, pH, etc. KNAUER columns are produced in many different sizes and designs. A wide range of packing materials is available for a variety of applications. Please see our column products on the KNAUER website (www.knauer.net). The most common problem associated with analytical columns is deterioration. This is true regardless of whether the column contains a bonded reversed phase or normal phase, ion exchange, affinity, hydrophobic interaction, size exclusion, and resin/silica based packing material. Symptoms of deterioration are poor peak shape, split peaks, shoulders, loss of resolution, decreased retention times, and high back pressure. These symptoms indicate that contaminants have accumulated on the frit or column inlet, or there are voids, channels, or a depression in the packing bed. Deterioration is more evident in higher efficiency columns. For example, a 3 micron packing retained by 0.5 micron frits is more susceptible to plugging than a 5 or 10 micron packing retained by 2 micron or larger frits. Proper column protection and sample preparation are essential to getting the most from each column. Overloading a column can cause poor peak shape and other problems. Column capacity depends on many factors, but typical values are:

Analytical column (250 mm x 4 mm) 0.02 - 2.0 mg
Semi-preparative column (250 mm x 8 mm) 0.08 - 8.0 mg
Preparative column (250 mm x 20 mm) 0.5 – 50.0 mg

Column end fitting problems

Leaks are a common problem in HPLC and UHPLC analyses. To minimize leaks in the system, avoid interchanging hardware and fittings from different manufacturers. Incompatible fittings can be forced to initially fit but repeated connections may leak. If interchanging is absolutely necessary, use appropriate adapters and check all connections for leaks before proceeding. Another occurring problem when hardware is interchanged is the occurrence of additional dead volume (see fig. 2). Especially in UHPLC dead volume has to be minimized to obtain the high performance of the system.

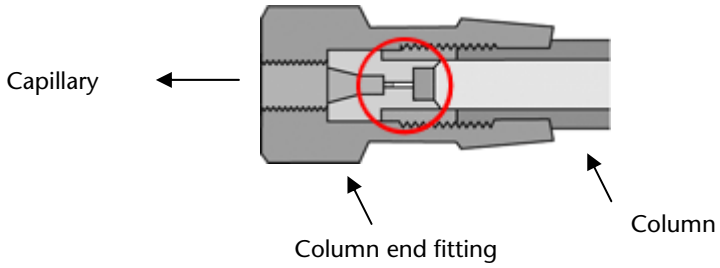


Figure 2: Column End Fitting

A clogged column inlet is another common HPLC problem. To minimize this problem from the start, use a guard column. To clean the inlet, first disconnect and reverse the column. Connect it to the pump (but not to the detector!), and pump solvent through at low flow rates (0.5 ml/min). About 100 ml of solvent should be sufficient to dislodge small amounts of particulate material from the inlet frit. If this does not work, extend the flow rate carefully to about 1 ml/min. Evaluate the performance of the cleaned column using the test mixture supplied.

Special recommendations for LC/LC - coupling of columns

Coupling of columns becomes more and more important as it is a useful tool for complex separation problems. In a conventional HPLC or UHPLC system, tandem LC can be easily arranged without any additional equipment. But there are some facts that have to be observed: Especially in UHPLC the minimization of dead volume is really important. When two columns are coupled, normally an additional capillary is needed to connect them. It is obvious that the capillary applied has to be as short as possible and with the smallest inner diameter acceptable. If you do not account for this, the separation from the first column may get mixed again in the capillary. To prevent this counterproductive issue, keep especially here capillaries as short as possible with small inner diameter (0.12 mm ID for example is typical for UHPLC).

Detector problems

A number of different detectors is available for HPLC systems. The most common are fixed and variable wavelength ultraviolet spectrophotometers, refractive index, and conductivity detectors. Electrochemical and fluorescence detectors are less frequently used since they are more selective. Detector problems fall into two categories – electrical and mechanical/optical. For electrical problems, you should contact the instrument manufacturer. Mechanical or optical problems can usually be traced to the flow cell. Detector-related problems include leaks, air bubbles, and cell contamination. These usually produce spikes, baseline noise or drift in the chromatograms or low sensitivity. Some flow cells – especially those used in refractive index detectors – are sensitive to pressure. Flow rates or back pressures that exceed the manufacturer's recommendation will break the cell window. Old or defective lamps as well as incorrect detector rise time, gain, or attenuation will reduce sensitivity and peak height. Faulty or reversed cable connections can also be the source of problems.

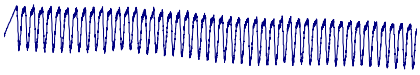
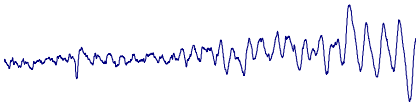
Further recommendations

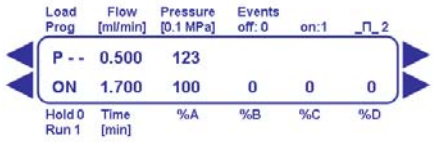
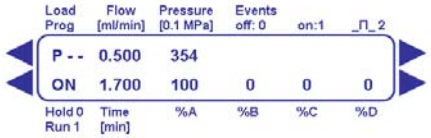
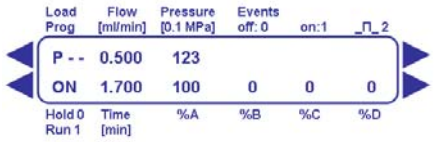
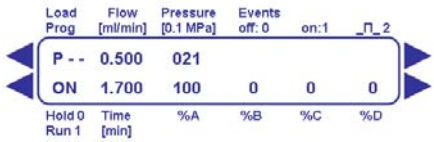
The HPLC troubleshooting table provides a systematic approach to isolate and correct common HPLC problems. We also suggest referring to the maintenance and troubleshooting sections of your instrument manual. For persistent problems relating to the KNAUER HPLC system or column, please contact our Technical Service Department or the KNAUER Column and Application Department. Finally, phone +49 (0)30-809727-0 to request additional literature about KNAUER HPLC and column products or visit our website: www.knauer.net for immediate access to all our free application and technical literature.

Problem Index

Table 1 – HPLC troubleshooting

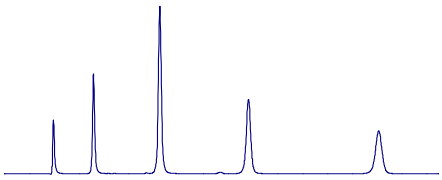
Problem	Probable cause	Troubleshooting
Problem No. 1: Baseline drift		
Regular: Problem:	1. Fluctuation of column temperature. (Even small changes cause cyclic baseline rise and fall. RI- and conductivity detectors and UV-detectors at high sensitivity are most often affected.)	1. Control temperature of column and mobile phase, use heat exchanger before detector.
	2. Mobile phase is inhomogeneous. (Drift usually to higher absorbance, rather than cyclic pattern from temperature fluctuation.)	2. Use HPLC grade solvents, high purity salts and additives. Degas mobile phase before use and apply a degasser or expel other gases by constantly bubbling the solvents with helium.
	3. Contaminant or air buildup in detector cell.	3. Flush cell with methanol or other strong solvent. If necessary clean cell with 1 N HNO_3 (never with HCl).
	4. Plugged outlet line after detector. (High pressure breaks cell window, producing noisy baseline.)	4. Unplug or replace line. Refer to detector manual to replace window.
	5. Mobile phase mixing problem or change in flow rate.	5. Correct composition/flow rate. Routinely monitor composition and flow rate to avoid problem.
	6. Slow column equilibration, especially when changing mobile phase.	6. Flush column with intermediate strength solvent, run 10-20 column volumes of new mobile phase through column before analysis.
	7. Mobile phase contaminated, deteriorated or prepared from low quality materials.	7. Check make-up of mobile phase.

	8. Strongly retained materials in sample (high k´) can elute as very broad peaks and appear to be a rising baseline. (Gradient analyses can aggravate problem.)	8. Use guard column. If necessary flush column with strong solvent between injections or periodically during analysis.
	9. Mobile phase recycled but detector not adjusted.	9. Reset baseline. Use new materials when dynamic range of detector is exceeded.
	10. Detector (UV) not set at absorbance maximum but at slope of curve.	10. Change wavelength to UV absorbance maximum.
	11. At higher lab temperatures (28°C) more baseline instabilities comparing to lower lab temperatures (22°C) when using ACN/Water or –buffer gradients and mixtures.	11. Higher temperatures can enhance the polymerization of ACN resulting in building of polymers. Filtration of ACN-eluent with Empore SDB-XC Polystyroldivinylbenzol filter.
Problem No. 2: Baseline noise - regular		
Regular:	1. Air in mobile phase, detector cell or pump.	1. Degas mobile phase. Flush system to remove air from detector cell or pump.
	2. Incomplete mobile phase mixing.	2. Mix mobile phase by hand or use less viscous solvent.
	3. Temperature effect (column at high temperature, detector unheated.)	3. Reduce differential or add heat exchanger.
Problem:	4. Pump pulsations.	4. Clean or exchange check valves of the pump head. If problem still persists, incorporate pulse dampener into system.
	5. Pressure close to maximum.	5. Minimize back pressure by reducing flow rate or heating the column.
Problem No. 2: Baseline noise - irregular		
Regular:	1. Leak.	1. Check system for loose fittings. Check pump for leaks, salt build-up and unusual noises. Change pump seals if necessary.
	2. Gradient mode: Mobile phase contaminated, deteriorated or prepared from low quality materials.	2. Check make-up of mobile phase.
	3. Detector electronics.	3. Isolate detector electronically. Refer to instruction manual to correct problem.
Problem:	4. Air trapped in system.	4. Flush system with strong solvent.
	5. Air bubbles in detector.	5. Purge detector. Install back pressure device after detector. Check the instrument manual, particularly for RI-detectors. Excessive backpressure can cause the flow cell to crack.

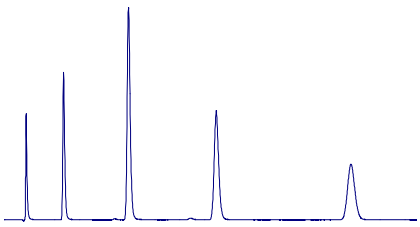
	6. Detector cell contaminated. (Even small amounts of contaminants can cause noises.)	6. Clean cell.
	7. Weak detector lamp.	7. Replace lamp.
	8. Column leaking packing material.	8. Replace column and clean the system.
Problem No. 3: Column back pressure too high/higher than usual		
Usual: 	1. Problem in pump, injector, in-line-filter or tubing.	1. Disconnect column from system and replace with unions 0.010'' ID or larger tubing to reconnect the injector to the detector. Run pump at high flow rate (2 - 5 ml/min). If pressure is minimal, see cause 2. If not, isolate cause by systematically eliminating system components. Start with the detector and work back to the pump.
Problem: 	2. Obstructed column.	2. Remove guard column if present and check pressure. Replace guard column if necessary. If column is obstructed, reverse and flush the column while disconnected from the detector. If problem persists, use appropriate restoration procedure. If problem still persists, replace column.
	3. Wrong mobile phase.	3. Check mobile phase. Check make-up of mobile phase: Even small changes in composition can affect back pressure.
Problem No. 4: Column back pressure too low		
Usual: 	1. Leak.	1. Check the system for loose fittings. Check the pump for leaks, salt build up and unusual noises. If necessary, change the pump seals.
	2. Mobile phase flow interrupted or obstructed.	2. Check mobile phase level in reservoirs. Check flow throughout the system. Especially examine sample loop for obstruction or air lock. Make sure that mobile phase components are miscible and that the mobile phase is degassed.
Problem: 	3. Air trapped in pump head, revealed by pressure fluctuations.	3. Disconnect tubing at column inlet and check for flow. Purge pump at high flow rate (e.g. 10 ml/min), prime system if necessary.
	4. Leak at column inlet end fitting.	4. Reconnect column and pump solvent through column. If pressure is still low check for leaks at column inlet and end fitting.

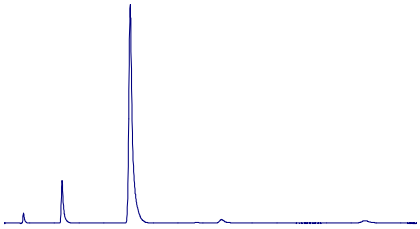
	5. Air trapped elsewhere in system.	5. Disconnect column and purge system. Reconnect column. If problem still persists, flush system with 100 % methanol or isopropanol.
	6. Worn pump seal causing leaks around pump head.	6. Replace seal. If problem persists, replace piston and seal.
	7. Wrong mobile phase.	7. Check mobile phase. Check make-up of mobile phase: Even small changes in composition can affect back pressure.

Problem No. 5: Ghost peak (Carry over peak)

Previous sample:  Regular (Blank): Problem (Blank): 	1. Contamination in injector or column.	1. Flush injector between analyses. If necessary, run strong solvent through column to remove late eluters. Include final wash step in gradient analyses to remove strongly retained compounds. Whenever it is possible, use the mobile phase as injector flushing solvent. Also carryover of the flushing solvent can cause ghost peaks.

Problem No. 6: Change in peak height for one or more peaks

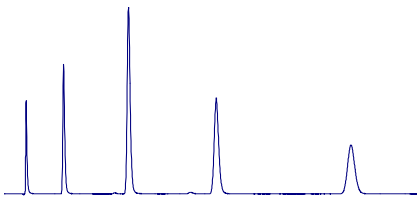
Regular:  Problem:	1. One or more sample components deteriorated or column activity changed.	1. Use fresh sample or standard to confirm sample as source of problem. If some or all peaks are still smaller than expected, replace column. If new column improves analysis, try to restore the old column, following appropriate procedure. If performance does not improve, discard old column.
	2. Changes in the sample preparation process. Differences in the matrix can affect peak heights.	2. Check sample preparation process and eliminate matrix effects as cause of problem.
	3. Leak, especially between injector port and column inlet. (Retention also would change.)	3. Check system for loose fitting. Check pump for leaks, salt build-up, and unusual noises. Change pump seals if necessary.



4. Inconsistent sample volume.
   4. Be sure samples are consistent. For fixed volume sample loop, use 2-3 times loop volume to ensure the loop is completely filled. Be sure automatic sampler vials contain sufficient sample. Check syringe-type injectors for air. In systems with wash or flushing step, be sure wash solution does not precipitate sample components.
5. Detector or detector setting changed.
   5. Check settings.
6. Weak detector lamp.
   6. Replace lamp.
7. Contamination in detector cell.
   7. Clean cell.

Problem No. 7: No peaks/Very small Peaks

Regular:

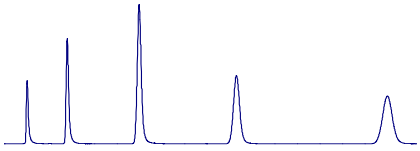


Problem:

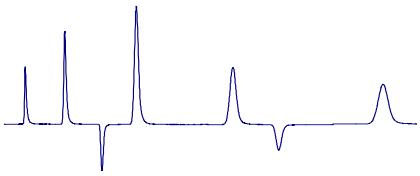
1. Detector lamp off.
   1. Turn lamp on.
2. Loose/broken wire between detector and Computer.
   2. Check electrical connections
3. No mobile phase flow.
   3. Start pump. Check mobile phase level in reservoir(s). Check flow throughout system. Examine sample loop for obstruction or air lock. Make sure mobile phase components are miscible and mobile phase is properly degassed. Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary. Disconnect tubing at column inlet. Check for flow. Purge pump at high flow rate (e.g. 5 ml/min), prime system if necessary (prime each pump head separately). If system has check valve, loosen valve to allow air to escape. If problem persists, flush system with 100% methanol or isopropanol. If problem still persists, contact system manufacturer.
4. No sample/deteriorated sample.
   4. Be sure automatic sampler vials have sufficient liquid and injector valve works well. Evaluate system performance with fresh standard to confirm sample as source of problem.
5. Settings too high on detector/Software.
   5. Check attenuation or gain settings.

Problem No. 8: Negative peaks

Regular:



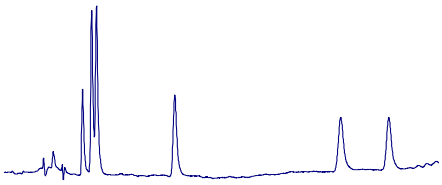
Problem:



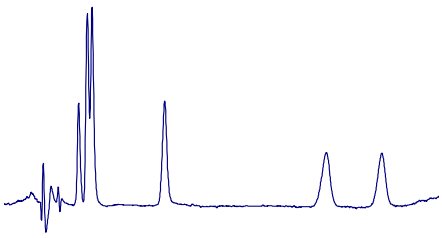
1. Recorder/analog signal lead reversed.	1. Check polarity.
2. Refractive Index of solute less than of mobile phase (RI detector).	2. Use mobile phase with lower refractive index or reverse recorder leads.
3. Sample solvent and mobile phase differ greatly in composition (UV-detector).	3. Adjust or change sample solvent. Dilute sample in mobile phase whenever possible.
4. Mobile phase more absorptive than sample components to UV wavelength (vacancy peaks).	4. Change UV wavelength or use mobile phase that does not adsorb chosen wavelength.

Problem No. 9: Loss of resolution

Regular:



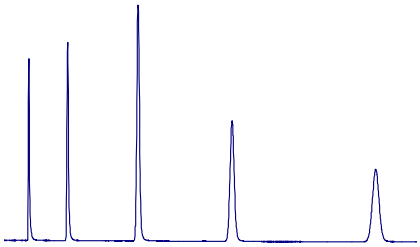
Problem:



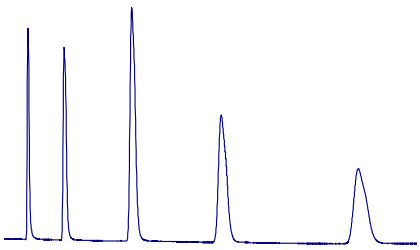
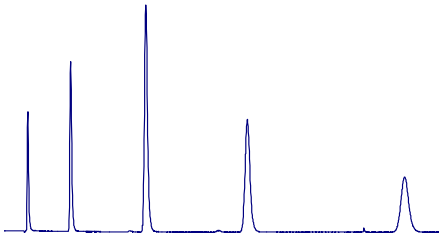
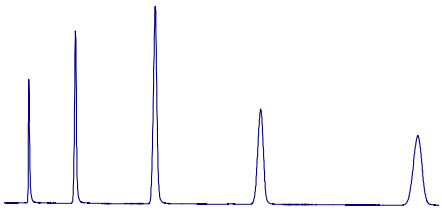
1. Mobile phase contaminated/ deteriorated (causing retention times to change).	1. Check make-up of mobile phase.
2. Obstructed guard or analytical column.	2. If present, remove guard column and attempt analysis. Replace guard column if necessary. If analytical column is obstructed, reverse and flush. If problem persists, column may be clogged with strongly retained contaminants. Use appropriate restoration procedure. If problem still persists, change column.

Problem No. 10: Broad peaks

Regular:

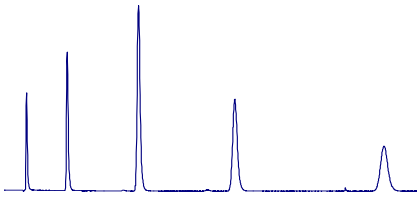


1. Mobile phase composition changed.	1. Prepare new mobile phase.
2. Mobile phase flow rate too low. Not in the optimum of van Deemter curve.	2. Adjust flow rate.
3. Leak, especially between column and detector. Peaks will be broad with lower peak height!	3. Check system for loose fittings. Check pump for leaks, salt build-up and unusual noises. Change pump seals if necessary.
4. Detector settings incorrect.	4. Adjust settings

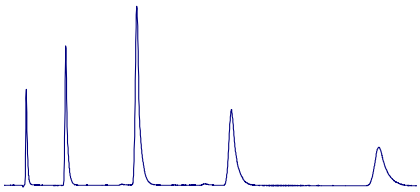
Problem: 	5. Extra-column effects: a Column overloaded. Peaks will be broad with high peak height. b Detector response time or cell volume too large. c Tubing between column and detector too long or ID too large. d Response time of the software too high.	5. Comments: a Inject smaller volume or dilutions of sample (e.g. 1:10, 1:100). b Reduce response time or use smaller cell. c Use as short a piece of 0.007 – 0.010'' ID tubing as practical. d Reduce response time.
	6. Buffer concentration too low. Peaks will be broad without tailing etc.	6. Increase concentration.
	7. Guard column contaminated.	7. Replace guard column.
	8. Column contaminated/worn out.	8. Replace column with new one of same type. If new column provides symmetrical peaks, flush old column and retest.
	9. Void at column inlet.	9. Replace column.
	10. Peak represents two or more poorly resolved compounds.	10. Change column type to improve separation.
	11. Column temperature too low.	11. Increase temperature. Do not exceed recommended temperature (45 °C for Eurospher and 90 °C for Eurokat).
Problem No. 11: Fronting peaks		
Regular: 	1. Interference in sample.	1. Check column performance with standards.
	2. Shoulder or gradual baseline rise before a main peak may be another sample component.	2. Increase efficiency or change selectivity of system to improve resolution. Try another column type if necessary.
	3. Column overloaded.	3. Inject smaller volume or dilutions (e.g. 1:10 or 1:100) of sample.
	4. Sample solvent incompatible with mobile phase.	4. Adjust solvent: Whenever possible, inject samples solved in mobile phase. Flush polar bonded phase column with 200 ml HPLC grade ethyl acetate, then with intermediate polarity solvent prior analyses.
Problem: 		

Problem No. 12: Tailing Peaks

Regular:



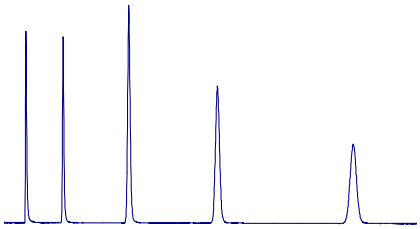
Problem:



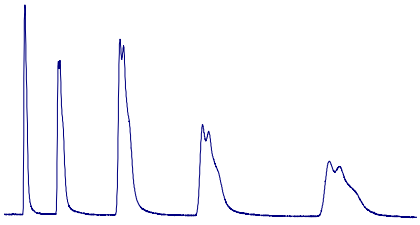
1. Sample reacting with active sites.	1. First check column performance with standards. If results for test mix are good, add salts or competing base or acid modifier.
2. Wrong column type.	2. Try another column type (e.g. endcapped column for basic compounds).
3. Wrong mobile phase pH.	3. Adjust pH. For basic compounds, higher pH usually provides more symmetric peaks.
4. Wrong injection solvent.	4. Peaks can tail when sample is injected in stronger solvent than mobile phase. If possible, dissolve sample in mobile phase.
5. Small void at column inlet.	5. Repack top of column with particles of same bonded phase functionality. Continue using column in reversed flow direction.

Problem No. 13: Split peaks

Regular:



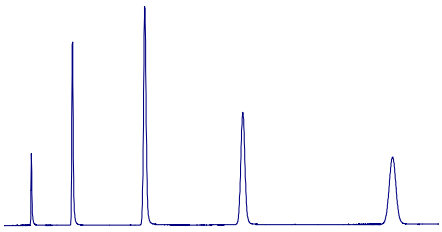
Problem:



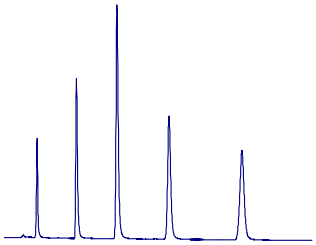
1. Contamination on column or guard column.	1. Remove guard column if present and attempt analysis. Replace guard column if necessary. If analytical column is obstructed, reverse and flush. If problem persists, column may be clogged with strongly retained contaminants. Use appropriate restoration procedure. If problem still persists, replace column.
2. Sample solvent incompatible with mobile phase.	2. Adjust solvent. Whenever possible, inject samples in mobile phase.
3. Small void at column inlet.	3. Repack top of column with particles of same bonded phase functionality. Continue using column in reversed flow direction.
4. Partially blocked frit.	4. Replace frit.
5. Column bed is broken.	5. Replace column.

Problem No. 14: Variable retention times

Regular:



Problem:



1. Leak.	1. Check system for loose fittings. Check pump for leaks, salt build-up, and unusual noises. Change pump seals if necessary.
2. Change in mobile phase composition. Small changes can lead to large changes in retention times.	2. Check make-up of mobile phase. If mobile phase is machine mixed, hand mix and supply from one reservoir.
3. Air trapped in pump. Retention times increase and decrease at random times.	3. Purge air from pump head or check valves. Change pump seals if necessary. Be sure mobile phase is degassed.
4. Column temperature fluctuations.	4. Use reliable column oven or insulate column.
5. Column overloading. Retention time usually decreases as mass of solute injected on column exceeds column capacity.	5. Inject smaller volume or dilutions of the sample.
6. Sample solvent incompatible with mobile phase.	6. Adjust solvent. Whenever possible, inject samples in mobile phase.
7. Column problem. Not a common cause of erratic retention. As a column ages, retention times gradually decrease.	7. Substitute new column of same type to confirm column as cause. Discard old column if restoration procedures fail.

Recommended HPLC/UHPLC accessories

	Part Number	Description
Spare parts	A1071	HPLC Standard Accessory Kit
	A0910	HPLC Start-up Kit, analytical
	A1039	HPLC Start-up Kit, biocompatible
	A0130	HPLC 1/16" stainl. steel capillaries ID 0.10 mm (300 cm length)
	A0131	HPLC 1/16" stainl. steel capillaries ID 0.25 mm (300 cm length)
	A0132	HPLC 1/16" stainl. steel capillaries ID 0.50 mm (300 cm length)
	A0685	HPLC 1/16" PEEK capillaries ID 0.25 mm (150 cm length)
	A0691	HPLC 1/16" PEEK capillaries ID 0.50 mm (150 cm length)
	A0692	HPLC 1/16" PEEK capillaries ID 0.70 mm (150 cm length)
	A0569	Tube cutter for plastic tubes and PEEK capillaries
	A0809	Cutting pliers for 1/16" stainl. steel capillaries
	A64383	HPLC Plus flexible stainl. steel capillary kit (OD 0.5 mm; ID 0.25 mm)
	A64382	UHPLC PLATINblue flexible stainl. steel capillary kit (OD 0.5 mm; ID 0.12 mm)
	A64460	PEEK fitting for HPLC Plus and UHPLC PLATINblue capillaries (OD 0.5 mm) 20 pc.
Sample loops	A0331	HPLC 5 µl sample loop, stainless steel
	A0561	HPLC 10 µl sample loop, stainless steel
	A0562	HPLC 20 µl sample loop, stainless steel
	A0563	HPLC 50 µl sample loop, stainless steel
	A0564	HPLC 100 µl sample loop, stainless steel
	A64716	UHPLC Sample loop 1 µl 1/32" with nuts, stainless steel
	A64717	UHPLC Sample loop 2 µl 1/32" with nuts, stainless steel
	A64718	UHPLC Sample loop 5 µl 1/32" with nuts, stainless steel
	A64719	UHPLC Sample loop 10 µl 1/32" with nuts, stainless steel
System suitability	A66110	UHPLC PQ test kit (including column and test solution Y10171)
Column performance (Test solutions)	Y1014	Test solution for RP columns (alkyl benzoates, 5 compounds)
	Y1015	Test solution for NP columns (Benzene, Nitrobenzene)
	Y1017	Test solution for RP columns (Thiourea, Benzene,Naphthalene)
	Y10171	Test solution for RP columns (Uracil, Naphthalene, Fluorene, Anthracene)
	Y10172	Test solution for UHPLC-RP-Columns (Theophyline, p-Nitroaniline, Methyl Benzoate, Phenetole, o-Xylene)
Column protection	AZ0109XA	Solvent Inline Filter, stainl. steel, recommended for HPLC columns
	A0015	Set of spare parts for A0109 (6 sieves 3 µm; 6 sieves 7 µm; 12 glass filters, 6 PTFE gaskets)
	A0037-2	Precolumn holder Vertex Plus (5 x 3-4.6mm ID)
	B1	UHPLC solvent inline filter with 5 filter pc.
	B100	UHPLC BlueOrchid precolumn holder (for 10x2 mm precolumns)
	B101	UHPLC Bluespher precolumn holder (for 5x2 mm precolumns)
Solvent filtration	A0950	HPLC solvent vacuum filtration unit (made of glass)
	A0951	PTFE membrane filter, 47 mm OD, pore size 0.45 µm (100 pc.)
	A0917	reg. cellulose membrane filter, 47 mm OD, pore size 0.45 µm
	A1149	HPLC PTFE 5 µm mobile phase filter with tubing
Sample syringe filtration	A3366	UHPLC stainl. steel 2 µm mobile phase filter
	A0693	disposable polypropylene syringe 5 ml 12 pc.
	A1036	disposable polypropylene syringe 2 ml 50 pc
	A1264	Syringe filter polypropylene-reinforced PTFE 0.45 µm 10 µl 500 pc.
	A0960	Syringe filter polypropylene-reinforced cellulose 0.45 µm 10 µl 500 pc

Column care and use
Silica based phases (Eurospher, Eurospher II)

The proper care of an HPLC column is extremely important for the lifetime of the column and, consequently, for the quality of your HPLC analysis. The following pages will give you some guidelines for the use, cleaning and storage of HPLC columns. These guidelines will depend on the nature of the chromatographic support (silica, polymers or others) and on the surface chemistry of the corresponding stationary phase.

General guidelines

Silica is the ideal support for HPLC columns. It offers good mechanical stability, excellent physicochemical surface properties, a wide range of bonding chemistry and is compatible with a broad range of organic solvents. However, the following points are extremely important when working with silica based HPLC columns.

pH stability

In general silica based HPLC columns are stable within a pH range of 2 to 8. When measuring pH, the measurement should be done in the aqueous media before mixing the eluent with organic solvents. This will give a more accurate and consistent measurement of pH than taking a measurement in a mixed aqueous/organic media. Some modern HPLC columns can be used outside that pH range. New bonding chemistry allows for operating as low as pH 1 with some stationary phases. However, you should check vendor's product information first before using silica based column outside the pH range of 2 to 8. Stationary phases based on ultra pure silica gel can also be used at a pH as high as 11, depending on the chemical nature of the modifier used in the mobile phase. Large bases (such as pyrolidine) are not able to attack the surface of the silica and, therefore, can be used as mobile phase modifiers when higher pH values are required. If you are working at pH values above 8 using small bases as the modifier (such as ammonia), we highly recommend using stationary phases based on polymers or zirconium dioxide.

mechanical stability

Stationary phases based on silica are mechanically very stable, and well-packed columns can be used at more than 40 MPa (6000 psi) without any problem. However, pressure shocks to the column should be avoided. Pressure shocks can lead to channeling in the bed column, which may result in peak splitting in the corresponding chromatogram.

mobile phases (eluents)

Silica based stationary phases are compatible with all organic solvents in the above mentioned pH range. For best results, the highest quality solvents available, such as HPLC grade solvents, should be used. Also, all prepared buffers should be filtered through a 0.45 µm filter before using them in your HPLC system. Always keep in mind that your column will collect any particulate material that enters the flow stream.
The use of non-pure solvents in HPLC causes irreversible adsorption of impurities on the column head. These impurities block adsorption sites, change the selectivity of the column and eventually lead to peak splitting in the chromatogram. In gradient elution, they cause so-called "ghost peaks". "Ghost peaks" are peaks that always appear at the same position in the chromatogram. Their origin is not the sample, but the impurities from the solvents or solvent additives. Therefore, it is highly recommended to run a gradient without injecting a sample at the beginning of each method to determine if ghost peaks will be a problem.
To avoid irreversible adsorption at the head of the column, you should always use a precolumn. The use of a precolumn increases the lifetime of a column dramatically. In addition to that, a precolumn can filter particulate material coming from pump seals or injection rotors. An alternative to a precolumn is an in-line filter. These filters are placed between the column and the injector and newer versions can be mounted directly on columns. These filters are great for removing particulate material from the eluent, but they will not take the place of precolumns by removing organic impurities that may irreversibly adsorb to the column.

Proper storage of silica based HPLC columns

- Silica based columns should be stored in an aprotic solvent. The best solvent for storage of RP packings (C18, C8, C4, C1, C30, CN, NH_2 and Phenyl) is acetonitrile/water. The water content should not be greater than 50%. The best solvent for storage of NP packings (Silica, Diol, Nitro, Cyano and Amino) is hexan/isopropanol 90:10 (v/v).

- Caution! Even for short-term storage, flush out all buffer solution from the column to prevent algal growth. Make sure that all buffers are washed out of the column before exchanging aqueous mobile phases by organic solvents. Buffer salts are not soluble in acetonitrile and can block capillary tubing and the column.

Equilibration time

The equilibration time of a column depends on the column dimensions. In general, a column is equilibrated after 20 column volumes are flushed through it. The equilibration times for the most important column dimensions are summarized in the following table. You can reduce the equilibration time by simply increasing the flow rate. However, make sure to flush the column with at least 20 column volumes to make sure the column is equilibrated.

Column Dimension	Column Volume [ml]	Flow Rate [ml/min]	Equilibration Time [min]
250 x 4.6 mm	2.91	1.00	58
150 x 4.6 mm	1.74	1.00	35
100 x 4.6 mm	1.16	1.00	23
50 x 4.6 mm	0.58	1.00	12
250 x 4.0 mm	2.20	1.00	44
125 x 4.0 mm	1.10	1.00	22
250 x 2.0 mm	0.55	0.25	44
150 x 2.0 mm	0.33	0.25	26
50 x 2.0 mm	0.11	0.25	9

Regeneration of a column

Impurities from the sample or mobile phase can adsorb to the head of a column and cause changes in selectivity or peak splitting. Often these "dirty columns" can be regenerated by applying the following protocols:

Regeneration of RP packings
(C18, C8, C4, C1, C30, CN and Phenyl stationary phases):

- Flush the column with 20 column volumes of Water
- Flush the column with 20 column volumes of Acetonitrile
- Flush the column with 5 column volumes of Isopropanol
- Flush the column with 20 column volumes of Heptane
- Flush the column with 5 column volumes of Isopropanol
- Flush the column with 20 column volumes of Acetonitrile

Regeneration of NP packings
(Silica, Diol, Nitro, Cyano and Amino stationary phases):

- Flush the column with 20 column volumes of Heptane
- Flush the column with 5 column volumes of Isopropanol
- Flush the column with 20 column volumes of Acetonitrile
- Flush the column with 20 column volumes of Water
- Flush the column with 20 column volumes of Acetonitrile
- Flush the column with 5 column volumes of Isopropanol
- Flush the column with 20 column volumes of Heptane

Column Care and Use - Polymer based phases (Eurokat®)

Eurokat is a sulfonated cross-linked styrene-divinylbenzene copolymer. This particular cation exchanger is characterized by 6% (8%) cross-linking and a very high density of functional groups. In contrast to silica materials, polymer resins are extremely stable in aqueous media over the complete pH range. This is one striking advantage compared with silica where lifetime, especially in the higher pH range, is limited.
Eurokat is available in three different ionic species (H, Ca, Pb). Eurokat H (8 % crosslinking) can be used for the determination of organic acids and complex mixtures of acids, carbohydrates and alcohols, as well as sugar alcohols. Eurokat Ca and Pb (6% crosslinking) are suitable predominantly for carbohydrate analysis. Higher carbohydrates (DP > 4) are completely excluded from the pores. In order to preserve the highest possible performance of your Eurokat column, the following points should be followed:

Column maintenance tips

- The maximum pressure limit during operating should not be exceed 100 bar.
- Forceful mechanical handling (bumps, shocks) as well as sudden temperature changes should be strictly avoided to conserve the homogeneity of the packed column bed.
- Water used in preparation of the mobile phase should be either fresh double-distilled or HPLC-grade.
- All reagents used in sample preparation (solvents, reference compounds, etc.) should be of p.a. grade. Particulate matter and precipitates must be removed from the sample by filtration before injection.
- Changes in column temperature should only be undertaken under continuous eluent flow. As a principle, drastic temperature changes should always be carried out in gradual steps.
- The optimal temperature range for the analysis of carbohydrates is between 60 and 90°C. It is additionally recommended that the complete HPLC system be maintained at this temperature and at a low flow rate (e.g. 0.1 ml/min) when not in use.
- Flow rate changes should also only be carried out stepwise. Optimal flow rates are typically between 0.3 – 0.6 ml/min for 4 mm diameter columns and 0.4 – 1.2 ml/min for 8 mm diameter columns.
- If the column is not to be used for a longer period, the inlet and outlet should be sealed with appropriate blind fittings to prevent the polymer material from drying out. For longer term storage, the column should be kept at 4°C to avert bacterial growth.

Column regeneration procedure

Eurokat columns can be regenerated in their corresponding ionic form. Regeneration of the polymer resin is important to maintaining the selectivity and lifetime of the column material. If metal ions or organic components are present in the sample, these materials may settle on the resin material or even react with the polymer, resulting in a gradual loss of column performance. Through periodic cleaning of the column, lifetime and performance can be significantly prolonged. To clean the resin, Eurokat Pb and Ca columns should be flushed for at least 4 hours (preferably overnight) with double-distilled water at a flow rate between 0.1 – 0.2 ml/min in the reverse direction at an appropriate temperature. Eurokat H columns can be cleaned in a similar manner but require 0.01 N sulfuric acid.
The column should then be rinsed for an additional hour with the same cleaning eluent in the normal flow direction and gradually cooled to ambient temperature. Maintaining this flow direction, Eurokat Pb and Ca columns should then be purged with a mixture of 20 % acetonitrile and 80 % water (vol./vol.). Eurokat H columns should be purged with 20 % acetonitrile and 80 % 0.01 N sulfuric acid (vol./vol.). After this cleaning process, the columns are to be regenerated as follows:

Eurokat Pb: purge with 0.25 M lead nitrate at 60 °C at a flow rate of 0.2 ml/min for about 4-6 hours
Eurokat Ca: purge with 0.25 M calcium nitrate at 60 °C at a flow rate of 0.2 ml/min for about 4-6 hours
Eurokat H: purge with 0.05 N sulfuric acid at 60 °C at a flow rate of 0.2 ml/min for 4-6 hours

Once this procedure has been completed, the desired flow rate may be resumed gradually. The column is now ready for further analyses and can be put back into normal use once having gradually reached the working temperature.

Column using tips

In general it is recommended that a precolumn (30 x 8 mm or 30 x 4 mm) be used. In order to eliminate undissolved particles or precipitates, the sample should be filtered through a 0.45 µm filter unit. Particulate matter in the eluent is removed by installing a column inlet filter between the injector and the column. To avoid contaminating the detector's measurement cell, neither the cleaning solution nor the regenerant should pass through the measurement cell.

UHPLC Column Care and Use -

Column operating guidelines

KNAUER BlueOrchid and Bluespher columns are based on high purity spherical silica with a 1.8 µm/2 µm particle size for ultra high performance liquid chromatography (UHPLC). The UHPLC column hardware and stationary phases are designed for operation at up to 1000 bar (15000 psi). Each column is individually packed and tested to ensure reliable performance. The enclosed test certificate includes a test chromatogram and specific column data concerning performance. The serial number of your column is noted on the column certificate as well as on the column label. Please retain this information. To ensure that your column provides you with reliable chromatography results, please adhere to the guidelines below.

Column connections

It is highly recommended that only capillaries with accurately cut straight ends be used for column installation to avoid excessive dead volume. Micro and narrow bore columns require HPLC equipment specifically designed with low volume components. We recommend that capillaries with an inner diameter of 0.12 mm ID be used and that the connections between the injector and column, as well as the column and detector, be kept as short as possible. The end fittings on KNAUER UHPLC columns are compatible with all UHPLC systems, however we recommend using the KNAUER PEEK compression screw if using flexible stainless steel capillaries from KNAUER (0.5 mm OD).

Column installation

Please handle the column with care, every drop or shock to the column can damage the packed column bed. The column is shipped with PEEK end plugs. Please loosen and remove the plugs before installation. Flush all capillaries with compatible eluent before use with the column. When the column is shipped it contains the solvent listed on the column test certificate (the column is also safely stored in this solvent.) Be sure that your mobile phase is compatible with this storage solvent. If not, flush the column with an intermediate solvent which is compatible with both solvents. We recommend isopropanol. The flow direction is given by an arrow on the column label. Firstly, connect the column only at the injector, flush the system and column at low flow rates and gradually increase the flow rate up to the optimum value. Finally after about 10 mins, connect the column to your detector. This procedure helps to avoid air bubbles from being introduced into the flow cell. Before starting any analysis, check for leak tightness by observing the backpressure or using a flow control unit.

Equilibration, regeneration and storage

Equilibration

The period of equilibration depends on the flow rate; we recommend using a minimum of 10-20 column volumes.

Column dimensions (length x ID)	Column volume	Equilibration time at 250 µl/min	Equilibration time at 500 µl/min
50 x 2 mm	157 µl	6 min	3 min
100 x 2 mm	314 µl	12 min	6 min

Regeneration

We recommend that the column be regenerated if changes in peak form, retention time, resolution or an increase in back pressure is observed. If the system pressure begins to rise, remove the column and check the system to find whether the pressure increase is being caused by the system or the column. Pressure increase caused by system: flush system, exchange eluent filters, frits and/or blocked capillaries. Pressure increase caused by column: backflush the column carefully to remove particle buildup from the inlet frit (connect the column outlet to the pump/injector and flush). *Do not connect the column to the detector.* If the column still has a high backpressure, flush the column according to the following regeneration scheme.

Regeneration scheme for RP columns (C18, C18A, C8, PFP, Phenyl)	Regeneration scheme for NP columns (Si, CN, NH_2, Diol, C4)
20 column volumes water	20 column volumes heptane
20 column volumes acetonitrile	5 column volumes isopropanol
5 column volumes isopropanol	20 column volumes acetonitrile
20 column volumes heptane	20 column volumes water
5 column volumes isopropanol	20 column volumes acetonitrile
20 column volumes acetonitrile	5 column volumes isopropanol
	20 column volumes heptane

Storage

Reversed phase columns can be safely stored for longer terms in at least 50% organic solvent (e.g. acetonitrile or methanol). For short-term or overnight storage, use a mixture of water with acetonitrile or methanol (not buffer). For long-term storage of normal phase columns, use nonpolar solvents such as heptane/dioxane 90:10 (v/v) or hexane/isopropanol 90:10 (v/v). To avoid drying out of the stationary phase during storage, close both column ends with blind plugs.

Additional recommendations

We highly recommend that you keep a record of the column's operating pressure. Sudden changes in pressure (from either direction) can damage the column, so please avoid rapid increases or drops in pressure. Although working pressures up to 1000 bar (15000 psi) are possible, we advise that you work at pressures < 800 bar for a longer column lifetime. If using buffered eluents, the pH of the eluent used will be dependent on the application and packing material. We recommend using eluents between pH 2 and 8 (pH values at range limits reduce column lifetime). All eluents should be filtered through a 0.45 µm filter and degassed. Use only HPLC-grade eluent (high quality/high purity). Filter all samples before injection (0.45 µm membrane filter unit) to prevent blocking the column. For dirty samples or those with unknown purity, we recommend the use of a column pre-filter and/or precolumn. If possible, dilute samples in the same eluent which is to be used for the analysis start conditions. We recommend a temperature limit up to max. 60°C. *Because every UHPLC system is unique, especially in regards to the dwell volume, your results may vary from those obtained in our laboratory. Please don't hesitate to call our column specialists to assist you in optimizing your separation.*
Failure to follow these precautions may void the column warranty. Technical data are subject to change without notice.

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