Bulletin 826B

HPLC Troubleshooting Guide

How to identify, isolate, and correct the most common HPLC problems

Although HPLC method development has been improved by advances in column technology and instrumentation, problems still arise. In this guide we offer you a systematic means of isolating, identifying, and correcting many typical problems.

The important segments of an HPLC system are the same, whether you use a modular system (See Figure A, page 2) or a more sophisticated unit. Problems affecting overall system performance can arise in each component. Some common problems are discussed here. Solutions to these problems are presented in easy-to-use tables.

997-0218

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Isolating HPLC Problems

In an HPLC system, problems can arise from many sources. First define the problem, then isolate the source.

Use Table 1 (page 5) to determine which component(s) may be causing the trouble. A process of elimination will usually enable you to pinpoint the specific cause and correct the problem.

How to Prevent Mobile Phase Problems

Low sensitivity and rising baselines, noise, or spikes on the chromatogram can often be attributed to the mobile phase. Contaminants in the mobile phase 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 use only high purity distilled or deionized water when formulating mobile phases. However, several common deionizers introduce organic contaminants into the water. To remove these contaminants, pass the deionized 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. These trace contaminants 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 make them up fresh. Prevent microorganism growth by adding about 100ppm of sodium azide to aqueous buffers. Alternatively, these buffers may also be mixed with 20% or more of an organic solvent such as ethanol or acetonitrile.

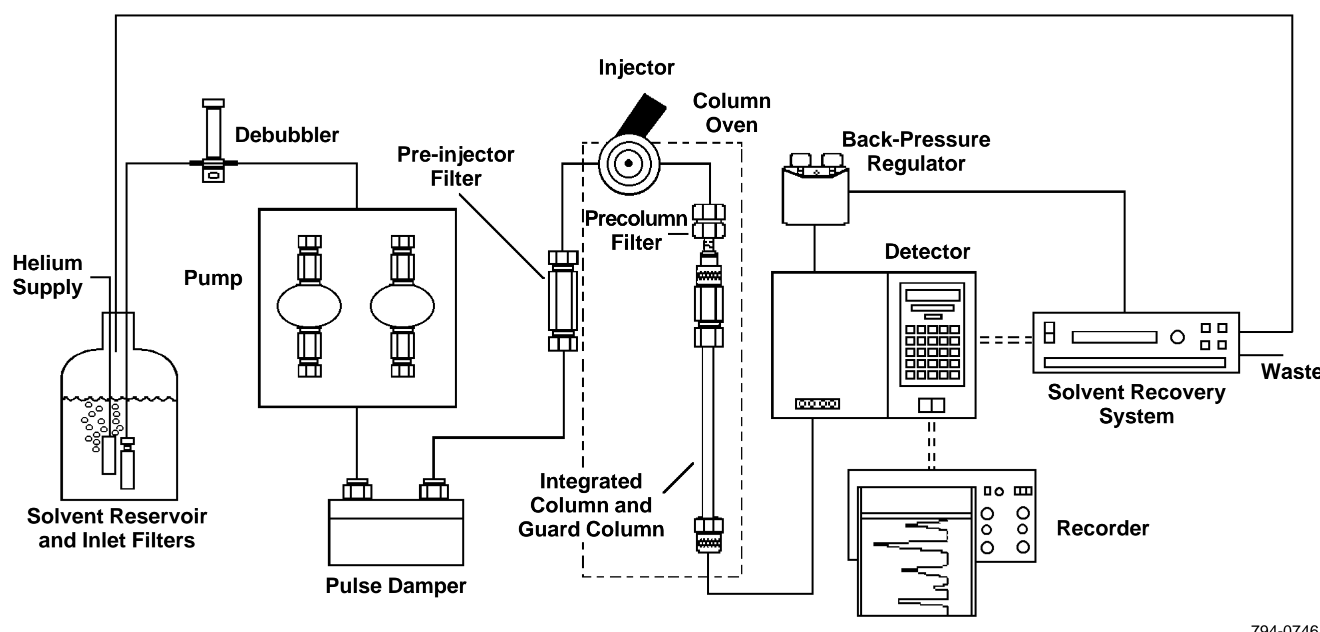
To prevent bubbles in the system, degas the mobile phase prior to use. Sparge it with helium (3-5psi) during use. Filtering the mobile phase through a 0.2 or 0.45µm filter using a vacuum filtration apparatus eliminates dissolved gas. This will also remove particles that could produce noisy baselines 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 10mM. 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 trifluoracetic 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.

Recycling the mobile phase used for isocratic separations has become more popular in recent years as a means of reducing the cost of solvents, their disposal, and mobile phase preparation time. A simple apparatus, such as the Supelco SRS-3000 Solvent Recovery System, uses a microprocessor controlled switching valve to direct the solvent stream to waste when a peak is detected. When the baseline falls under the selected threshold, uncontaminated solvent is directed back to the solvent reservoir.

Figure A. Components of an HPLC System



Isolating Pump Problems

The pump must deliver a constant flow of solvent to the column over a wide range of conditions. Modern HPLC pumps incorporate single or dual piston, syringe, or diaphragm pump designs.

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 buildup of salts at a pump connection. Buffer salts should be flushed from the system daily with fresh DI water. To isolate and repair specific problems related to your apparatus, 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.

Injector and Injection Solvents

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. These are activated manually, pneumatically, or electrically.

Mechanical problems involving the injector (e.g., leaks, plugged capillary tubing, worn seals) are easy to spot and correct. Use a pre-column filter to prevent plugging of the column frit due to physical degradation of the injector seal. Other problems, such as irreproducible injections, are more difficult to solve.

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 mobile phase. Otherwise, be sure the injection solvent is of lower eluting strength than the mobile phase (Table 3). Be aware that some autosamplers use separate syringe washing solutions. Make sure that the wash solution is compatible with and weaker than the mobile phase. This is especially important when switching between reversed and normal phase analyses.

Column Protection

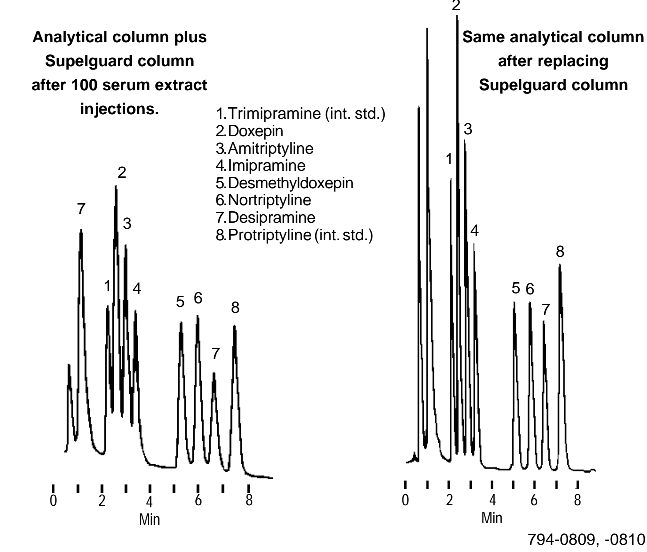
Although not an integral part of most equipment, mobile phase inlet filters, pre-injector and pre-column filters, saturator columns, and guard columns greatly reduce problems associated with complex separations. We recommend that all samples be filtered through 0.45µm or 0.2µm syringe filters (see page 18). The use of guard columns is also strongly recommended.

Filters and guard columns 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 life of these disposable products depends on mobile phase composition, sample purity, pH, etc. As these devices become contaminated or plugged with particles, pressure increases and peaks broaden or split. As an example, Figure B presents a clear case for the use of guard columns. For more about column protection, see the product pages of this guide and request Bulletin 781.

Figure B. Supelguard Columns Prolong the Lifespan of Your Analytical Columns

Column: **SUPELCOSIL LC-PCN, 25cm x 4.6mm ID, 5µm particles (with Supelguard LC-PCN guard column)**
Cat. No.: **58378**
Mobile Phase: 0.01M potassium phosphate (pH to 7 w/85% phosphoric acid):acetronitrile:methanol, 25:60:15
Flow Rate: 2mL/min
Temp.: 30°C
Det.: UV, 215nm
Inj.: 100µL reconstituted SPE eluant (20ng/mL each analyte and int. std. in serum)



Getting the Most from Your Analytical Column

Columns are available in many different sizes and designs. A wide variety of packings are also available, but all have the same purpose — to perform the separation. The most common problem associated with analytical columns is deterioration. This is true regardless of whether the column contains a bonded reversed or normal phase, ion exchange, affinity, hydrophobic interaction, size exclusion, and resin/silica based packing.

Symptoms of deterioration are poor peak shape, split peaks, shoulders, loss of resolution, decreased retention times, and high back pressure. These symptoms indicate 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 shapes and other problems. Column capacity depends on many factors, but typical values are:

Analytical column (25cm x 4.6mm) <500µg
Semi-preparative column (25cm x 10mm) <100mg
Preparative column (25cm x 21.2mm) <500mg

Solving Detector Problems

More than 20 types of detectors are 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. Improvements in detector cell technology have made them more durable and easier to use.

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 or baseline noise on the chromatograms or low sensitivity.

Some 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.

Column Heater, Recorder

These components seldom cause problems with the system. They will be discussed in the troubleshooting table (Table 1).

Keeping Accurate Records

Most problems don't occur overnight, but develop gradually. Accurate record keeping, then, is vital to detecting and solving many problems.

Evaluate every column you receive, when you receive it and at regular intervals thereafter. By keeping a written history of column efficiency, mobile phases used, lamp current, pump performance, etc., you can monitor your system's performance.

Records also help prevent mistakes, such as introducing water into a silica column, or precipitating buffer in the system by adding too much organic solvent. Many analysts modify their HPLC systems in some way. Reliable records are the best way to ensure that a modification does not introduce problems. For problems relating to pumps, detectors, automatic samplers, and data systems, consult your instrument manual's troubleshooting guide.

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Trademarks

FPLC — Pharmacia Biotech AB

Iso-Disc, Pelliguard, Sigma-Aldrich, Supelco, SUPELCOSIL, Supelguard, Trizma — Sigma-Aldrich Co.

LO-Pulse — Scientific Systems, Inc.

Rheodyne — Rheodyne, Inc.

Swagelok — Crawford Fitting Co.

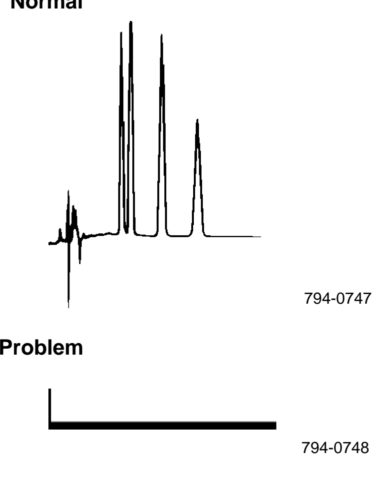
Teflon — E.I. du Pont de Nemours & Co., Inc.

Table 1. HPLC Problems, Probable Causes, and Remedies

Problem | **Probable Cause** | **Remedy/Comments**

Problem No. 1: No Peaks/Very Small Peaks

Normal



794-0747

Problem

794-0748

Problem

794-0749

Probable Cause

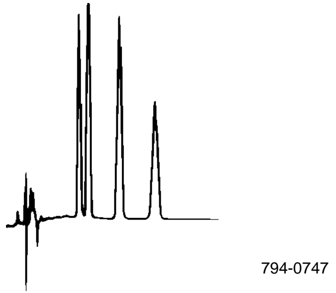
1. Detector lamp off.
2. Loose/broken wire between detector and integrator or recorder.
3. No mobile phase flow.
4. No sample/deteriorated sample/ wrong sample.
5. Settings too high on detector or recorder.

Remedy/Comments

1. Turn lamp on.
2. Check electrical connections and cables.
3. See “No Flow” (Problem No. 2).
4. Be sure automatic sampler vials have sufficient liquid and no air bubbles in the sample. Evaluate system performance with fresh standard to confirm sample as source of problem.
5. Check attenuation or gain settings. Check lamp status. Auto-zero if necessary.

Problem No. 2: No Flow

Normal



794-0747

Problem

794-0748

Probable Cause

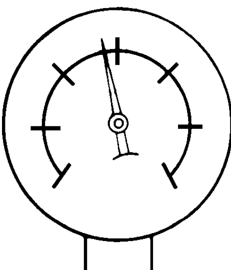
1. Pump off.
2. Flow interrupted/obstructed.
3. Leak.
4. Air trapped in pump head. (Revealed by pressure fluctuations.)

Remedy/Comments

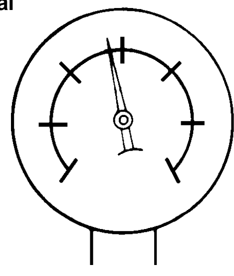
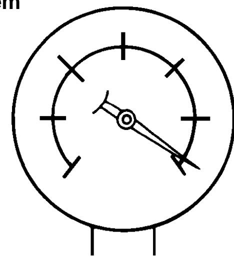
1. Start pump.
2. Check mobile phase level in reservoir(s). Check flow throughout system. Examine sample loop for obstruction or air lock. Make sure mobile phase components are miscible and mobile phase is properly degassed.
3. Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary.
4. Disconnect tubing at guard column (if present) or analytical column inlet. Check for flow. Purge pump at high flow rate (e.g., 5-10mL/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.

Problem	Probable Cause	Remedy/Comments

Problem No. 3: No Pressure/Pressure Lower Than Usual

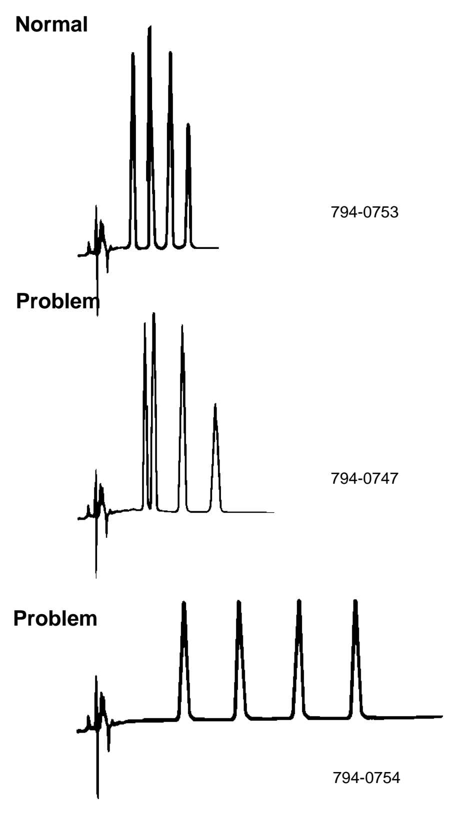
Problem	Probable Cause	Remedy/Comments
Normal  794-0750	1. Leak.	1. Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary.
	2. Mobile phase flow interrupted/ obstructed.	2. Check mobile phase level in reservoir(s). Check flow throughout system. Examine sample loop for obstruction or air lock. Make sure mobile phase components are miscible and mobile phase is properly degassed.
Problem	3. Air trapped in pump head. (Revealed by pressure fluctuations.)	3. Disconnect tubing at guard column (if present) or analytical column inlet. Check for flow. Purge pump at high flow rate (e.g., 10mL/min), prime system if necessary. (Prime each pump head separately.) If system has check valve, loosen valve to allow air to escape.
	4. Leak at column inlet end fitting.	4. Reconnect column and pump solvent at double the flow rate. If pressure is still low, check for leaks at inlet fitting or column end fitting.
	5. Air trapped elsewhere in system.	5. Disconnect guard and analytical column and purge system. Recon nect column(s). If problem persists, flush system with 100% methanol or isopropanol.
794-0751	6. Worn pump seal causing leaks around pump head.	6. Replace seal. If problem persists, replace piston and seal.

Problem No. 4: Pressure Higher Than Usual

Problem	Probable Cause	Remedy/Comments
Normal  794-0750	1. Problem in pump, injector, in-line filter, or tubing.	1. Remove guard column and analytical column from system. Replace with unions and 0.010" ID or larger tubing to reconnect injector to detector. Run pump at 2-5mL/min. If pressure is minimal, see Cause 2. If not, isolate cause by systematically eliminating system components, starting with detector, then in-line filter, and working back to pump. Replace filter in pump if present.
Problem  794-0752	2. Obstructed guard column or analytical column.	2. Remove guard column (if present) and check pressure. Replace guard column if necessary. If analytical column is obstructed, reverse and flush the column, while disconnected from the detector (page 14). If problem persists, column may be clogged with strongly retained contaminants. Use appropriate restoration procedure (Table 2, page 14). If problem still persists, change inlet frit (page 16) or replace column.

Problem	Probable Cause	Remedy/Comments

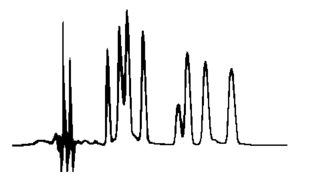
Problem No. 5: Variable Retention Times



Probable Cause	Remedy/Comments
1. Leak.	1. Check system for loose fittings. Check pump for leaks, salt buildup, 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 using proportioning values, 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 (especially evident in ion exchange systems).	4. Use reliable column oven. (Note: higher column temperatures increase column efficiency. For optimum results, heat eluant before introducing it onto column.)
5. Column overloading. (Retention times usually decrease as mass of solute injected on column exceeds column capacity.)	5. Inject smaller volume (e.g., 10µL vs. 100µL) or inject the same volume after 1:10 or 1:100 dilutions of 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 (see page 14).

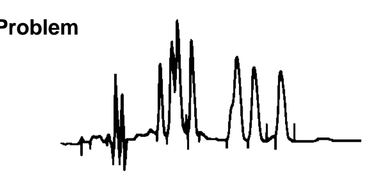
Problem No. 6: Loss of Resolution

Normal



794-0755

Problem

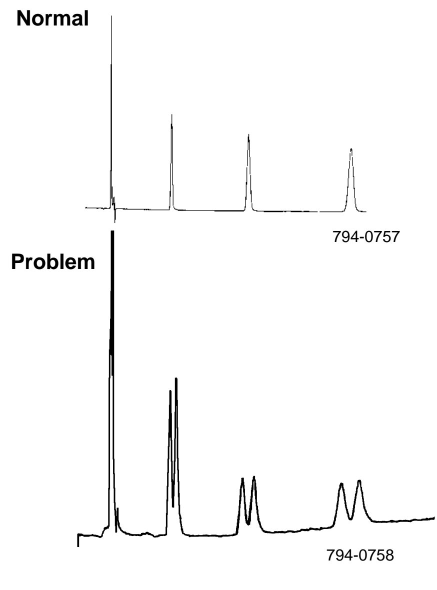


794-0756

Probable Cause	Remedy/Comments
1. Mobile phase contaminated/deteriorated (causing retention times and/or selectivity to change).	1. Prepare fresh mobile phase (page 2).
2. Obstructed guard or analytical column.	2. Remove guard column (if present) and attempt analysis. Replace guard column if necessary. If analytical column is obstructed, reverse and flush (page 14). If problem persists, column may be clogged with strongly retained contaminants. Use appropriate restoration procedure (Table 2, page 14). If problem still persists, change inlet frit (page 16) or replace column.

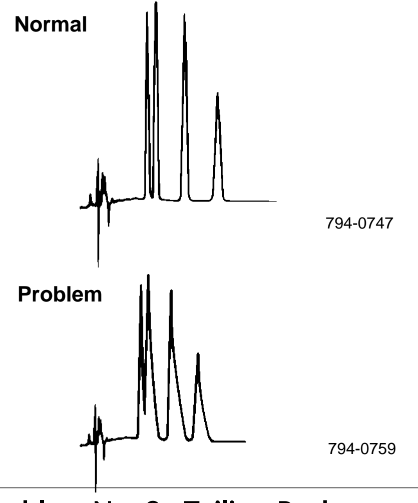
Problem	Probable Cause	Remedy/Comments

Problem No. 7: Split Peaks



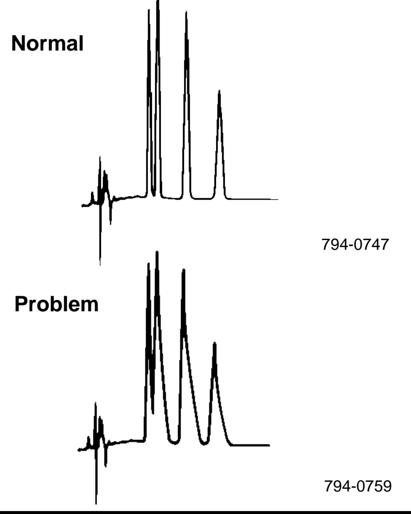
Probable Cause	Remedy/Comments
1. Contamination on guard or analytical column inlet.	1. Remove guard column (if present) and attempt analysis. Replace guard column if necessary. If analytical column is obstructed, reverse and flush (page 14). If problem persists, column may be clogged with strongly retained contaminants. Use appropriate restoration procedure (Table 2, page 14). If problem still persists, inlet frit is probably (partially) plugged. Change frit (page 16) or replace column.
2. Partially blocked frit.	2. Replace frit (see above)
3. Small (uneven) void at column inlet.	3. Repack top of column with pellicular particles of same bonded phase functionality. Continue using the column in reverse flow direction.
4. Sample solvent incompatible with mobile phase.	4. Adjust solvent. Whenever possible, inject samples in mobile phase.

Problem No. 8: Peaks Tail on Initial and Later Injections



Probable Cause	Remedy/Comments
1. Sample reacting with active sites.	1. First check column performance with standard column test mixture. If results for test mix are good, add ion pair reagent or competing base or acid modifier (page 2).
2. Wrong mobile phase pH.	2. Adjust pH. For basic compounds, lower pH usually provides more symmetric peaks.
3. Wrong column type.	3. Try another column type (e.g., deactivated column for basic compounds).
4. Small (uneven) void at column inlet.	4. See Problem No. 7.
5. Wrong injection solvent.	5. Peaks can tail when sample is injected in stronger solvent than mobile phase. Dissolve sample in mobile phase.

Problem No. 9: Tailing Peaks

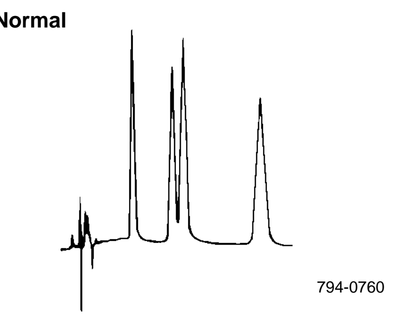


Probable Cause	Remedy/Comments
1. Guard or analytical column contaminated/worn out.	1. Remove guard column (if present) and attempt analysis. Replace guard column if necessary. If analytical column is source of problem, use appropriate restoration procedure (Table 2, page 14). If problem persists, replace column.
2. Mobile phase contaminated/deteriorated.	2. Check make-up of mobile phase (page 2).
3. Interference in sample.	3. Check column performance with standards.

Problem	Probable Cause	Remedy/Comments

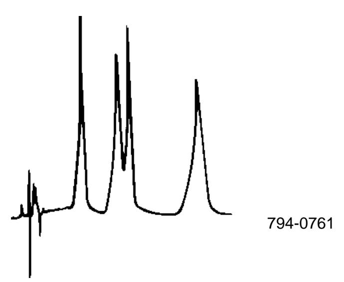
Problem No. 10: Fronting Peaks

Normal



794-0760

Problem

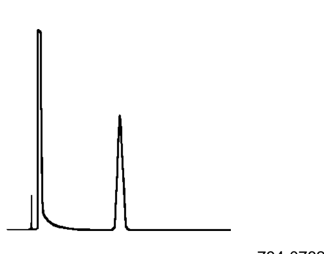


794-0761

Probable Cause	Remedy/Comments
1. Column overloaded.	1. Inject smaller volume (e.g., 10µL vs. 100µL). Dilute the sample 1:10 or 1:100 fold in case of mass overload.
2. Sample solvent incompatible with mobile phase.	2. Adjust solvent. Whenever possible, inject samples in mobile phase. Flush polar bonded phase column with 50 column volumes HPLC grade ethyl acetate at 2-3 times the standard flow rate, then with intermediate polarity solvent prior to analysis.
3. Shoulder or gradual baseline rise before a main peak may be another sample component.	3. Increase efficiency or change selectivity of system to improve resolution. Try another column type if necessary (e.g., switch from nonpolar C18 to polar cyano phase).

Problem No. 11: Rounded Peaks

Normal



794-0762

Problem

794-0763

Probable Cause	Remedy/Comments
1. Detector operating outside linear dynamic range.	1. Reduce sample volume and/or concentration.
2. Recorder gain set too low.	2. Adjust gain.
3. Column overloaded.	3. Inject smaller volume (e.g., 10µL vs. 100µL) or 1:10 and 1:10 dilution of sample.
4. Sample-column interaction.	4. Change buffer strength, pH, or mobile phase composition. If necessary, raise column temperature or change column type. (Analysis of solute structure may help predict interaction.)
5. Detector and/or recorder time constants are set too high.	5. Reduce settings to lowest values or values at which no further improvements are seen.

Problem	Probable Cause	Remedy/Comments

Problem No. 12: Baseline Drift

Normal

794-0748

Problem

794-0764

Probable Cause	Remedy/Comments
1. Column temperature fluctuation. (Even small changes cause cyclic baseline rise and fall. Most often affects refractive index and conductivity detectors, UV detectors at high sensitivity or in indirect photometric mode.)	1. Control column and mobile phase temperature, use heat exchanger before detector.
2. Nonhomogeneous mobile phase. (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, sparge with helium during use.
3. Contaminant or air buildup in detector cell.	3. Flush cell with methanol or other strong solvent. If necessary, clean cell with 1N HNO_3 (*never* with HCl and never use concentrated nitric acid with PEEK tubing or fittings.)
4. Plugged outlet line after detector. (High pressure cracks 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. To avoid problem, routinely monitor composition and flow rate.
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 not prepared from high quality chemicals.	7. Check make-up of mobile phase (page 2).
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. Detector (UV) not set at absorbance maximum but at slope of curve.	9. Change wavelength to UV absorbance maximum.

Problem No. 13: Baseline Noise (regular)

Normal

794-0748

Problem

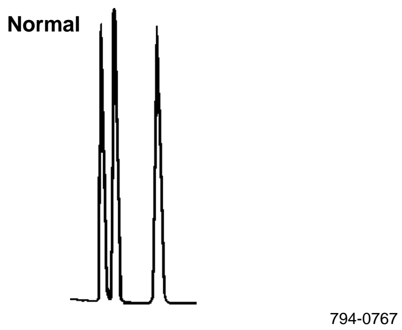
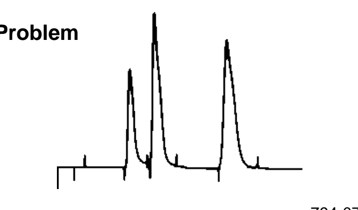
794-0765

Probable Cause	Remedy/Comments
1. Air in mobile phase, detector cell, or pump.	1. Degas mobile phase. Flush system to remove air from detector cell or pump.
2. Pump pulsations.	2. Incorporate pulse damper into system.
3. Incomplete mobile phase mixing.	3. Mix mobile phase by hand or use less viscous solvent.
4. Temperature effect (column at high temperature, detector unheated).	4. Reduce differential or add heat exchanger.
5. Other electronic equipment on same line.	5. Isolate LC, detector, recorder to determine if source of problem is external. Correct as necessary.
6. Leak.	6. Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary.

Problem No. 14: Baseline Noise (irregular)

Problem	Probable Cause	Remedy/Comments
Normal 794-0748	1. Leak.	1. Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary.
	2. Mobile phase contaminated, deteriorated, or prepared from low quality materials.	2. Check make-up of mobile phase. (page 2).
Problem	3. Detector/recorder electronics.	3. Isolate detector and recorder electronically. Refer to instruction manual to correct problem.
	4. Air trapped in system.	4. Flush system with strong solvent.
794-0766	5. Air bubbles in detector.	5. Purge detector. Install backpressure regulator 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 noise.)	6. Clean cell.
	7. Weak detector lamp.	7. Replace lamp.
	8. Column leaking silica or packing material.	8. Replace column and clean system.

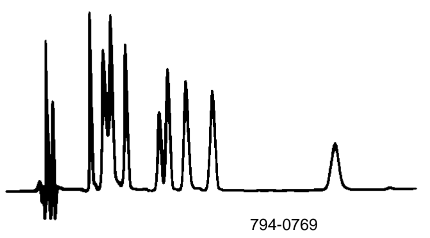
Problem No. 15: Broad Peaks

Problem	Probable Cause	Remedy/Comments
Normal  794-0767	1. Mobile phase composition changed.	1. Prepare new mobile phase.
	2. Mobile phase flow rate too low.	2. Adjust flow rate.
	3. Leak (especially between column and detector).	3. Check system for loose fittings. Check pump for leaks, salt buildup, and unusual noises. Change pump seals if necessary.
	4. Detector settings incorrect.	4. Adjust settings.
	5. Extra-column effects:	5.
	a. Column overloaded	a. Inject smaller volume (e.g., 10µL vs. 100µL) or 1:10 and 1:100 dilutions of sample.
	b. Detector response time or cell volume too large.	b. Reduce response time or use smaller cell.
	c. Tubing between column and detector too long or ID too large.	c. Use as short a piece of 0.007-0.010" ID tubing as practical.
	d. Recorder response time too high.	d. Reduce response time.
Problem  794-0768	6. Buffer concentration too low.	6. Increase concentration.
	7. Guard column contaminated/worn out.	7. Replace guard column.
	8. Column contaminated/worn out.	8. Replace column with new one of same type. If new column does not provide narrow peaks, flush old column (Table 2, page 14), then retest.
	9. Void at column inlet.	9. Replace column or open inlet end and fill void (page 16).
	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 75°C unless higher temperatures are acceptable to column manufacturer.

Problem	Probable Cause	Remedy/Comments

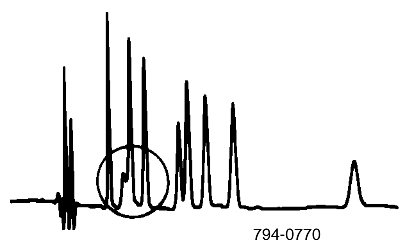
Problem No. 16: Change in Peak Height (One or More Peaks)

Normal



794-0769

Problem

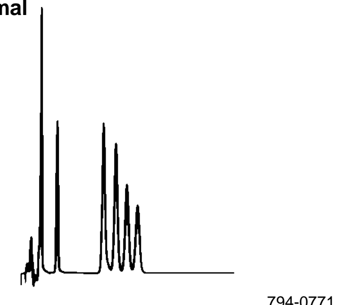


794-0770

Probable Cause	Remedy/Comments
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 (Table 2, page 14). If performance does not improve, discard old column.
2. Leak, especially between injection port and column inlet. (Retention also would change.)	2. Check system for loose fittings. Check pump for leaks, salt buildup, unusual noises. Change pump seals if necessary.
3. Inconsistent sample volume.	3. Be sure samples are consistent. For fixed volume sample loop, use 2-3 times loop volume to ensure loop is completely filled. Be sure automatic sampler vials contain sufficient sample and no air bubbles. Check syringe-type injectors for air. In systems with wash or flushing step, be sure wash solution does not precipitate sample components.
4. Detector or recorder setting changed.	4. Check settings.
5. Weak detector lamp.	5. Replace lamp.
6. Contamination in detector cell.	6. Clean cell.

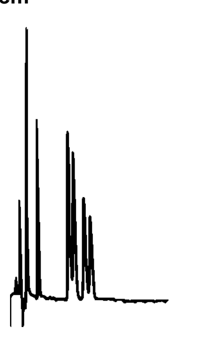
Problem No. 17: Change in Selectivity

Normal



794-0771

Problem



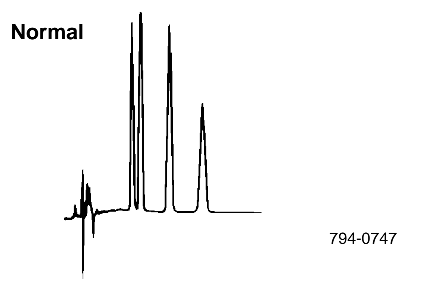
794-0772

Probable Cause	Remedy/Comments
1. Increase or decrease solvent ionic strength, pH, or additive concentration (especially affects ionic solutes).	1. Check make-up of mobile phase (page 2).
2. Column changed, new column has different selectivity from that of old column.	2. Confirm identity of column packing. For reproducible analyses, use same column type. Establish whether change took place gradually. If so, bonded phase may have stripped. Column activity may have changed, or column may be contaminated.
3. Sample injected in incorrect solvent or excessive amount (100-200µL) of strong solvent.	3. Adjust solvent. Whenever possible, inject sample in mobile phase.
4. Column temperature change.	4. Adjust temperature. If needed, use column oven to maintain constant temperature.

Problem	Probable Cause	Remedy/Comments

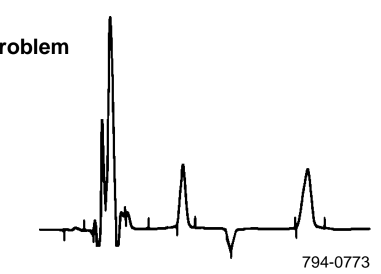
Problem No. 18: Negative Peak(s)

Normal



794-0747

Problem

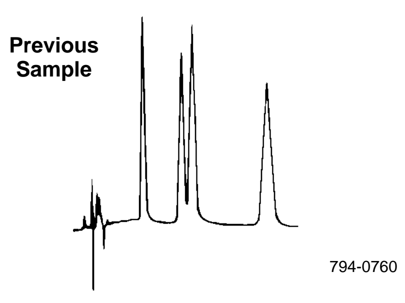


794-0773

Probable Cause	Remedy/Comments
1. Recorder leads reversed.	1. Check polarity.
2. Refractive index of solute less than that 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 (vacancy peaks).	3. Adjust or change sample solvent. Dilute sample in mobile phase whenever possible.
4. Mobile phase more absorptive than sample components to UV wavelength.	4. a. Change polarity when using indirect UV detection, or b. Change UV wavelength or use mobile phase that does not adsorb chosen wavelength.

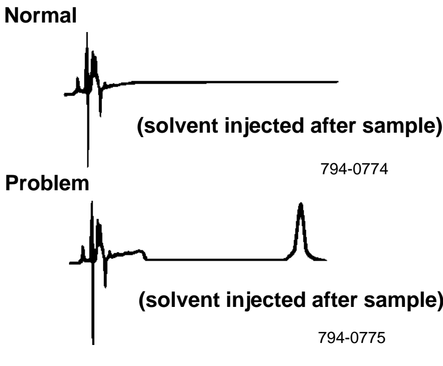
Problem No. 19: Ghost Peak

Previous Sample



794-0760

Normal



(solvent injected after sample)

794-0774

Problem

(solvent injected after sample)

794-0775

Probable Cause	Remedy/Comments
1. Contamination in injector or column.	1. Flush injector between analyses (a good routine practice). If necessary, run strong solvent through column to remove late eluters. Include final wash step in gradient analyses, to remove strongly retained compounds.
2. Late eluting peak (usually broad) present in sample.	2. a. Check sample preparation. b. Include (step) gradient to quickly elute component.

Further Recommendations

Table 1, above, 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. Modern HPLC systems often have self-diagnostic capabilities that help isolate the problem area within the instrument. For persistent problems relating to the column or your particular analysis, please contact Supelco's Technical Service Department.

The remaining chapters in this Troubleshooting Guide include detailed procedures for *Restoring Your Column's Performance* following loss in resolution, retention or selectivity (pages 14-15).

Suggestions on how to *Prevent and Solve Column Hardware Problems* are discussed on page 16, including tips on how to prevent leaks, unclog a frit, replace a frit and fill a void at the column top.

This guide concludes with a *Selection of Column Protection Products* from the Supelco catalog. Please refer to the latest edition of the catalog for our complete line of accessories that prolong column life and, in general, simplify or improve your HPLC or FPLC analysis.

Finally, phone 800-247-6628 or 814-359-3441 to request additional literature about Supelco's HPLC and FPLC® products or use our ChromFax service for immediate access to all our free technical literature.

Restoring Your Column's Performance

The following procedures should rejuvenate a column whose performance has deteriorated due to sample contamination.

Disconnect and reverse the column. Connect it to the pump, but not the detector. Follow the appropriate flushing procedure in this table, using a flow rate that results in column back pressure of 1500-4500psi, but never higher than the maximum recommended pressure in the manufacturer's instruction manual. If you have a SUPELCOSIL column, analyze with the test mix and the conditions listed on the data sheet. Efficiency, symmetry, and capacity should be within 10-15% of the test sheet values. If not, repack the column inlet (page 16) or replace the column.

Note: Volumes listed are for 25cm x 4.6cm ID columns, which have a column volume of 4.15mL. When using columns of shorter length, multiply the recommended volume by the ratio of the column lengths. For columns of different internal diameter, multiply the recommended volume by the ratio of the square of IDs.

Table 2. Column Restoration Procedures

Silica Column

Flush with the following:
1. 50mL hexane
2. 50mL methylene chloride
3. 50mL 2-propanol
4. 50mL methanol
5. 25mL methylene chloride
6. 25mL mobile phase

Evaluate column performance

Note: See also the Silica Column Regeneration Solution listed on page 15 for rejuvenating a deactivated silica column.

Silica-Based Reversed Phase Column
(alkyl•, phenyl, or diphenyl column, SUPELCOSIL LC-PAH or DB column)

A. Water Soluable Samples

Flush with the following:
1. 50mL hot (40-60°C) distilled water
2. 50mL methanol
3. 50mL acetonitrile
4. 30mL tetrahydrofuran
5. 25mL methanol
6. 25mL mobile phase

Evaluate column performance according to conditions specified by the manufacturer.

B. Samples Not Soluble in Water

Flush with the following:
1. 50mL 2-propanol
2. 50mL tetrahydrofuran
3. 50mL methylene chloride
4. 50mL hexane
5. 25mL isopropanol
6. 25mL mobile phase

Evaluate column performance

Polar-Bonded Phase Column
(amino, cyano, or diol column, SUPELCOSIL LC-PCN, or Pirkle-type chiral columns).

For a column used in the reversed phase mode (e.g., drug analyses using an organic solvent/aqueous buffer mobile phase), follow the same cleanup procedure as for nonpolar columns. For a column with nonaqueous mobile phase, use the following scheme:

Flush with the following:
1. 50mL chloroform
2. 50mL methanol
3. 50mL acetonitrile
4. 25mL methylene chloride
5. 25mL methanol
6. 25mL mobile phase

Evaluate performance.

Silica-Based Ion Exchange Columns
(strong or weak anion or cation exchange)

Most analyses involving ion exchange systems use ionic mobile phases. Compounds that may affect column performance are usually insoluble or only slightly soluble in water. The following procedure should be sufficient to remove these compounds.

Flush with the following:
1. 50mL hot (40-60°) distilled water
2. 50mL methanol
3. 50mL acetonitrile
4. 25mL methylene chloride
5. 25mL methanol
6. 25mL mobile phase

Evaluate column performance

Silica-Based Columns for RPLC of Proteins and Peptides

Flush with the following:
1. 50mL 0.1% trifluoroacetic acid (TFA)
2. 50mL 0.1% TFA in 2-propanol
3. 50mL 0.1% TFA in acetonitrile
4. 50mL mobile phase

Evaluate column performance

•C8, C18, etc.

Table 3. Properties of Organic Solvents Commonly Used in HPLC

Solvent	Polarity	Miscible with Water?	UV Cut Off•	Refractive Index at 20°C	Solvent Strength, ϵ_o (silica)	Viscosity at 20°C, cP
Hexane	nonpolar	no	200	1.3750	0.00	0.33
Isooctane		no	200	1.3910	0.01	0.50
Carbon tetrachloride		no	263	1.4595	0.14	0.97
Chloroform		no	245	1.4460	0.31	0.57
Methylene chloride	↓	no	235	1.4240	0.32	0.44
Tetrahydrofuran		yes	215	1.4070	0.35	0.55
Diethyl ether		no	215	1.3530	0.29	0.23
Acetone		yes	330	1.3590	0.43	0.32
Ethyl acetate		poorly	260	1.3720	0.45	0.45
Dioxane		yes	215	1.4220	0.49	1.54
Acetonitrile		yes	190	1.3440	0.50	0.37
2-Propanol		yes	210	1.3770	0.63	2.30
Methanol		yes	205	1.3290	0.73	0.60
Water		yes	—	1.3328	>0.73	1.00
	polar					

•Typical values.

Nonbonded Silica Columns Exposed to Polar Solvent

Samples and mobile phases containing very strongly polar solvents, such as water or alcohols, can deactivate uncoated silica HPLC columns. This can drastically affect column performance, particularly solute retention and selectivity. (Figure C2). Even prolonged column flushing with a nonpolar solvent only partially restores column performance, while wasting chemicals.

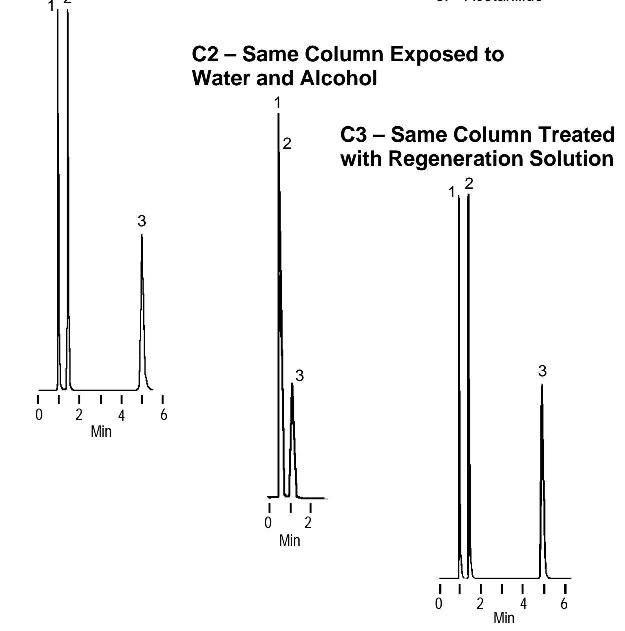
A silica regeneration solution quickly and inexpensively restores silica column performance by removing trapped polar material. Pump the solution through the affected column for 10 minutes at a rate of 4mL/minute, then flush with mobile phase for 10 minutes at a rate of 2mL/minute. Evaluate column performance by using the test mixture for evaluating silica columns (Cat. No. 58281). Performance should be virtually the same as before the polar solvent was introduced (Figure C3).

Silica Column Regeneration Solution, 200mL **33175**

Figure C. Regeneration Solution Restores Silica Column Performance

Column: **SUPELCOSIL LC-Si, 15cm x 4.6mm ID, 3µm packing**
Cat. No.: **58981**
Mobile Phase: methylene chloride:methanol:water, 99.4:0.5:0.1 (Fig. C1)
2-propanol:water, 50:50 (Fig. C2)
Silica Column Regeneration Solution, 4mL/min for 10 min, then methylene chloride:methanol:water, 99.4:0.5:0.1, 2mL/min for 10min (Fig. C3)
Temp.: ambient
Flow Rate: 2mL/min
Det.: UV, 254nm
Inj.: 10µL (injected after methylene chloride:methanol:water equilibration for Fig. C3).

1. Benzene
2. Benzanilide
3. Acetanilide



794-0787, -0788, -0789

Preventing and Solving Common Hardware Problems

Preventing Leaks

Leaks are a common problem in HPLC analyses. To minimize leaks in your system, avoid interchanging hardware and fittings from different manufacturers. Incompatible fittings can be forced to fit initially, but repeated connections may eventually leak. If interchanging is absolutely necessary, use appropriate adapters and check all connections for leaks before proceeding.

Highly concentrated salts (>0.2M) and caustic mobile phases can reduce pump seal efficiency. The lifetime of injector rotor seals also depends on mobile phase conditions, particularly operation at high pH. In some cases, prolonged use of ion pair reagents has a lubricating effect on 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.

Unclogging the Column Frit

A clogged column frit is another common HPLC problem. To minimize this problem from the start, use a precolumn filter and 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 twice the standard flow rate. About 5-10 column volumes of solvent should be sufficient to dislodge small amounts of particulate material on the inlet frit. Evaluate the performance of the cleaned column using a standard test mixture.

Filling a Void/Replacing a Frit at the Column Inlet

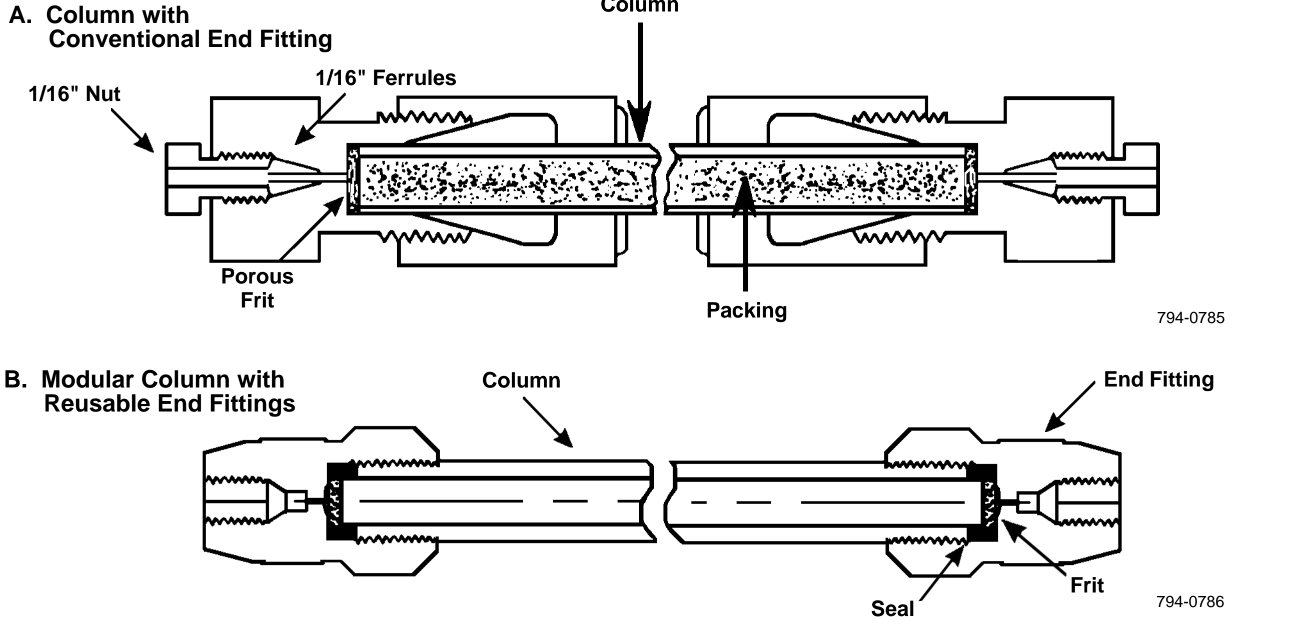
Sometimes neither solvent flushing (see above) nor restoration procedures (see Table 2) restore a column's performance. If you've isolated the column as the problem source, and other restorative procedures have failed, a void in the packing or a persistent obstruction on the inlet frit may exist.

As a last resort, open the *inlet* end of the column. **Caution:** opening the inlet, and more so outlet end, can permanently damage the packing bed. Before opening columns, consult the manufacturer's literature. (Never open either end of a *resin-filled* column).

Use the following procedure to open a column.

1. Disconnect the column from the system. To prevent the packing from oozing out of the column, perform subsequent steps as quickly as possible.
2. Using a vise and wrench, or two wrenches, carefully remove the inlet end fitting (see Figure D). If the frit remains in the fitting, dislodge it by tapping the fitting on a hard surface. If the frit stays on the column, slide it off rather than lift it off. This will help preserve the integrity of the packing bed.
3. Examine the old frit. Compression of the frit against the stainless steel tubing will leave a ring around the edge on the column side of a properly seated frit. No ring can mean the ferrule is seated too near the tubing end. The resulting loose connection can leak silica or act as a mixing chamber.
4. Examine the packing bed. If it is depressed or fractured, add pellicular material of the same bonding type to fill the void. Although this won't completely restore column efficiency, it will eliminate split peaks or shoulders caused by a void.
5. Clean any loose silica away from the ferrule and column and install a new frit (2µm pores for 4µm or larger packings, 0.5µm pores for smaller packings).
6. Replace the end fitting. Screw it down fingertight, then tighten 1/4 turn with a wrench.
7. Modular columns may require a special tool to remove the frit cap.

Figure D. Typical HPLC Column Designs



A Selection of Column Protection Products

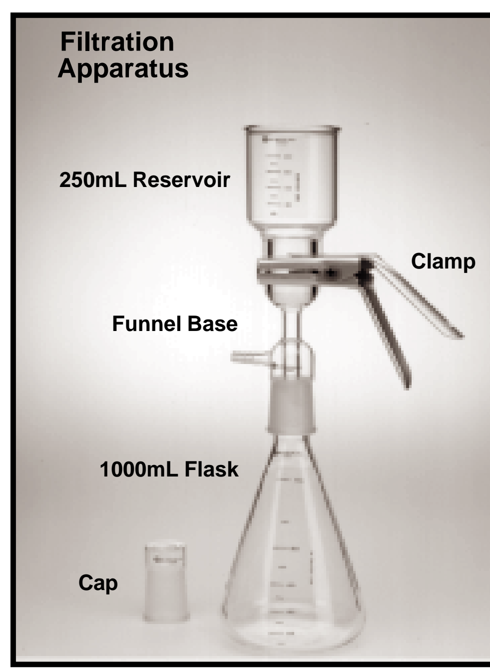
Mobile Phase Filtration Apparatus

(connects to aspiration line)

Includes 250mL glass reservoir, Ŧ34/45 tapered funnel base, Ŧ34/45 tapered top, 1000mL flask and glass cap, clamp, SS holder and screen, 10 Teflon gaskets, 50 Nylon-66 filters (47mm, 0.45µm pores). **58062-U**

Replacement Glass Parts

Reservoir, 100mL	**64675**
Reservoir, 250mL	**58063**
Reservoir, 500mL	**58074**
Tapered funnel base, Ŧ34/45	**58068**
Tapered flask, Ŧ34/45, 1000mL	**58070-U**
Tapered flask, Ŧ34/45, 2000mL	**58075**
Cap for flask	**58071**



910-0113

Mobile Phase Inlet Filters

2µm porous filter, SS

1/8" tube connection	**58267**
1/8" Swagelok® fitting	**58268**

10µm porous filter, SS

Universal slip-on filter for 1/16" ID (1.5mm) tubing, 2.2mm ID(0.085", for Varian pumps) tubing, and 3.0mm ID (0.119", for Waters pumps) tubing. **59277**

Injectors

Supelco offers the complete line of Rheodyne® injectors and parts, including popular models 7725 and 7725i injectors shown below. Request our Rheodyne® price list (Pub. No. 895007).

996-0109

Pump Replacement Parts

See the current Supelco catalog for an extensive listing of pump seals, pistons, and check valves.

Pulse Damper

A pulse damper controls pump pulsations for a more stable baseline. The SSI LO-Pulse® damper is a patented unit compatible with single piston reciprocation HPLC pumps (Altex 110A, Eldex pumps, LDC Mini-Pump VS, SSI Models 200 and 300, etc.). At pressures to 6000psi (420kg/cm^2), it improves precision of quantitative analyses and detection limits for trace sample components. Fittings and instructions included.

Pulse Damper	**58455**
Pulse Damper without Cabinet	**58442**

Pre-Injector Filter

Placed between the pump and injector, this 0.5µm porosity SSI filter provides final filtration for the mobile phase. Easily replaced 316 stainless steel filter element. Maximum operating pressure: 15,000psi (100MPa). For 1/16" OD tubing, 10-32 threads.

Pre-Injector Filter	**59262-U**

Replacement Filter Elements and Seals (pk. of 5)

0.5µm pores	**59264**
2µm pores	**59265**

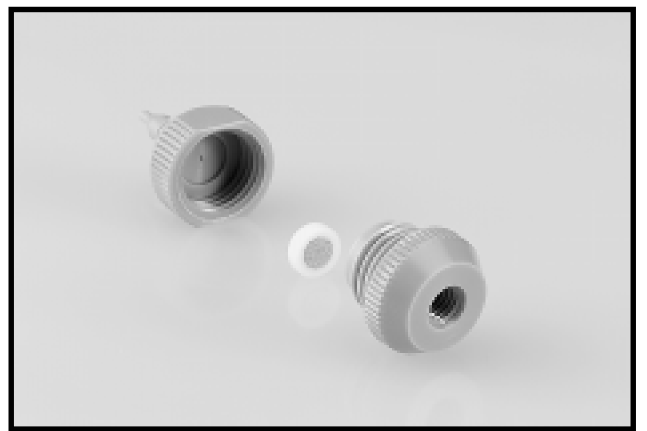
Refer to the current Supelco catalog for many additional products, and for prices.

Sample Filtration: Iso-Disc™ Syringe Filters Remove Particles from Samples Prior to Injection.

Filter Unit	Membrane	Diameter (mm)	Pore Size (µm)	Color Coding	Quantity	Cat. No.
Iso-Disc N-254	Nylon	25	0.45	green	50	**59230-U**
Iso Disc N-252	Nylon	25	0.2	purple	50	**59231-U**
Iso-Disc N-34	Nylon	3	0.45	green	100	**59238**
Iso Disc N-32	Nylon	3	0.2	purple	100	**59239**
Iso-Disc P-255	PTFE	25	5.0	gray	50	**59244**
Iso-Disc P-254	PTFE	25	0.45	yellow	50	**59234-U**
Iso-Disc P-252	PTFE	25	0.2	blue	50	**59235-U**
Iso-Disc P-34	PTFE	3	0.45	yellow	100	**59240-U**
Iso-Disc P-32	PTFE	3	0.2	blue	100	**59241**
Iso-Disc CA-254	Cellulose acetate	25	0.45	red	50	**59242**
Iso-Disc CA-252	Cellulose acetate	25	0.2	white	50	**59243**

Column Protection

We offer a selection of pre-column filters that are installed between the injector and the guard or analytical column.



997-0213

Sigma-Aldrich® Pre-Column Filter

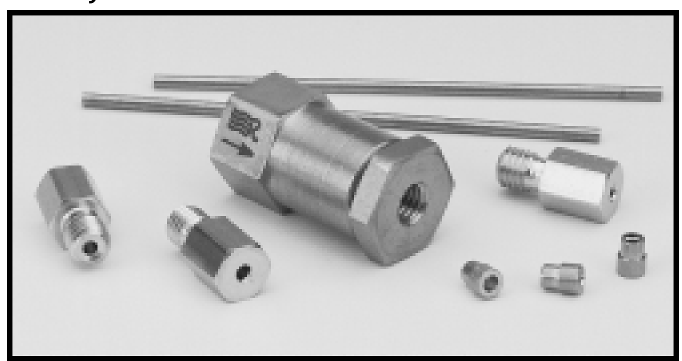
PEEK Body with SS frit. Connects directly to columns with standard 10-32 threads.

Pre-column filter kit, with 2µm frit **Z227323**

Replacement Frits, 2µm **Z227331**

Replacement Frits, 0.5µm **Z290874**

Rheodyne Pre-Column Filter



910-0142

Column Inlet Filter

3mm frit, (for 4.6mm ID columns) **59124**

1.5mm frit, (for 2.1mm ID columns) **59123**

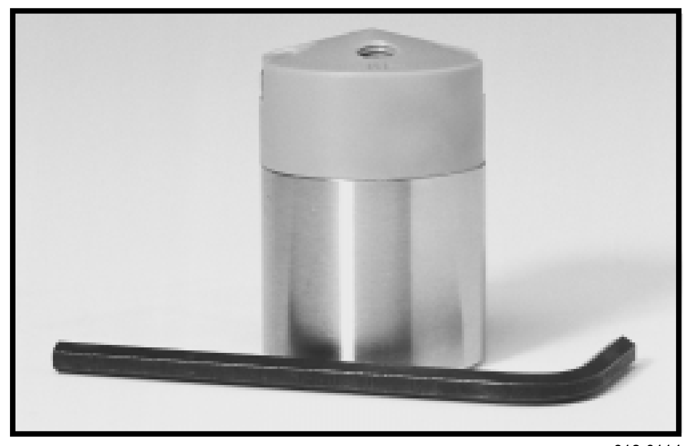
Replacement Frits, Pk. of 5

3mm frits, 0.5µm pores **59126**

1.5mm frits, 0.5µm pores **59125**

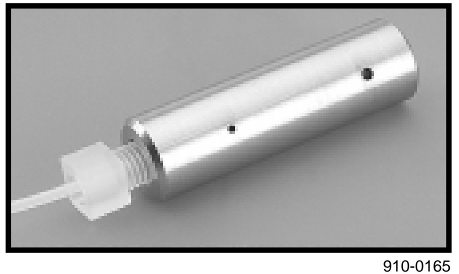
Back-Pressure Regulators

Flow through design (inlet and outlet fittings).
Adjustable for 0.3-5 atmospheres **59284**

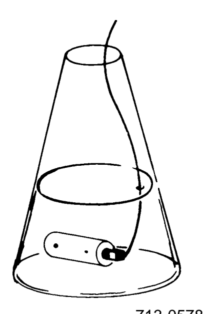


912-0114

Standard design (no outlet fittings). Adjustable from 1-4 atmospheres. Non-clog design. **58788**



910-0165



713-0578

Troubleshooting Book

Troubleshooting LC Systems by J.W. Dolan and L.R. Snyder (Humana Press, 1989)

Chapters describe reservoirs/degassing, pumps, tubing, fittings, injectors and autosamplers, columns, detectors, and recorders/ data systems. 515 pages. 23538

Contact our Technical Service Department for expert answers to your questions. Call 800-359-3041 or 814-359-3041, or FAX 800-359-3044 or 814-359-5468. In other countries contact your local Supelco subsidiary or dealer.

Solvents and Reagents

The quality of a liquid chromatography separation is closely linked to the purity of your solvent system. Supelco offers a large selection of HPLC grade solvents for your separation needs. Following is a brief selection of the more common solvents. See current catalog for listings of smaller and larger unit volumes.

Common HPLC Solvents (1 liter volume)

Solvent	Purity %	Cat. No.
1,4-Dioxane	99.8	**27,053-9B**
Acetone	99.9+	**27,072-5B**
Acetonitrile	99.9+	**27,071-7B**
Chloroform	99.9+	**27,063-6B**
Dichloromethane	99.9	**27,056-3B**
Ether	99.9	**30,996-6B**
Ethyl acetate	99.8	**27,052-0B**
Heptane	99+	**27,051-2B**
Hexane	95+	**27,050-4B**
Methanol	99.9+	**27,047-4B**
N,*N*-Dimethylacetamide	99.9+	**27,055-5B**
N,*N*-Dimethylformamide	99.9+	**27,054-7B**
Tetrachloroethylene	99.8+	**27,039-3B**
Tetrahydrofuran (THF)	99.9+	**27,038-5B**
Toluene	99.8	**27,037-7B**
Water	—	**27,073-3B**

Buffers

In addition to high quality solvents, HPLC separations often require the addition of buffers or mobile phase modifiers. These materials serve to stabilize the pH or ionic strength, or improve compound peak shape or resolution by acting as competing acids or bases in the separation system. The key quality characteristic of such materials is purity, and purity is central to Supelco's line of chromatography chemicals. Several of the most frequently used buffers, ion pair reagents, and mobile phase modifiers are listed below.

Common HPLC Buffers

Buffer Component*	Weight	Cat. No.
Perchloric acid, 69-72%	100g	**24,425-2A**
Phosphoric acid, 85%	100g	**21,510-4A**
Potassium phosphate, dibasic, 98+%	100g	**23,450-8B**
Potassium phosphate, monobasic, 99.99%	25g	**22,130-9A**
Sodium acetate, 99+%	500g	**23,650-0B**
Sodium citrate, trisodium salt, dihydrate, 99+%	25g	**39,807-1A**
Sodium phosphate, monobasic, monohydrate, 98+%	25g	**22,352-2A**
Trizma base, reagent grade, 99.8%	100g	**T1503B**
Trizma HCl, reagent grade, 99+%	100g	**T3253B**
Acetic acid, 99.7+%	100g	**24,285-3A**

*ACS Reagent grade unless otherwise noted

Fluka Ion Pair Reagents

	Purity %	Weight	Cat. No.
for analyzing basic compounds			
1-Decanesulfonic acid, sodium salt, monohydrate	99.9+	10g	**30631**
1-Heptane sulfonic acid	99.9+	10g	**51832**
1-Octanesulfonic acid, sodium salt, monohydrate	99+	10g	**74882**
Dodecyl sulfate, sodium salt	99	10g	**71726**
for analyzing acidic compounds			
Hexadecyltrimethyl ammonium bromide	99.9+	10g	**52347**
Tetrabutyl ammonium chloride hydrate	99+	10g	**86852**
Tetraethyl ammonium hydrogen sulfate	99+	10g	**86626**
Tetrahexyl ammonium hydrogen sulfate	99+	10g	**87299**
Tetraoctyl ammonium bromide	99+	10g	**87996**

Mobile Phase Modifiers

	Purity %	Weight	Cat. No.
Trifluoroacetic acid (protein sequencing grade, anhydrous)	99+	100g	**T1647D**
Triethylamine	99+	50g	**23,962-3**

Supelco™ Solvent Recovery Systems

SRS-3000 SRS-1000

995-0148, 997-0035

Recover and reuse clean mobile phase, dispose of only contaminated mobile phase.

- Reduce solvent purchase and disposal costs
- Save money, mobile phase preparation time, and the environment

Supelco SRS-3000 and SRS-1000 Solvent Recovery Systems can save money and time in any isocratic analysis. A microprocessor-controlled solvent switching valve monitors detector output and directs the solvent to the waste reservoir only when a peak is detected (Figure E). When the baseline falls below the threshold you select, the uncontaminated solvent is directed back to the mobile phase reservoir. Figure F shows where these systems save mobile phase. In a typical isocratic analysis, 80 to 90% of the mobile phase is uncontaminated and can be recycled. Settings for threshold, detection range, and delay time enable you to precisely control the switching valve.

In addition to the basic features mentioned above, the SRS-3000 unit offers validation output (included), an Autoclean option (see below), and storage for up to 10 method files. The validation output provides a continuous, auditable data trail of the solvent recycling valve position, for GMP, GLP, or ISO-9000 protocols. The valve position is recorded by superimposing tick marks over a separate copy of the chromatographic signal.

The SRS-3000 system also is available with a valve that enables the user to select a different solvent to flush the HPLC system. The Autoclean valve is especially useful if you are using a single pump with mobile phases containing buffer or other salts. The Autoclean valve installs between the mobile phase reservoir and the pump. It has two inlet lines, one for the mobile phase and one for the wash solvent. The valve can be factory installed, or ordered as a user-installed option.

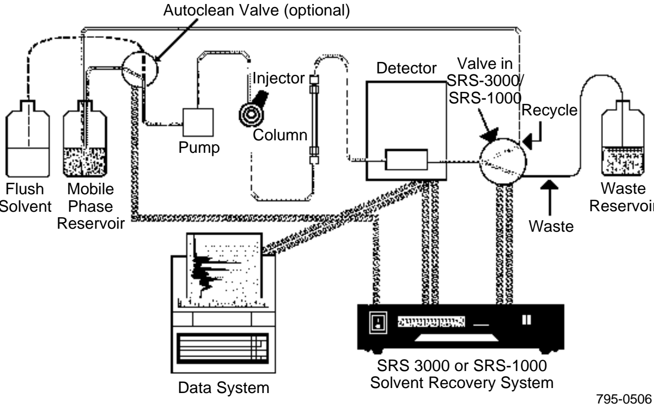
The economically priced SRS-1000 includes the same solvent-saving features as the SRS-3000 unit. A simpler display and no advanced features (no validation output, Autoclean option, or method storage memory) enable us to keep the price substantially lower.

Both solvent recovery systems include a control unit with switching valve, a power cord, a 2-lead signal cable (+/–), Teflon tubing and fittings, and an instruction manual.

The SRS-3000 system with the Autoclean valve contains the components listed above, plus the wash valve, additional tubing and fittings, a wash start cable, and a pump remote stop cable.

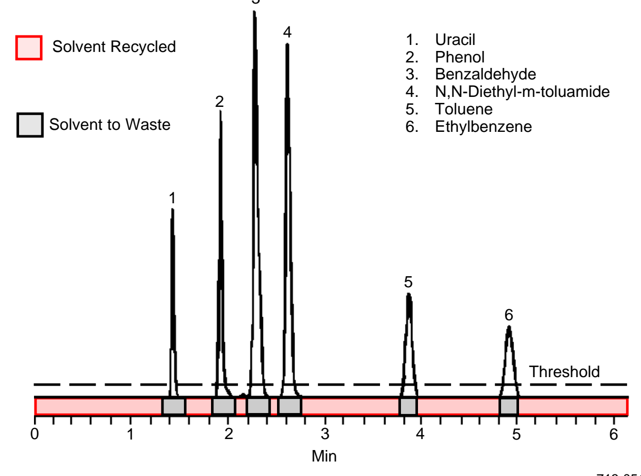
The SRS-3000 unit and the SRS-1000 unit meet all CE requirements. The SRS-1000 unit also meets UL and CSA requirements.

Figure E. Installation Is Simple



1. Connect SRS-3000 or SRS-1000 unit to detector signal output (cable is included).
2. Connect SRS-3000 or SRS-1000 unit to mobile phase and waste reservoirs and detector (Teflon tubing is included).
3. Set the threshold value and begin saving time and money.

Figure F. Recover 80% or More of the Mobile Phase Used In an Isocratic Analysis



Description	Cat. No.
SRS-3000 Solvent Recovery System	**57431**
SRS-3000 System with Autoclean	**57432**
Autoclean retrofit kit	**57433**
Switching valve assembly for SRS-3000 unit	**57435**
Override cable for SRS-3000 unit	**57434**
Switching valve for SRS-2000 unit	**57426**
2-Lead signal cable (+/-)	**57427**
3-Lead signal cable (+/-/ground)	**57428**
Replacement fuse	**57429**
SRS-1000 Solvent Recovery System	
110VAC	**506125**
220VAC	**506133**

For more information, or current prices, contact your nearest Supelco subsidiary listed below. To obtain further contact information, visit our website (www.sigma-aldrich.com), see the Supelco catalog, or contact Supelco, Bellefonte, PA 16823-0048 USA.

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