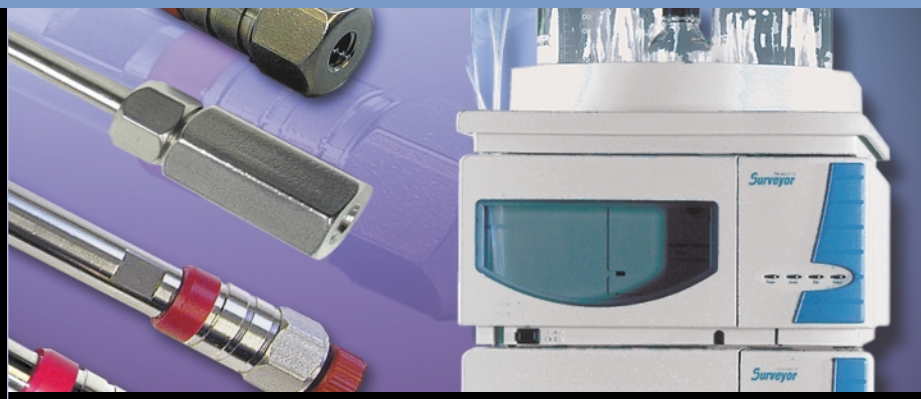
CHROMATOGRAPHY

Successful HPLC Operation



A Troubleshooting Guide – Version 1.1

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ELECTRON CORPORATION

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1. Introduction

1.1 Purpose

This guide is intended as an aid in troubleshooting problems that occur in the day to day running of your HPLC system. It is also intended as a guide to best practice in systems that are free from problems.

Its primary aims are to provide a logical process when troubleshooting, to maximize system operation time and to promote good chromatographic practices.

Wherever possible, diagrams or schematics are used for clarity. This enables the guide to be used quickly and efficiently by operators with varying experience. "Quick Tips" at the end of each section are included for rapid troubleshooting and less experienced HPLC users.

1.2 Content

Each troubleshooting chapter contains information on the most likely cause(s) of the observed problem and ways in which they can be rectified. Each table has the most probable cause of the observed fault as it's first entry.

The scope of this guide has been extended where necessary to include LC/MS column and instrument troubleshooting. The focus of the bulk of the causes of problems and their corrective action is the UV and other flow-through detectors.

2. Troubleshooting Strategy And Processes

2.1 Strategy

Any troubleshooting strategy involves five steps:

1. **Identification of the problem**
2. **Awareness of the cause(s) of the problem**
3. **Isolation of the exact cause of the problem**
4. **Rectifying the problem if able**
5. **Returning the unit to routine use OR referring the problem to your maintenance manager.**

2.2 Troubleshooting Process

To execute the strategy a systematic approach, which will work for any problem, is required. The systematic approach should follow a logical sequence, so that the exact cause of the problem can be found.

1. **Gather the facts – not theories.**
2. **Check the simplest things first – it's easier.**
3. **Compare the performance obtained to the expected performance.**
4. **List possible causes.**
5. **Work through the possible causes in a step-by-step manner checking the outcome from any changes made.**
6. **As a last resort – get help from elsewhere, for example your instrument supplier help desk or your local technical support department.**

It is important to remember that once the problem is defined and possible corrective action is identified, only one change at a time should be made; after each change, the whole system should be checked again to determine whether the problem still exists or whether the change corrected the problem.

All problems and corrective action should be documented in the instrument log. An example instrument log is available in Appendix F for your convenience.

3. Troubleshooting Isolation & Corrective Action

Before starting any troubleshooting, whether it is related to instruments or columns, it is essential that safe laboratory practices be observed. The chemical and physical properties of any solvents used should be known and the Material Safety Data Sheet (MSDS) for these solvents should be readily available.

All electrically powered instruments should be powered down and unplugged from the main supply before the removal of their covers etc.

Wear eye protection when troubleshooting the detector (with the cover removed) as ultraviolet light is emitted during UV and fluorescence detector operation and this will damage the eye's cornea irreversibly.

There are many areas in a HPLC instrument that can give rise to system and chromatographic problems. This guide will deal with each one in the following sections:

3.1 Visual Inspection
3.2 Pressure
3.3 Baseline Irregularities
3.4 Changes in Chromatography
3.5 Qualitative Results
3.6 Quantitative Results

Chromatography Data Systems (CDS) and in-depth hardware investigations will not be included in this guide as they are outside its scope. We recommend troubleshooting these systems with your supplier.

3.1 Visual Inspection

When a problem occurs, it is advisable to perform a quick visual check of the instrument and column. This will pick up leaks, loose or disconnected tubing, changes in instrument settings etc. These problems are easy to rectify and will save time.

3.2 Pressure

System pressure is affected by a number of variables including the viscosity of the solvent used, column variables, flow rate and temperature. It is important to have a reference point when comparing high or low pressures to the norm. This reference point should be the pressure generated in the system when everything is functioning correctly. It is a good idea to note the system pressure under normal operating conditions each day or each time your HPLC is used. This will allow you to spot any pressure trends that otherwise might go unnoticed.

Pressure problems fall into one of three categories: high, low or fluctuating pressure. They can occur suddenly or be a gradual process. Sudden pressure rises tend to be due to particles from the sample, blocked or damaged tubing or column packed bed collapse. Gradual pressure rises can also be due to particles in the sample, but they can also arise from particles generated in the instrument, for example, debris from vial septa or degrading seals.

Before releasing any high pressure build-up in a system, be aware that the solvent may form an aerosol or spray when loosening connections. Eye protection should be worn and ideally the connection to be loosened should be positioned above an adsorbent material to soak up all released solvents.

The simplest way to troubleshoot pressure problems is using a systematic approach, as highlighted in the following tables for high, low and fluctuating pressure.

Note: *Subsequent tables will be displayed using a cause and effect layout with the most common "fault" entered first into the table.*

3.2.1 High Pressure

High Pressure Reading

Question	Yes	No
1. Has the ambient temperature changed?	Stabilize the operating environment temperature	Go to next question
2. Is the flow rate correct?	Set the correct flow rate. Refer to the table in section 5.2.1 or your method.	Go to next question
3. Is the eluent viscous?	Calculate/check the viscosity. Viscous solvents do produce higher system pressures. If possible, dilute or change to a less viscous solvent mix.	Go to next question
4. Is the pressure transducer operating correctly?	Go to next question	Loosen transducer output fitting – set the flow to zero. Does the pressure fall to zero?
		Does adjusting the transducer zero the pressure?
		Replace the transducer as per the pump manual.
5. Loosen detector waste outlet fitting. Does the pressure return to normal?	Replace blocked tubing as per the detector manual	Go to next question
6. Loosen detector inlet fitting. Does thepressure return to normal?	Flow cell fluid path blockage. Refer to the detector manual for cleaning instructions	Go to next question.
7. Loosen column outlet fitting. Does the pressure return to normal?	Blocked outlet tubing. Replace.	Go to next question
8. Loosen column inlet fitting. Does the pressure drop to <100psi/7 Bar?	Voided or blocked column.	Go to next question
9. Loosen fitting at guard or in-line filter. Does the pressure return to normal?	Blocked guard or filter. Replace the disposable unit.	Go to next question
10. Loosen injector outlet fitting. Does the pressure return to normal?	Injector or connecting tubing blocked. Unblock as per injector manual instructions. Check that the vials are not coring and that samples are particulate free/soluble	Go to next question
11. Loosen pump outlet fittings. Does the pressure return to normal?	Outlet connecting tubing blocked. Replace tubing as per pump operating manual. Verify solvent miscibility.	Go to next question
12. Pump problem – Contact your maintenance provider.		

Quick Tip

The most common causes of high pressure are blocked tubing around the injector and column inlet.

3.2.2 Low Pressure

No/Low Pressure Reading

Question	Yes	No
1. Is the pump fuse in working order?	Go to next question	Replace fuse and re-test
2. Is the pump on?	Go to next question	Turn on the pump
3. Is there solvent flow?	Go to question 10	Go to next question
4. Is there solvent in the reservoir?	Go to next question	Refill the reservoir and re-test
5. Is the low pressure cut-off higher than the operating pressure?	Reset the low pressure cut-off to a value below the operating pressure.	Go to next question
6. Does solvent flow out of the purge valve when opened?	Go to next question	In-line filter blocked. Clean as per the pump manual instructions
7. Was the pump primed?	Go to next question	Prime as per the pump instruction manual
8. Is air visible in the solvent lines?	Remove the air – check for loose connections	Go to next question
9. Are the pump heads functioning correctly?	Go to next question	Refer to pump head maintenance in the pump manual.
10. Is the flow rate set correctly?	Go to next question	Set the correct flow rate
11. Is the column temperature constant?	Go to next question	Maintain column at a constant temperature
12. Are there any leaks?	Check for pools of liquid, buffer crystals and loose connections. Clean up and stop leaks where necessary	Go to next question
13. Is the correct solvent composition being used?	Go to next question	Use the correct solvent composition
14. Was the purge valve closed after priming?	Go to next question	Close the purge valve
15. Is the auto injector in prime mode?	Take the auto injector out of prime mode refer to injector operation manual	Go to next question
16. Is the flow rate delivered the same as the rate entered?	Go to next question	Check the actual versus theoretical flow for each solvent line. Refer to the pump maintenance manual
17. Pump problem – Contact your maintenance provider		

Quick Tip

The most common causes of no/low pressure are the solvent inlet lines not being immersed in solvent, no solvent in the reservoir and leaks.

3.2.3 Fluctuating Pressure

Fluctuating Pressure Reading

Question	Yes	No
1. Is the pressure transducer functioning correctly?	Go to next question	Set the flow to zero. Is the pressure stable? Adjust the transducer. Can the pressure be zeroed? Defective transducer. Refer to the pump maintenance manual.
2. Was the pump primed properly?	Go to next question	Prime the pump again
3. Are you performing a gradient analysis?	If pressure changes follow the gradient, then this may be normal, depending on the individual solvent viscosities	Go to next question
4. Are the pump heads functioning correctly?	Go to next question	Refer to the pump maintenance manual
5. Are all the solvents degassed?	Go to next question	Bubble with an inert gas or place in an ultrasonic bath to remove dissolved gases
6. Are all solvents miscible?	Go to next question	Check the solvent miscibility using section 5.3 of this guide
7. Are the solvents volatile?	Ensure that the operating temperature is suitable for the particular solvent used. Degas thoroughly	Go to next question
8. Pump problem – Contact your maintenance provider		

QUICK TIP

The most common cause of fluctuating pressure is poorly primed lines with badly degassed solvents.

3.3 Baseline Irregularities

Baseline irregularities can be non-cyclic (erratic) or cyclic (follow a pattern). They can originate from electrical interferences, detector faults, solvent impurities, column contamination etc.

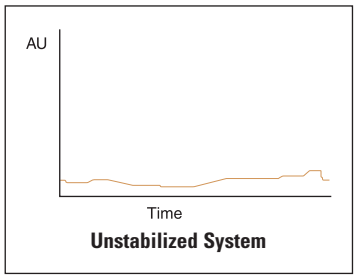
To isolate the source of a baseline irregularity, it is important to determine whether the problem lies with the fluid path, detector or electrical connections. This can be achieved by following the simple steps below:

1. Turn off the instrument pump – fluid flow must be zero
2. Monitor the baseline for 5 to 10 minutes. Note if there is any improvement in the baseline's appearance. If yes, then the problem lies within the instrument fluid path. If no, the problem is either electrical or detector related.
3. Disconnect the detector electrical cables from the A/D interface with the PC, integrator and chart recorder, i.e. the data handling devices. Attach a jump source to the input terminals on the data- handling device (a crocodile clip, paper clip etc). If the noise ceases, then the problem is within the detector or it's electrical connections. If the noise continues, then the problem is within the data-handling device.

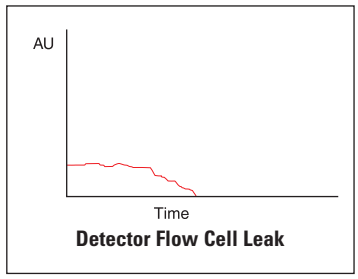
Data-handling device troubleshooting is beyond the scope of this guide.We recommend that you contact your instrument provider for this service.

The sections provide a quick reference guide for typical baseline irregularities, their causes and corrective action that can be taken to cure the problem.

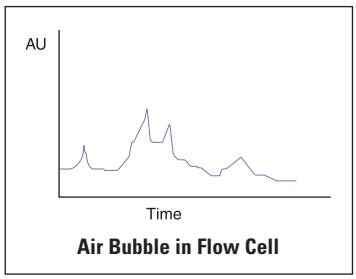
3.3.1 Non-Cyclic Noise – Fluid Path Problems



Unstabilized System



Detector Flow Cell Leak



Air Bubble in Flow Cell

Possible Cause	Corrective Action
System Not Equilibrated	Allow the column, detector etc sufficient time to stabilize. If performing gradient analysis, allow sufficient time between analyses for the system to re-equilibrate.
	The first time ion pair reagents are used with a column, allow sufficient time and volume of solvent to adequately equilibrate the column.
Contaminated Mobile Phase	Do not use mobile phase that is contaminated or thought to be contaminated. Thoroughly wash the reservoir that contained the contaminated phase. Ensure that no traces of detergent remain in the vessel, as this will cause spurious peaks in the baseline.
	Clean all solvent inlet filters in a sonic bath using 6N nitric acid, followed by water, then finally methanol.
	Prepare fresh mobile phase and purge the solvent lines with this phase. Close the purge valve and pump the new phase around the HPLC system to flush out any remaining contaminated phase. Allow the system to equilibrate prior to use.
Column Contamination	To determine whether the column is contaminated, replace it with a new column or a column where the performance is known. Flush the column with mobile phase and monitor the baseline. A baseline free from the previous noise indicates that the original column was contaminated.
	To clean the contaminated column, refer to the guidelines in section 5.6 of this guide. Please be aware that not all columns can be cleaned and not all contaminants can be removed from column beds. In such circumstances, it is prudent to replace the column with a new one immediately.
	If the baseline contains the same level of noise, even after changing the column, then it indicates that the noise is due to another cause such as solvent miscibility, contaminated mobile phase or contaminated guards/ in-line filters.
Guard/In-Line Filter Contamination	Guard cartridges and in-line filters are designed to be disposable. We do not recommend attempting to clean up these items as the costs involved in time and materials out-weights the cost of part replacement.
Air Bubble Trapped in Detector Flow Cell	To remove the air bubble, either purge the detector flow cell or apply a slight pressure to the detector waste outlet. The air bubbles usually originate from poorly degassed mobile phase, so once the bubble is removed, it is advisable to thoroughly degas the phases again.
	To stop air bubbles forming in the flow cell, attach a 30 to 90cm length of 0.23 mm ID/1.58 mm OD tubing to the detector water outlet. The tubing acts as a flow restrictor, increasing backpressure in the cell. When adding the tubing, please be aware of the backpressure limits of the flow cell.
	Note: *90 cm of tubing will produce 30 to 50 psi of backpressure at 1 mL/min*
Electrochemical Detectors Only; Air Bubble in Reference Electrode	Remove the reference electrode from the instrument and gently shake it to dislodge the air bubble.
Air Bubbles in the Flow Path	Prime the pump once again and ensure that all solvents are thoroughly degassed.

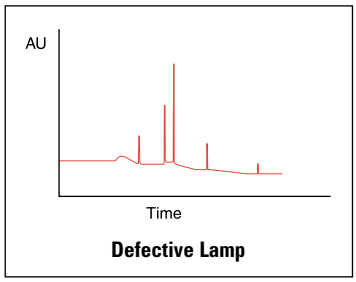
Quick Tip

The most common cause of non-cyclic baseline noise related problems is air in the system. To overcome this, all solvents should be thoroughly degassed prior to use, all lines should be purged with solvent and the pump should be thoroughly primed.

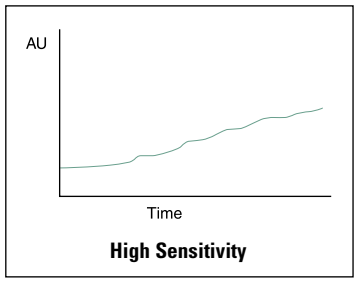
Quick Tip

Air bubbles can obscure the detector flow cell and cause baseline noise – be aware that from time to time, the cell may require cleaning and/or removal of air bubbles.

3.3.2 Non-Cyclic Noise – Detector Electronics Problems



Defective Lamp



High Sensitivity

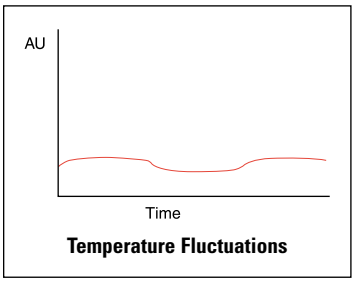
Possible Cause	Corrective Action
Detector Not Stable	After turning the detector on, allow it sufficient time to stabilize. The baseline will be stable once the detector is stabilized.
	Different detectors and conditions will require different stabilization times. We recommend referring to your detector manual for guidance.
Detector Lamp Malfunction	Check that the lamp energy and reference energy are within specification limits for normal detector operation – refer to your detector maintenance manual for guidance.
	If the lamp energy is below that recommended for normal detector operation, replace the lamp.
	Note: *Some models of detector allow the lamp energy level to be manipulated when the lamp nears the end of its useful lifetime. Refer to your detector maintenance manual for details, if applicable.*
Contaminated Detector Flow Cell	Clean the flow cell as per the maintenance instructions given in your detector manual.
	Alternatively, remove the analytical column and replace with a union. Flush the system with water, followed by methanol, then water to remove any excess buffers (ensure that water and methanol are compatible with the last solvent to pass through the flow cell).
	The detector flow cell can also be cleaned with a 50/50 v/v mixture of THF/water, then 100% THF if the system is used in normal phase. (Once again, ensure that the solvents used in the cleaning procedure are compatible with that last used in the flow cell.)
Detector Electronic Problem	Contact your maintenance provider.
Cables	Check that all cables are securely seated in their respective terminals. Ensure that all output switches have the correct setting are in the correct position. All cables should be well maintained and grounded where necessary.
Radio Interference	The detector should be isolated from all sources of radio interference or cycling equipment, for example, large electric motors.
	The detector should be adequately grounded.
	If necessary, move the detector away from the source of interference or position it within a Faraday Cage.
Gain/Sensitivity Setting Too High	Re-set to a lower value on the data handling device.
Reference Electrode Leak	ECD Only – Refer to the detector maintenance manual for repair or replacement instructions.
Dirty Reference Electrode	Replace electrode filling solution and frit.
Contaminated/Scratched Reference Electrode	Polish/clean the working electrode. If the problem remains, replace the working electrode.

Quick Tip

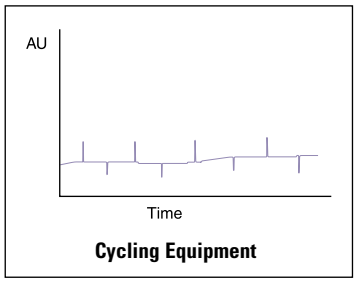
The most common cause of problems related to electronic baseline noise is the detector. Usually, if the detector is allowed insufficient time to equilibrate before an injection is performed, then the resultant chromatogram will contain spurious peaks and there will also be some evidence of baseline drift.

If the problem occurs after routine maintenance, check that all the cables are securely seated in their sockets and that the correct cable is in the correct socket. Also check that all settings have been returned to their positions prior to the routine maintenance.

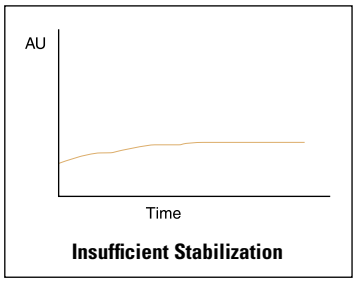
3.3.3 Cyclic Noise – Detector Related Problems and Others



Temperature Fluctuations



Cycling Equipment



Insufficient Stabilization

Possible Cause	Corrective Action
Sort Term Cycling Equipment or Radio Interference	Refer to the previous table.
Long Term Detector Temperature Problems	The heater cycles on and off to maintain the detector temperature. Change the regularity of the on/off frequency to avoid baseline noise.
Ambient Temperature Fluctuations	Stabilize the air temperature around the instrument and allow the system to return to equilibrium. If this is not possible, relocate the instrument to a laboratory position where the detector is thermally stable and/or avoid placing the instrument in direct sunlight.
Baseline Drift – Unstable Detector	Refer to the previous table.
Baseline Drift – Ambient Temperature Change	Refer to "Ambient Temperature Fluctuations" in this table.
Baseline Drift – Contaminated Detector Flow Cell	Refer to the previous table.
Baseline Drift – Dirty Reference Electrode	Refer to the previous table.
Baseline Drift – Scratched or Contaminated Reference Electrode	Refer to the previous table.
Noise Spikes – Detector Lamp Malfunction	Refer to the previous table.
Noise Spikes – Cycling Equipment and Radio Interference	Refer to the previous table.
Noise Spikes – Detector Electronics Problems	Contact your instrument maintenance provider.

Quick Tip

The most common cause of cyclic baseline noise is the detector. Usually, if the detector is allowed insufficient time to equilibrate before an injection is performed, then the resultant chromatogram will contain spurious peaks and there will also be some evidence of baseline drift.

3.4 Changes in Chromatography

The most common changes in chromatographic response are related to the shape and separation of peaks, their elution times and changes in established performance.

There are two stages to troubleshooting unacceptable chromatography. First, the evaluation of what you have, chromatographically speaking and secondly, isolation of the source of the problem.

The evaluation stage is best performed on a standard rather than sample injection. The nature and prior chromatographic performance of the standard should be recorded each time it is injected. This will provide historical data for any comparisons that you need to perform. A typical record sheet for recording standard data is shown in Appendix E.

The following sections will list each of the most common causes of change in chromatographic response and corrective action that can be made to return your chromatography to its previous state.

3.4.1 Retention Time Changes from Injection to Injection

Possible Cause	Corrective Action
System Not Equilibrated	Allow the column, detector etc. sufficient time to stabilize. If performing gradient analysis, allow sufficient time between analyses for the system to re-equilibrate. The first time ion pair reagents are used with a column, allow sufficient time and volume of solvent to adequately equilibrate the column.
Insufficient Equilibration – Gradient Analysis	A suitable period of time for equilibration must be allowed between gradient analyses. This allows the T_0 mobile phase composition to be pumping through the column as the injection occurs.
	Insufficient equilibrium time results in erratic retention times.
Pump Pressure/Action Problems	Refer to the flow chart in section 3.2.3 of this guide. Identify the problem and correct it.
Ambient Temperature Variations	Stabilize the ambient temperature around the instrument and allow it to come to equilibrium. If this is not possible or cannot be achieved, we recommend the use of a column heater/cooler. The unit should fully enclose the column and ideally be adjustable to compensate for multiple column dimensions.
	Ensure that all pre-mixed solvents are miscible and that the solution is homogeneous.
	***Note**: Some methods will also benefit from pre-heated solvents prior to passage through the column.*
Volume Injection/Concentration Too High	Either reduce the injection volume used or dilute the original sample.
	***Note:** Using a weaker solvent means that the injection volume can be increased to approximately 10% of the column void volume. Using a strong solvent means that the injection volume can only be up to 1% of the column void volume.*

Quick Tip

The most common cause of peak retention time drift is an un-equilibrated system. The detector and fluid system must be stable prior to starting an analysis.

Temperature changes during the analysis are another major cause of peak drift. If your analytical column is subject to fluctuations in temperature, then we recommend that the column is housed in a thermally controlled environment, such as a column oven/jacket etc.

Finally, where possible, we recommend the pre-mixing of all solvents used in isocratic methods.

3.4.1 Retention Time Changes from Injection to Injection (Continued)

Possible Cause	Corrective Action
Solvent Blending Problems	Check the miscibility of the solvents – if their miscibility is poor, consider changing one or all to give a miscible mix. Refer to section 5.3 for a solvent miscibility table.
	If the solvent is mixed manually, ensure that it is filtered and thoroughly degassed before use. The solvent line and pump should be thoroughly primed with this solvent to remove all traces of previous solvents and air. The column should have a minimum of 10 column volumes of solvent passed through it to allow equilibration. Finally, a series of standard injections should be performed to ensure that the system is performing reproducibly
	If the retention times are reproducible, the problem was due to insufficient preparation of the solvents and system.
	If the solvent is mixed automatically from two or more reservoirs, follow the same procedure as listed above, but this time more solvents will require filtering, more lines priming etc. It is also advisable to mix the solvents manually and repeat the procedure to check whether there is a problem in the pump mixing/proportioning cell.
	Automatic Mixing: If the retention times are reproducible, the problem was due to insufficient preparation of the solvents and system. If the times remain erratic, perform the manual mixing analysis.
	Manual Mixing: If the retention times are reproducible, there is a problem in the mixing/proportioning unit of your pump. Refer to the pump maintenance manual for repair/cleaning instructions.
	Note: *If the system is used for multiple methods, ensure that a gradient program is not being used in place of an isocratic program and vice versa.*
Column Contamination	Replace the column with one where the performance is known. Perform a series of standard injections and compare the peak retention times. If they were reproducible, then it would indicate that your original column is contaminated. It can be cleaned using the procedures listed in section 5.6 of this guide.If the erratic peak times continue, the problem could be due to solvent immiscibility, contaminated solvent or contaminated guards/inline filters.
	If guards are used, it is advisable to remove them prior to the calculation of column efficiency. Replace them with new units once the test is complete.

3.4.2 Continually Increasing or Decreasing Retention Times

QUICK TIP

The most common cause of peak retention time drift in one direction is poorly prepared or mixed solvents or a system leak.

If you are confident that the solvents were prepared correctly, then it is very important that you determine whether they are being mixed correctly (mixing cell problems). Where solvents are mixed manually prior to pumping, ensure that the solvent flow rate is correct and constant.

Possible Cause	Corrective Action
Ambient Temperature Changes	Refer to the previous table.
Flow Rate Changes	Ensure that the flow rate delivered is the same as that entered into the pump software. Also, ensure that the flow rate being used is correct for the application.
Insufficient Equilibration	Refer to the previous table.
Column Contamination	Refer to the previous table.
Column Degradation	Using a standard mixture, solvent and analysis conditions, as close to those used to generate your column's test certificate as possible, calculate the columns efficiency (N). If N is markedly lower than quoted on the manufacturers certificate of analysis, try cleaning the column using the procedures in section 5.6 of this guide. Repeat the efficiency calculation.
	If there is no increase in efficiency, replace the degraded column with a new one.
	You may wish to contact your column supplier if the degraded column is relatively new to discuss whether it was a handling error that caused the degradation.
Solvent Preparation	Ensure that all solvents are freshly prepared and free from microbial growth. Filter and degas thoroughly prior to use.
	Discard old solvents and thoroughly wash all dirty reservoirs prior to re-use. Prime the pump and solvent lines with freshly prepared solvents and allow the column/system to equilibrate.
Solvent Delivery System Blockage	Check the solvent lines and inlet filters for blockages. Refer to the pump maintenance manual for cleaning/replacement of blocked or dirty parts.
System Leaks	Check all fittings and unions for leaks.
	Tighten any loose fittings, but do not be tempted to over tighten as this may damage the fitting's threads and cause leaks.
	Where leaks occur between tightened fittings, replace the fitting and ferrule since they may be damager or misaligned.

3.4.3 Increasing/Decreasing to a New Constant Retention Time

Possible Cause	Corrective Action
Mobile Phase Inconsistencies	Check that the solvents in use are the correct ones, of the correct strength and are present in the correct composition. If additives, for example, preservatives are used, ensure that they are the correct ones and do not interfere with the analyte in your sample.
	Prepare fresh solvents
	Note: *It is always worth checking the pH of any buffers used. If the buffer is not freshly prepared, then it may have been contaminated or absorbed CO_2 from the atmosphere etc. These factors would affect the buffer pH and ultimately your sample chromatography.*
Changes in Solvent Flow Rate	Ensure that the flow rate delivered is the same as that entered into the pump software. Check all solvents lines that are in use for the method.
	If the measured flow rate is not the same as that entered into the pump software, refer to the pump maintenance manual for further assistance or contact your maintenance provider.
	Also, ensure that the flow rate being used is correct for the application.
Incorrect Column	Check that the column in place in the system is the correct one for the method. Dimensions and packing materials will make a considerable difference between chromatograms using the same chromatographic conditions.
Temperature Changes	Refer to the table in section 3.4.1
Column Contamination	Refer to the table in section 3.4.1
Gradient Delays	Check the maintenance schedule for the pump/fluid system to find out whether there have been any changes since the system was last used. If a change has been made, re-calculate the new gradient delay volume. Refer to the pump operation manual for details of this calculation or contact the instrument manufacturer.

QUICK TIP

The most common cause of peak retention time drift to a new constant value direction is poorly prepared or mixed solvents.

If you are confident hat the solvents were prepared correctly, then it is very important that you determine whether they are being mixed correctly (mixing cell problems).

Where solvents are mixed manually prior to pumping, ensure that the solvent flow rate is correct and constant.

The last most common cause of retention time change is a leak in the system or build up of contaminants.

3.4.4 Abnormal Peak Shape

Abnormal peak shape encompasses a range of possible peak shape problems.

- **No peaks**
- **Fronting or tailing peaks**
- **Smaller than expected peaks**
- **Double peaks/shouldering peaks**
- **Broad peaks – early eluting analytes or all analytes**
- **Flat topped peaks**
- **Negative peaks**

If all the peaks in a chromatogram are affected, then it suggests that the problem is related to either the system or the column. If only early eluting peaks are affected then it suggests that the problem lies within the fluid path – perhaps with incorrect ID tubing, fittings etc.

If single peaks are affected, then it suggests that there might be a specific chemistry problem. The method in use should be examined for areas where the chemistry may not be correct.

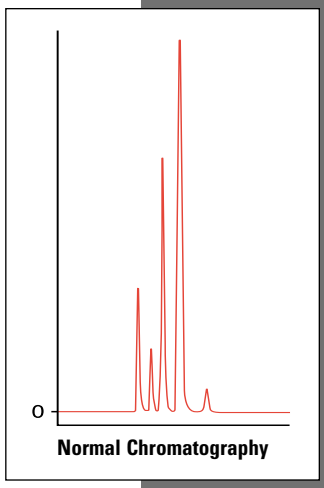
Gradient methods, where early eluting peaks are abnormal and later peaks are acceptable may be suffering from pre-column band broadening. If all the peaks are abnormal, then post-column band broadening or other changes in the system are the most likely causes.

Isocratic methods, where the early eluting peaks are abnormal and the later peaks are acceptable may be suffering from extra-column band broadening, injector problems, incorrect detector time constant or incorrect A/D sampling time. If all the peaks are abnormal, then extra-column band broadening or other changes to the system are the most likely causes.

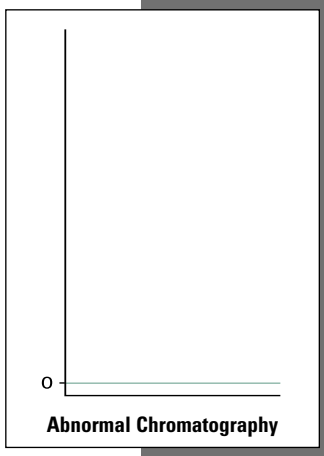
Each table in this section will show an example of the type of chromatography being investigated; it's cause(s) and any corrective action that can be taken.

No Peaks

Possible Cause	Corrective Action
Injector Problem	**No vial:** Fill autosampler position with a vial.
	Empty vial: Fill the vial with sample.
	Over full vial: Replace with a vial where there is an air gap between the top of the fluid and vial cap.
	Wrong vial: Check that the correct sample was injected.
	Insufficient sample: Inject a more concentrated sample solution or a larger volume of the same sample solution.
	Incorrect injection volume: Inject the correct volume of sample.
	Needle blocked: Remove the injector needle from its support, as per the autosampler maintenance manual and either clean or replace it. The blockage may be due to the vial septa degrading or coring. Ideally replace all vial septa/caps with a non-coring variety. Other sources of blockage include particulates in the sample. Ensure that all samples are filtered prior to injection.
Fluid Path Problem	Typically, no flow or very low flow.
	Ensure that there is power to the pump and that it is switched on. Check that solvent comes out of the detector waste line when the pump is set running. Check that the solvent reservoirs contain solvent and that the solvent inlets are at a suitable height to draw any liquid present. Also check that the fluid inlet filters and lines are not blocked.
	If no solvent flow occurs, refer to the pump maintenance manual or contact your maintenance provider.
Column Problem	Typically, incorrect or missing column. Check that there is a column in the system, that it is the correct dimension and that it is packed with the correct media for the application.
Detector Problem	Ensure that the detector is set to the correct wavelength and that sensitivity and auto zeros are also correctly set.
	Zero the detector baseline if necessary.
	Check all power cables and connections between the detector and data-handling devices. Ensure that all output signal switches are in the correct position.
Incorrect Solvent/Sample	Prepare new solvents, prime all lines and the pump and allow the system to reach equilibrium.
	Make sure that the sample injected is the correct one, that it is of the correct strength and that it has not degraded. Replace where possible.
	If sample preparation techniques involved an extraction or similar, ensure that the correct sample and solvents were used. Where possible, repeat the sample preparation and check that all reagents/extraction equipment are correct and within their shelf lives.
	Note: *Precipitation of the sample because of incompatibility with solvents will also result in no peaks being detected. There is often a rise in system backpressure accompanying this problem.*



Normal Chromatography



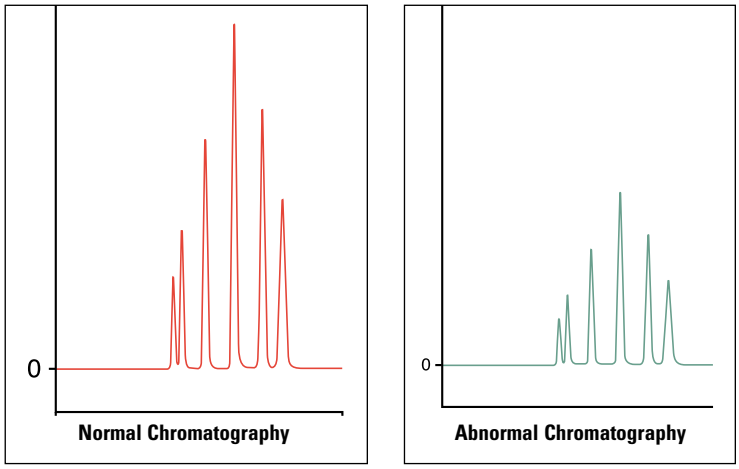
Abnormal Chromatography

Quick Tip

Lack of chromatogram peaks is often due to either the wrong sample being injected, the detector not being switched on or a blockage between the injector and detector lines.

The next most common reason for a lack of peaks is that some part of the sample or mobile phase preparation has been performed incorrectly, so it is always worth revisiting to check that the correct buffer has been used, the sample/solvent pH is correct etc.

Smaller Than Expected Peaks



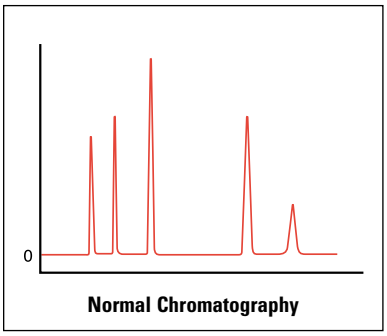
Quick Tip

Smaller than expected peaks are often due to either the wrong sample being injected, an incorrect sample volume being injected, or a blockage between the syringe needle and detector. Problems with the syringe plunger sticking in the barrel can occur if the sample contains particulates.

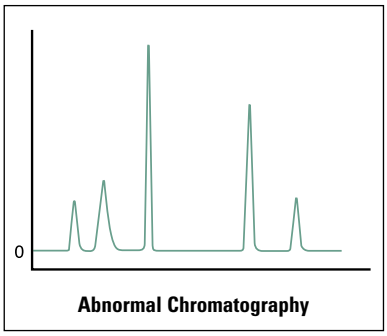
Note: *Viscous samples will require a longer draw time. Insufficient draw time will result in a lower volume of sample being injected onto the column and smaller peaks will result.*

Possible Cause	Corrective Action
Vial Problem	Check that the vial contained enough of the correct sample to perform the injection – if not, replace with a fresh one.
	Make sure that the vial seats correctly in the autosampler and that the needle is not obstructed when performing an injection.
	Note: *If the vial were over-full, a vacuum would form as the needle tried to draw solvent from it. This would result in smaller than normal peaks. Ensure that all sample vials contain up to two-thirds liquid and one-third air and that the caps (where possible) are not over tightened.*
Syringe Malfunction	Check the syringe for cracks, dirty or worn barrels and plungers, sticking etc. Remove and clean the syringe where possible. Where this is not possible, remove and replace.
	Check the needle for blockages and deformities. Replace where necessary.
Sample Loop Incorrect	Change the sample loop to the correct volume if the one in-situ is incorrect.
Wrong Injection Volume	Inject the correct volume.
Detector Problem	Zero the detector output.
	Check the output signal between the detector and data-handling device. If the detector flow cell is contaminated, clean the flow cell windows as per the instructions in your detector maintenance manual.
	Check that the detector lamp energy is within specified limits for operation. If the energy is markedly lower than when the lamp was new, replace it.
	Note: *Some models of detector allow the lamp energy level to be manipulated when the lamp nears the end of its useful lifetime. Refer to your detector maintenance manual for details, if applicable.*
	Finally, ensure that the detector wavelength setting is optimized for the analyte chromophores. Refer to section 5.5 of this guide for chromophore detection wavelengths.
Sample Too Viscous	Dilute the sample or decrease the rate at which the syringe draws the sample.

Early Eluting Peaks Broad



Normal Chromatography



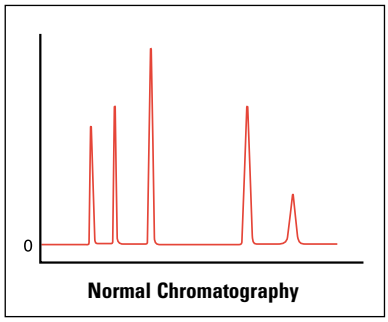
Abnormal Chromatography

Possible Cause	Corrective Action
Sample Overload	Dilute the sample or inject a lower volume to stop equilibrium disruption.
	Note: *Using a weaker solvent means that the injection volume can be increased to approximately 10% of the column void volume. Using a strong solvent means that the injection volume can only be up to 1% of the column void volume.*
Blockage Before the Column – Associated Pressure Increase	Check the guard, in-line filter, column inlet and all associated tubing for blockages.
	Replace any blocked tubing, filters or guard units. If the column inlet frit has been blocked, gently back flush the column with a wash solvent at very low flow rate (preferably over night for best effect). Re-invert the column and equilibrate with test solvent. If this procedure has not removed the blockage, please contact your column supplier for further technical advice.
Incorrect Tubing ID	Measure the level of band spreading throughout the system. Refer to section 5.1.6 of this guide for details of the calculation.
	If the level of band spreading is greater than the limits specified for the system, ensure that all tubing is appropriate for the job it is intended for – for example, all internal diameters are as narrow as possible. Also check that all tubing is cut with a flat-end. This will enable it to fit into unions or fittings with far greater efficiency and minimize excess dead volume.
Injector Problem	With reference to the autosampler maintenance manual, check that the valves in the injector system are not sticking or leaking. Clean or replace as necessary.
	Also check that the needle and seat are not blocked or damaged. Once again, clean or replace as necessary.
Detector Time Constant Incorrect	Determine the detector time constant setting and adjust accordingly. Details of how to determine the time constant can be found in the detector operator manual.
Late Eluting Peaks/Carry-over	Ensure that there is no analyte carry over from previous injections, especially from previous injections where gradient analyses are being performed. Determine whether the broad peaks are due to strongly retained analytes from a previous injection by performing a single injection of sample and increasing the run time.
	Important: *Insufficient equilibration time between injections where a gradient analysis is perform can also affect peak shape. Check that all wait times between injections are sufficient to allow reproducible chromatography.*

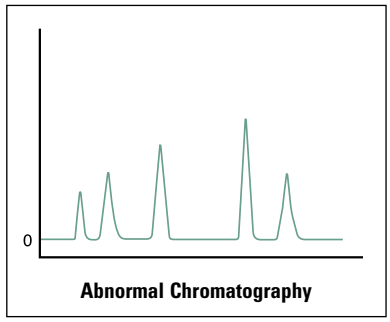
Quick Tip

Broad early eluting peaks are most commonly associated with sample overload or incorrect system plumbing.

All Peaks Broad



Normal Chromatography



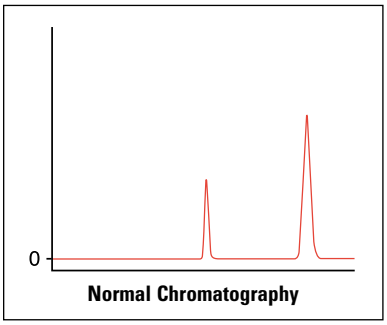
Abnormal Chromatography

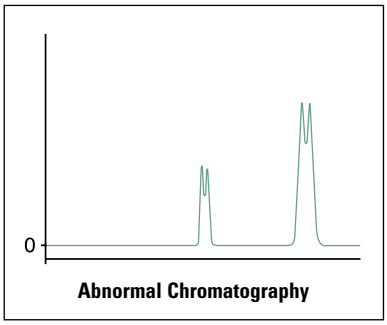
Quick Tip

Broad peaks (all) are most often due to errors in instrumentation or column. It is worthwhile investigating the column and guards first as they often are the critical part of the system

Possible Cause	Corrective Action
Sample Solvent Too Strong for the Mobile Phase	Dissolve the sample in mobile phase, use a weaker diluent or make a smaller injection.
Ambient Temperature Change – Column	Use a column heater or cooler unit to stabilize the temperature around the column.
System not at Equilibrium	Allow the column, detector etc. sufficient time to stabilize. If performing gradient analysis, allow sufficient time between analyses for the system to re-equilibrate.
	The first time ion pair reagents are used with a column, allow sufficient time and volume of solvent to adequately equilibrate the column.
Older Systems Only – Chart Speed Incorrect	Set the chart speed to a faster rate.
Column Problems	Contamination: Clean the column following the guidelines in section 5.6 of this guide. If the problem remains after cleaning, replace the column.
	Column degradation: Replace the column with one where the performance is known. Perform a series of standard injections and compare the peak retention times. If they were reproducible, then it would indicate that your original column is contaminated. It can be cleaned using the procedures listed in section 5.6 of this guide.
	If the erratic peak times continue, the problem could be due to solvent immiscibility, contaminated solvent or contaminated guards/inline filters.
	If guards are used, it is advisable to remove them prior to the calculation of column efficiency.
	The guard may also be the source of contamination, so it is worth changing the guard at the same time as the column.
Incorrect Column	Check that the column in use is of the correct dimension and is packed with the correct media for the application.
Guard Problems	Replace contaminated or degraded guards and check the system performance. It should improve. If there is no improvement, check for column contamination/degradation, as previously discussed.

All Peaks Doubling



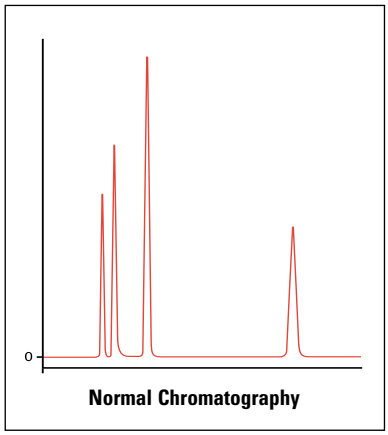


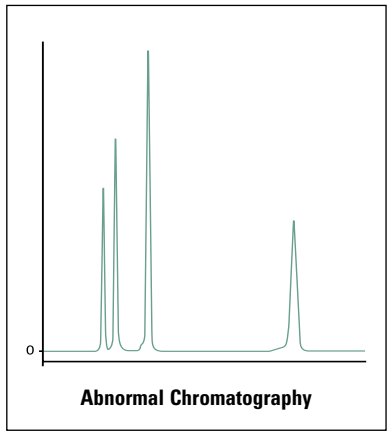
Possible Cause	Corrective Action
Blockage/Partial Blockage Before the Column	Check the guard, in-line filter, column inlet and all associated tubing for blockages.
	Replace any blocked tubing, filters or guard units. If the column inlet frit has been blocked, gently back-flush the column with a wash solvent at very low flow rate (preferably over night for best effect). Re-invert the column and equilibrate with test solvent. If this procedure has not removed the blockage, please contact your column supplier for further technical advice.
Column Voiding	Using a standard mixture, solvent and analysis conditions, as close to those used to generate your column's test certificate as possible, calculate the columns efficiency (N). If N is markedly lower than the manufacturer's test certificate value, try cleaning the column using the procedures in section 5.6 of this guide. Repeat the efficiency calculation.
	If there is no increase in efficiency, replace the degraded column with a new one.
	You may wish to contact your column supplier if the degraded column is relatively new to discuss whether it was a handling error that caused the degradation.
Guard Column Voiding	Remove the defective guard and replace with a new one. Allow the system to reach equilibration and repeat the sample injection.
	If the problem persists, remove the guard and holder and perform an injection. If the problem remains, it is not related to the guard. If the problem disappears, check the connections between the guard unit and the column. Ensure that the connector adds zero dead-volume to the system and that the guard cartridges fit snugly into the holder. Ideally, use an integral guard.
Injection Disrupting Equilibrium	Dissolve the sample in mobile phase, use a weaker diluent or make a smaller injection.

Quick Tip

The most common cause of peak doubling can be either blockage prior to the column or column or guard voiding.

Fronting Peaks



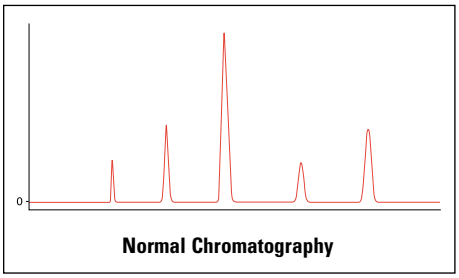


Possible Cause	Corrective Action
Injection Disrupting Equilibrium	Dissolve the sample in mobile phase, use a weaker diluent or make a smaller injection.
Column Voiding	Using a standard mixture, solvent and analysis conditions, as close to those used to generate your column's test certificate as possible, calculate the columns efficiency (N). If N is markedly lower than is quoted on the manufacturers certificate of analysis, try cleaning the column using the procedures in section 5.6 of this guide. Repeat the efficiency calculation.
	If there is no increase in efficiency, replace the degraded column with a new one.
	You may wish to contact your column supplier if the degraded column is relatively new to discuss whether it was a handling error that caused the degradation
Guard Column Degrading and Contamination	Replace contaminated or degraded guards and check the system performance. It should improve, if there is no improvement, check for column contamination/degradation, as previously discussed.

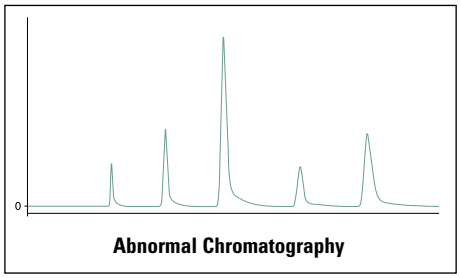
Quick Tip

Fronting peaks are very often due to large injection volumes of a sample that is dissolved in solvents that are incompatible with the mobile phase being used. The next most common cause of peak fronting is a voided or contaminated guard or column.

Tailing Peaks



Normal Chromatography



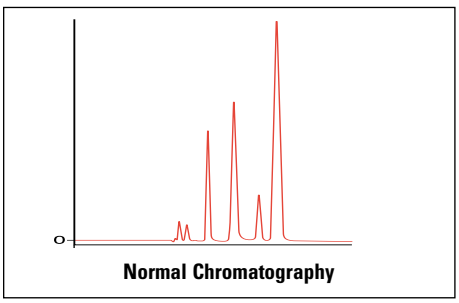
Abnormal Chromatography

QUICK TIP

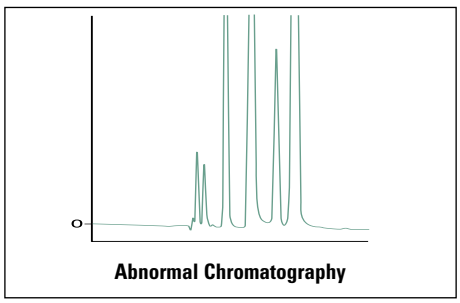
Tailing peaks are typically caused by column degradation or inlet contamination. Carefully maintained columns and guards will considerably reduce the incidence of tailing peaks.

Possible Cause	Corrective Action
Column Problems	**Column contamination:** Clean the column following the guidelines in section 5.6 of this guide. If the problem remains after cleaning, replace the column.
	Column degradation: Replace the column with one where the performance is known. Perform a series of standard injections and compare the peak retention times. If they were reproducible, then it would indicate that your original column is contaminated. It can be cleaned using the procedures listed in section 5.6 of this guide.
	If the erratic peak times continue, the problem could be due to solvent immiscibility, contaminated solvent or contaminated guards/inline filters.
	If guards are used, it is advisable to remove them prior to the calculation of column efficiency.
	The guard may also be the source of contamination, so it is worth changing the guard at the same time as the column.
Guard Problems	Replace contaminated or degraded guards and check the system performance. It should improve. If there is no improvement, check for column contamination/degradation, as previously discussed.
Injector Problem	The problem may be due to leaking or malfunctioning valves or a damaged or blocked needle.
	Refer to the injector maintenance manual.
Detector Problem	Check that the detector time constant setting is correct. Change if necessary.

Flat Topped Peaks



Normal Chromatography



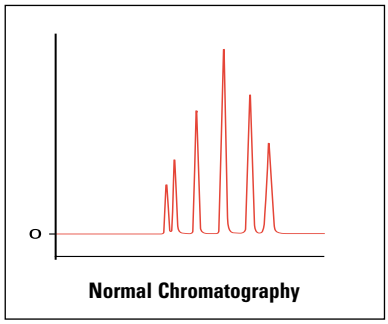
Abnormal Chromatography

QUICK TIP

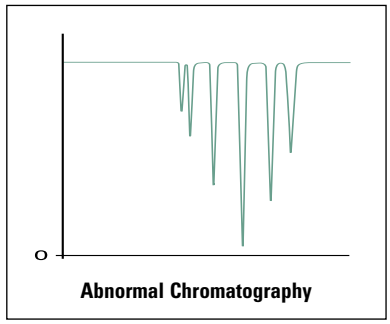
Flat-topped peaks are most often caused by either large injection volumes of dilute sample or by small injection volumes of strong sample solution.

Possible Cause	Corrective Action
Injection Problem	Ensure that the volume of sample injected is correct and that the sample is of the correct strength.
	Dilute the sample or perform a smaller injection.
Detector Setting Error	Check the wavelength, zero and sensitivity settings. Adjust if necessary.
Recorder Input Error	Adjust the recorder input voltage.

Negative Peaks



Normal Chromatography



Abnormal Chromatography

Possible Cause	Corrective Action
Signal Polarity Setting Incorrect (All Peaks)	Change the polarity setting and repeat the injection. The peaks should automatically invert. Refer to the detector operator manual for further details.
Cables Reversed (All Peaks)	Reverse the cables from the detector to the data-handling device.
RI Detector Only – Unbalanced Optics (All Peaks)	Balance the optics.Refer to the detector operator manual for further details.
Highly Adsorbing Mobile Phase (≥1 Peak)	Dissolve the sample in mobile phase. If the peak is due to contaminated solvents, replace all solvents.
Air Bubble Injected in System (≥1 Peak)	Refer to the injector system maintenance manual for troubleshooting.
RI of Analyte Lower than that of the Mobile Phase (≥1 Peak)	Determine whether the peak is due to sample or solvent contamination. If the peak is due to contaminated solvents, replace all solvents.
Ion Pair Separation Only – System Peak (≥1 Peak)	Dissolve the sample in mobile phase.

Quick Tip

Negative peaks are most often caused by differences in refractive index between the sample solvent, sample and mobile phase.

They are also caused after routine maintenance when the system has not been reconfigured correctly.

3.5 Qualitative Results

Qualitative assays do not measure exact quantities of an analyte in solution. Therefore, problems associated with these assays usually fall into one of two categories; a) missing or extra peaks and b) peak mis-identification.

The following tables will assist in tracing errors in qualitative methods.

Missing Peaks

Possible Cause	Corrective Action
Incorrect Solvents Used	Ensure that the solvents used are correct for the method/sample. Prepare fresh solvent if necessary.
	Note: *if the problem occurs with an increase in system pressure, then this would indicate sample precipitation within the LC system. Follow the flow chart in section 3.2.1 to determine where the increase in pressure originates.*
Sample Degradation	Perform one injection of a standard. If all the peaks are present and correct, the sample has degraded. Re-prepare the sample and re-inject.
Resolution Lost	Replace the column with one where the performance is known and re-inject the sample. If the missing peak(s) re-appear, then the efficiency of your original column may be lower than that of the replacement column.
	Check the efficiency of the original column using a test mix and conditions similar to those used by the column manufacturer to test efficiency when the column was new. For columns that have been in use for an extended period of time, perform a column clean up as per section 5.6 of this guide. Repeat the efficiency test to determine whether this has improved column performance. You may wish to contact your column supplier if the low efficiency column is relatively new to discuss whether it was a handling error that caused the problem.
Column Incorrect	Ensure that the correct media and dimension of column is used. Also check that the correct media particle size is used as this will have an impact on the resolution of closely eluting peaks – a 3 µm media will provide higher column efficiency than a 5 µm media.
Solvent Flow Program Inconsistencies	Verify that the analysis uses the correct proportions of solvents and that the correct gradient or isocratic path is being followed.
	Check that the solvent flow rate is accurate from all lines and that the correct flow rate is entered into the pump software.
	If a gradient is used, check that the time between analyses is sufficient to allow the system to re-equilibrate.
No Peaks Found	Refer to section 3.4.4 of this guide.

Quick Tip

Single or multiple missing peaks are usually due to the wrong sample being injected or the sample degrading.

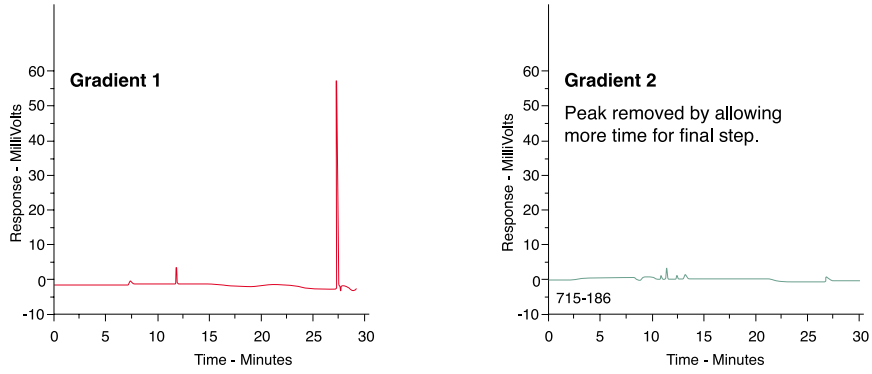
Equally likely though is a loss of resolution due to column/ solvent inconsistencies.

Extra Peaks

Possible Cause	Corrective Action
Contaminated Solvents	Discard all old solvents. Prepare fresh buffers etc. and place in clean dry reservoirs. Purge all lines and the pump with the new solvents. Allow the system to come to equilibrium and perform a standard injection. The spurious peaks should not be present.
Mobile Phase – Gradient Methods	Ghost peaks can be caused by changes in mobile phase composition during gradient methods. An example of such a peak is shown in the chromatograms that follow this table.
Contaminated/Degraded Sample	Perform one injection of a standard. If all the peaks are present and correct, the sample has been contaminated or has degraded. Re-prepare the sample and re-inject.
Fluid Path Noise	Refer to section 3.3 of this guide
Sample Loop Flush Inadequate	Ensure that the sample loop is thoroughly flushed with solvent between injections to avoid carry-over.
Contaminated Injector	Flush the injector with solvent. If necessary, replace all perishable parts, for example seals and filters.
Contaminated Column	Clean the column following the guidelines in section 5.6 of this guide. If the problem remains after cleaning, replace the column.
Contaminated Guard	Replace contaminated guard and check the system performance. It should improve. If there is no improvement, check for column contamination, as previously discussed.

Quick Tip

Extra peaks in chromatograms are more often than not due to contamination or degradation of the sample, mobile phase or column. To check if the extra peak(s) is/are in the sample alone, perform a blank injection of sample solvent. The peak(s) should be absent.



Gradient 1	Gradient 2	%A	%B
0	0	90	10
20	20	0	100
25	25	0	100
26	30	90	10

Column: 5 µm BetaBasic™ C18, 150 x 4.6 mm
Solvent A: 0.1M Ammonium Acetate, pH 4.7
Solvent B: Acetonitrile
Flow Rate: 1 mL/min
Detector: UV at 254nm

Peaks Misidentified

Possible Cause	Corrective Action
Data-Handling Inaccuracies	If a data-handling system identifies your peaks then ensure that all the peak retention time variables such as peak windows, threshold, integration and retention times are correctly entered into sample and calibration tables etc.
	Make any necessary changes and perform a standard injection to ensure that peaks are now correctly identified.
	Refer to the data handling operators manual for further details.
Peak Retention Time Variation	Refer to sections 3.4.1 to 3.4.3

Quick Tip

Peak mis-identification occurs most often in degradation samples or those in which related substance levels are being measured. This is because the software is "calibrated" using a standard mixture with specific concentrations of each impurity. Most "real-life" samples contain impurities at very low levels so the retention time of their peaks will be slightly different to those generated by the standard mix.

Identification windows should be set widely enough to take into account this time variation with respect to concentration.

3.6 Quantitative Results

Quantitative assays measure exact quantities of an analyte in solution. Therefore, problems associated with these assays usually fall into one of two categories; a) loss of precision and b) loss of accuracy.

The most common mistake made is to assume that accuracy and precision are the same. Accuracy is defined as the proximity of a result to the true value whereas precision is a measure of reproducibility of the result. It is important to determine whether your method is inaccurate or imprecise, as this will determine the steps taken to rectify the problem. In general, if the result generated is incorrect, but reproducible then the problem is accuracy based. If the results generated vary, then the problem is precision based.

The following tables will assist in tracing errors in quantitative methods.

Loss of Accuracy

Possible Cause	Corrective Action
Incorrect Sample/Sample Preparation	Check that the correct sample has been prepared and that the preparation has produced a solution that is of the correct strength. At the same time, check that any internal/external standards have been prepared correctly and are of the correct strength.
Solvent Evaporation	Samples prepared in volatile solvent, such as chloroform, DCM etc. are affected by changes in ambient temperature. They will evaporate more quickly in warmer temperatures, producing more concentrated sample solutions. Ensure that the ambient temperature around the sample remains constant and that the vial cap is sufficiently tight to stop evaporation, but not tight enough to create a vacuum when injections are performed.
Degraded/Contaminated Sample	Refer to the table detailing loss of precision.
Peak Integration Error	Refer to the table detailing loss of precision.

QUICK TIP

Loss of accuracy is most often related to the sample.

Loss of Precision

QUICK TIP

Loss of precision is most often caused by an injector error, by a sample that is mixed poorly, or a sample that is degrading.

Possible Cause	Corrective Action
Injection Error – External Standard	**Manual Injection** Using a fixed-loop system: load three times the loop volume before making the injection.
	Using a partial fill loop: Inject less than 50% of the sample loop volume. Syringe and injection valve: Ensure that the injection technique is as constant as possible. This method of performing injections is subject to human error in addition to instrumentation errors, so you must take this into account when determining an acceptable level of precision for the method. Check that the loop size and syringe are correct and uncontaminated. Use a syringe where not less than 20% of the full volume is injected. Finally, ensure that the injection port is not leaking and that any switches open and close to their full extent.
	Automatic Injection Check to make sure that air is not being injected, that the sample vial contains enough solvent to perform multiple injections from it and that there are no leaks in the system.
	Ensure that the sample loop is the correct size and that the injection system is undamaged and clean.
	Between injections, ensure that the injector purge is adequate to eliminate carry-over from previous injections.
Degraded/Contaminated Sample	Perform one injection of a standard. If all the peaks are present and correct, the sample has been contaminated or has degraded. Re-prepare the sample and re-inject.
Chromatographic Errors	Refer to the troubleshooting tables in section 3.4 of this guide.
Detector Response Inaccuracies	Refer to the detector operator's manual for instrument specific information on troubleshooting and corrective action.
Peak Integration Error	Ensure that the correct values of peak lift-off, touchdown, threshold etc. are entered into the data-handling device. Make any necessary changes.
	Note: *Many regulatory bodies insist that all chromatograms in a "run" are integrated using the same integration parameters. For this reason, it is considered best practice to set the integration parameters using a system suitability test mix that contains peaks at, or just above the limit of detection and larger.*

4. Good Laboratory Practice For HPLC

4.1 Preparation of Solvents

Correct solvent preparation is very important. It can save vast amounts of time spent troubleshooting spurious peaks, base-line noise etc.

4.1.1 Quality

All reagents and solvents should be of the highest quality. HPLC grade reagents may cost slightly more than lower grade reagents, but the difference in purity is marked. HPLC grade reagents contain no impurities to produce spurious peaks in a chromatogram baseline whereas AR grade reagents do contain trace levels of impurity, which may produce spurious baseline peaks.

Ensure that any water used in buffer preparation is of the highest purity. Deionized water often contains trace levels of organic compounds and so therefore is not recommended for HPLC use. Ultra pure HPLC water (18MΩ resistivity) is generated by passing deionized water through an ion exchange bed. Modern water purification instruments use this mechanism to produce water of suitable quality in high volumes. Alternately, HPLC grade water can be purchased from solvent suppliers.

Important: *Do not store HPLC grade water in plastic containers. Additives in the plastic may leach into the water and contaminate it. Always store HPLC grade water in glass containers.*

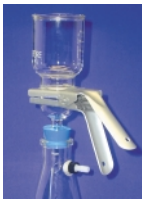
4.1.2 Buffers

All buffers should be prepared freshly on the day required. This practice ensures that the buffer pH is unaffected by prolonged storage and that there is no microbial growth present. Changes in pH and microbial growth will affect chromatography.

If buffer solutions are stored, be aware that they have a finite lifetime. Refer to pharmacopoeia monographs or similar for further guidance on buffer shelf life.

Buffer reagents can contain a stabilizing agent, for example, sodium metabisulphite. These stabilizing agents often affect the optical and chromatographic behaviour of buffer solutions, so it is often worth buying reagents that contain no stabilizer. Containers of solid reagent are easily contaminated by repeated use. For this reason, we recommend that regents be purchased in low container weights.

4.1.3 Filtration



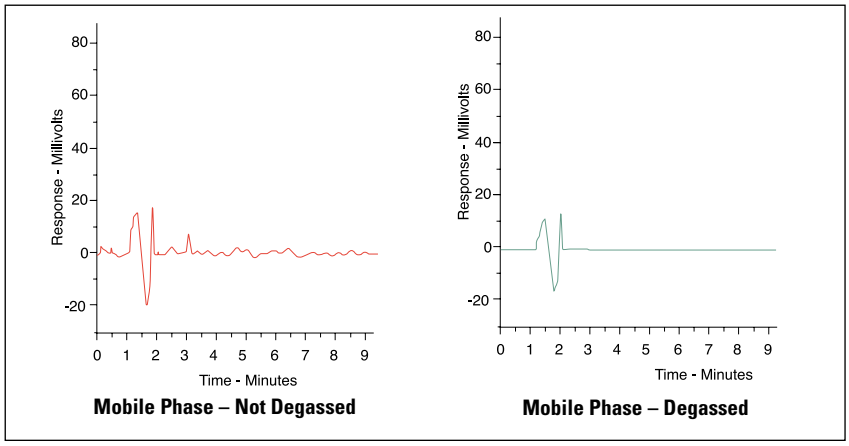
Ideally, all HPLC solvents should be filtered through a 0.45 µm filter before use. This removes any particulate matter that may cause blockages. After filtration, the solvents should be stored in a covered reservoir to prevent contamination with dust etc. Filtering HPLC solvents will benefit both your chromatography and the wear and tear of the HPLC system. Pump plungers, seals and check valves will perform better and lifetimes will be maximized.

4.1.4 Degassing

Before the freshly prepared mobile phase is pumped around the HPLC system, it should be thoroughly degassed to remove all dissolved gasses. Dissolved gas can be removed from solution by:

- **Bubbling with helium**
- **Sonication**
- **Vacuum filtration**

If the mobile phase is not degassed, air bubbles can form in the high-pressure system resulting in problems with system instability, spurious baseline peaks etc.

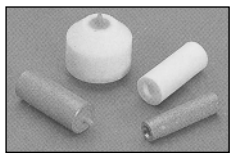


The most efficient form of degassing is bubbling with helium or another low solubility gas. If this method is available, we recommend that the mobile phase is continually degassed at very low levels throughout the analysis.This will inhibit the re-adsorption of gases over the analysis time.

Note: *Ensure that the solvent reservoir has a vent to the atmosphere to prevent the build up of pressure inside the reservoir.*

4.2 Solvent Use

4.2.1 Instrument



Each solvent line should be fitted with an inlet filter. This is the first line of system defence against particulate contamination from solvents. The filters should be kept clean to prevent cross contamination. When they are not being used, it is recommended that they are stored in a solution of 50% acetonitrile/50% water. This will inhibit microbial growth and stop dust and dirt from embedding in the filter pores.

The solvent lines should be clean, growth-free and should have no sharp bends or creases in them. Solvent reservoirs should be placed as high as possible on or in the instrument – always higher than the pump inlet manifold. The solvent lines and filters should be of sufficient length to reach the bottom of the solvent reservoir.

Important: *Before starting an analysis, calculate the total volume of mobile phase required. Prepare all the phases at the same time and place in reservoirs that are large enough to accommodate them. Insufficient mobile phase may cause the system to pump dry. This is undesirable because it will fill the system with air. If this does happen, purge the solvent lines and pump with fresh solvent then allow the system to pump solvent until it is equilibrated.*

4.2.2 Mobile Phase Properties

Do not use highly acidic or basic solvents unless your HPLC system and column have been engineered to accommodate them. Seals, plungers etc. can be damaged by extreme pH conditions. If in doubt, please contact your column or instrument supplier before using an aggressive solvent.

The use of highly aqueous mobile phases is becoming more popular as safety guidelines demand less exposure to organic solvents. Care should again be taken that the HPLC column has been engineered to accommodate highly aqueous solvents – traditional alkyl chain media can be prone to phase collapse in low organic composition solvent mixes, for example at less than 5% organic solvent.

Highly aqueous mobile phases are ideal breeding grounds for microbes. Ensure that an organic solvent is flushed through the HPLC system and column at least once every 48 hours to kill unwanted microbial growth. Alternatively add a small amount of sodium azide to the aqueous solvent to inhibit growth.

Note: *Never allow a HPLC column or system to stand with water or buffer in it for an extended period of time (for example, over a holiday break). Always flush with a solvent mix that contains a minimum of 20% organic in water.*

4.3 Changing Solvents

4.3.1 Buffered Phase to Wash or Storage Phase

Ensure that the buffer is soluble in the proposed wash or storage phase. If it is not, first flush the system with a solvent mix that is highly aqueous to remove the buffer from the system and column, then change to the proposed wash or storage solvent mix.

4.3.2 Normal to Reversed Phase and Vice Versa

There are few occasions where these solvent changes will be necessary. Such occasions include columns that can be used with both solvent types, for example Hypercarb™ columns or systems where both normal and reversed phase analyses are performed. Where possible, systems should be dedicated to normal or reversed phase chromatography.

To convert a normal phase system/column to a reversed phase system/column, flush with a solvent that is miscible with both the current normal phase solvents and ideally, the proposed reversed phase solvents. If the final reversed phase solvents include a buffer, then it is advisable to move from the 100% methanol flush to a 50% aqueous methanol flush. For example,

Normal Phase	**Hexane/Ethyl Acetate**
Flush	**IPA then Methanol**
	Finally 50:50 Methanol/Water
Reversed Phase	**Buffered Aqueous Methanol**

Important: *There are few columns that can be used in both normal and reversed phase. Check that your column has been engineered to be compatible with both phase types before you attempt any solvent changes.*

To convert a reversed phase system/column to a normal phase system/column, follow a similar path to the one listed previously, but in reverse, for example,

Reversed Phase	**Buffered Aqueous Methanol**
Flush	**50:50 Methanol/Water**
	Methanol then IPA
Normal Phase	**Hexane/Ethyl Acetate**

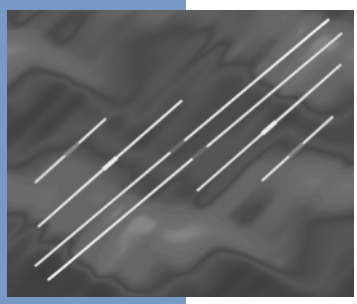
Refer to section 5.3 for solvent miscibilities and properties.

4.3.3 General

Before attempting any solvent change, ensure that the solvent already in the system and column is compatible with the new solvent.

If the miscibility or physical properties of the two solvents are unknown, then it is better to mix the solvents in a beaker to see the reaction than to go ahead and pump the second solvent into the first on the HPLC instrument. Mixing problems are easier to rectify before the HPLC.

4.4 System Plumbing and Fittings



The purpose of a well-plumbed HPLC system is to minimize dead volume between it's components and to eliminate leaks.

System tubing errors show themselves in many ways, for example as band broadening, baseline noise, etc. Detection of incorrect diameter tubing is often very difficult once it is in-situ. For this reason, we recommend that all plumbing changes be recorded in the instrument maintenance log. It is easier then to pinpoint the most recent changes. An example of an instrument maintenance log is shown in Appendix C of this guide.

The internal diameter of tubing used in a HPLC system varies with the position in the instrument. Refer to your system maintenance manuals to determine the recommended tubing for a specific application.

Note: *Most suppliers of HPLC tubing now color code their products for ease of internal diameter identification.*

The type of tubing used is determined by the application that is being performed. The two most common types of tubing are stainless steel and PEEK™, although others are also available. When changing tubing, make sure that the replacement is manufactured from a material that is compatible with any solvents that may be flushed through it.

4.4.1 Cutting Tubing

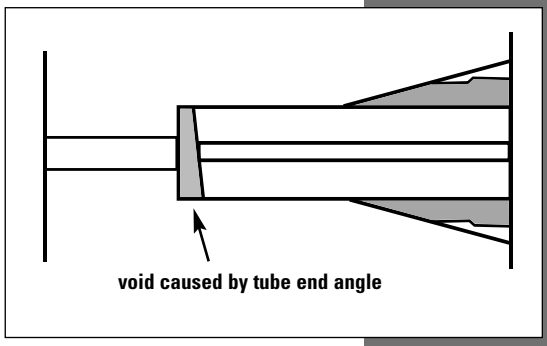
Tubing should always be cut using instruments that are designed for the purpose. The use of scissors and wire cutters is not recommended as they can deform the tubing bore and almost always produce angled cuts that are one of the biggest causes of leaks and increased dead volume.

Note: *Where possible, pre-cut tubing should always be used.*

Stainless Steel

Stainless steel can be effectively cut using the procedure outlined below:

1. Estimate the length required. Remember to allow extra length if the tubing is to go around corners as sharp bends in the tubing will distort the inside bore and hence the solvent flow through it.
2. Using a stainless steel cutter, score cleanly round the tubing. If a cutter is unavailable, use a knife-file to score the tubing. Care should be taken, whichever method is used, not to distort or damage the tubing.
3. Using two pairs of smooth jawed pliers, one above the score and one below it, gently bend the tubing back and forth until it snaps. Excessive bending should be avoided as this will damage the tubing and not give a clean break.
4. There will be one or two burrs present on the cut surface. These can be carefully filed away to give a smooth, flat cut surface. Care should be taken not to allow any filed material to block the tubing bore.



PEEK or Polymeric Tubing

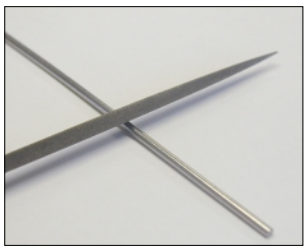
The simplest way to cut polymeric tubing is using a blade, for example a razor blade or craft knife. The polymeric tubing cutters that are supplied by many manufacturers are essentially a razor blade in a safety housing. The advantage of using one of these cutters is that they hold the tubing at 90° to the blade so a clean, flat cut is assured.

Polymeric tubing can be effectively cut using the procedure outlined below:

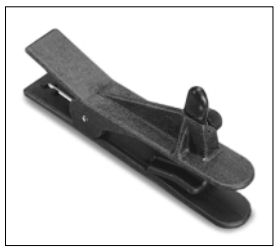
1. Estimate the length required. Remember to allow extra length if the tubing is to go around corners as sharp bends in the tubing will distort the inside bore and hence the solvent flow through it.
2. Use a sharp blade or specialist cutter to cut the tubing. Do not use a "sawing" action. This will give an uneven cut surface. Make the cut in a single action.
3. Inspect the cut surface for burrs. These can be carefully filed away to give a smooth, flat cut surface. Care should be taken not to allow any filed material to block the tubing bore.

As a final action, after cutting the tubing and before connecting it to the HPLC, flush it with solvent to remove any filed material, dust or other debris that could be in the tubing bore. The tubing is now ready to have fittings attached to it.

Metal tubing cutter



Metal tubing cutter



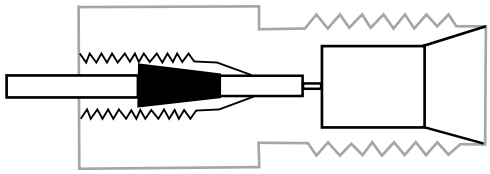
Polymeric tubing cutter

4.4.2 Fittings

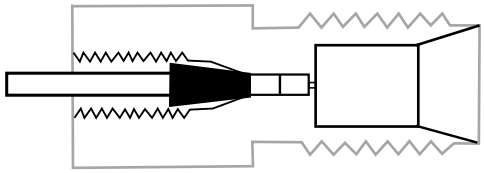
Fittings are available in stainless steel and a range of polymeric compounds. In general, you should use stainless steel fittings with stainless steel tubing, PEEK fittings with PEEK tubing etc.

We recommend that compression fittings and ferrules from different manufacturers are never interchanged. The dimensions of the fitting and ferrule vary between suppliers, so mixing them often leads to leaks, stripped threads and damage to female ports such as column inlet and outlets, detector inlets etc.

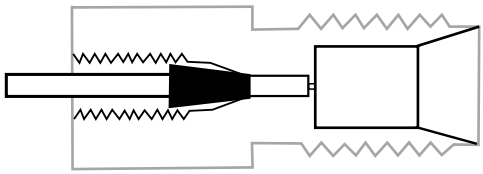
The amount of tubing that extends past the ferrule also varies with manufacturer. If the tubing does not seat properly within the female fitting, then leaks and increases in dead volume will occur.



Ferrule does not seat



Tubing is not seated causing unswept dead volume



Tubing and ferrule seated correctly

Laboratories that contain instruments from more than one manufacturer often find it useful to purchase "universal" fittings. These fittings are compatible with all instruments.

Note: *Care should always be taken when tightening fittings. Over-tightening can cause damage to the fitting threads, ferrules etc, causing the unit to leak, or in worse case scenarios, causing the fitting to break off in the housing.*

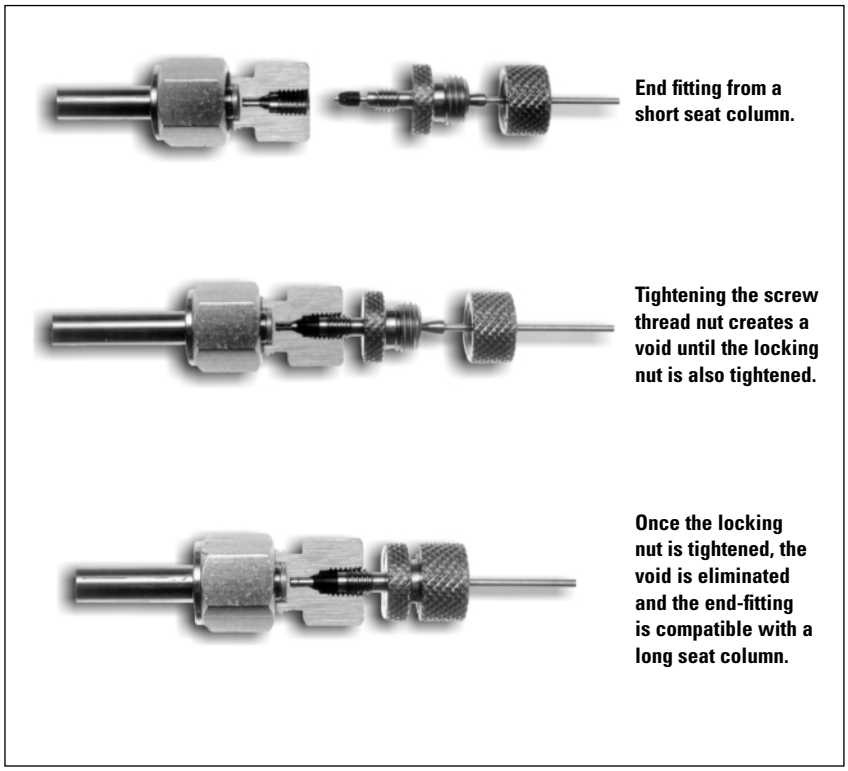
The use of fingertight fittings is recommended wherever possible. These require no additional tools to form a leak-proof seal.

Depth of Tubing Past Swaged Ferrule		Fitting Length, Excluding Ferrule & Head	
Waters®	3.3 mm	Waters	8.1 mm
Swagelok®	2.3 mm	Swagelok	5.7 mm
Parker™	2.3 mm	Parker	5.3 mm
Uptight®	2.3 mm	Uptight	6.4 mm
Valco®	2.0 mm	Valco	7.6 mm
Rheodyne®	4.3 mm	Rheodyne	5.6 mm

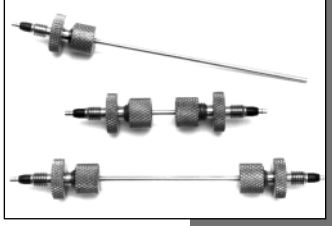
4.4.3 SLIPFREE™ Fittings

SLIPFREE fittings from Thermo ensure a void free connection on any HPLC system. They are compatible with all end-fitting depths and are fingertight to 10,000 psi. They can be used between the injector and column, between a guard and column or between the column and detector.

The SLIPFREE ferrule is firmly seated before the tubing depth beyond the ferrule is adjusted. This gives the best fit possible, as shown in the following images:



Single and Double end SLIPFREE® fittings



The locking nut allows the SLIPFREE tubing to extend into the fitting to ensure a zero dead volume seal in all column fittings.

For further information on these products, please contact your Thermo representative.

4.4.4 Blocked Tubing

Blocked tubing results in an increase in system pressure, deterioration of chromatographic performance and leaks from worn fittings. Blocked tubing can either be replaced or the blockage removed. If no replacement tubing is readily available, the blockage must be removed. This can be done in the following way:

1. Isolate the blockage, as described in section 3.2.1 of this guide.
2. Remove the piece of tubing from the instrument, reverse it and attach the reversed end directly to a pump.
3. Flush the tubing at approximately 0.5 mL/min with a suitable solvent to remove the blockage. If the material blocking the tubing is not known, use a 50% aqueous methanol solution.
4. There are occasions when this flush procedure will not remove the blockage, for example if it is due to a large insoluble piece of seal etc. In such circumstances, increase the flow rate to between 3 and 5 mL/min to force the blockage out. This flush should be used as a last resort because of the risk of formation of solvent aerosols as the tubing unblocks. If this flush is used, ensure that the waste end of the tubing is placed in a covered beaker or sealed waste bottle for safety.
5. If the tubing remains blocks after flushing, then it must be replaced.

4.4.5 Old and Leaking Fittings

Compression and finger tight fittings have a finite lifetime. Eventually, they will have to be replaced with new ones.

The ferrule of the worn fitting will be swaged to the tubing; i.e. it will be irremovable (stainless steel) or will be removable but will leave an indentation around the tubing (PEEK). This section of tubing cannot be used again as it would not seal properly and would leak.

To change a fitting, follow the guideline below:

Compression Fitting

1. Turn off all pumps – solvent should not be flowing through the system.
2. Remove the fitting from it's housing.
3. Cut the tubing between the ferrule and fitting. If this is not possible, cut the tubing before the fitting.
4. Ensure the tubing's cut end is flat and burr free. Inspect the old fitting; if it is worn, replace it. If it is not worn, it can be re-used with a new compatible ferrule.

Note: *Leaking fittings are often caused by damaged, mis-shapen ferrules, so it is not necessary to replace the whole fitting if only a part of it is damaged.*

5. Assemble the new fitting and ferrule on the tubing and tighten into it's housing to swage the ferrule onto the tubing. If the fitting still leaks after this, replace the tubing.

Fingertight Fitting

1. Turn off all pumps – solvent should not be flowing through the system.
2. Remove the fitting from it's housing.
3. Slip the fitting and ferrule (if it has a separate one) from the tubing.
4. Inspect the tubing for defects. Remove the end of the tubing if necessary.
5. Place the new fitting on the tubing and tighten it into it's housing. If the fitting still leaks after this, replace the tubing.

SLIPFREE Fitting

1. Turn off all pumps – solvent should not be flowing through the system.
2. Remove the fitting from it's housing.
3. Check the ferrule for damage – this is the most likely cause of leaks. The SLIPFREE ferrules do not swage onto the unit's tubing, unlike other ferrules, so they can easily be replaced.
4. Replace the ferrule and reassemble the SLIPFREE connector into it's housing. If the fitting still leaks, replace the entire SLIPFREE unit.

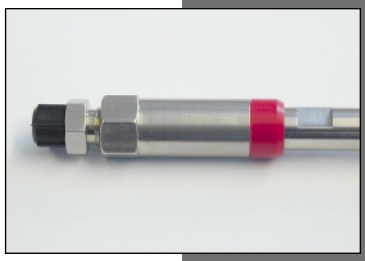
Important: *There are occasions where the fitting or ferrule is not the cause of solvent leaks. They can be generated by degeneration of the fitting housing. Where persistent leaks occur, even after fitting changes, it is worth while inspecting the housing for damaged threads, blockage etc.*

4.5 Column Selection

For applications where there is no specified column type, or in method development situations, column selection is vitally important. It can mean the difference between efficient and inefficient method development. There are many variables to consider such as the analyte properties and available chromophores. To make your selection choices easier, refer to pages 61 and 62, or contact your Thermo representative. Reference tables of hydrophobic character and wavelength selection for chromophores are included in Appendix A and section 5.5 of this guide, for your convenience.

4.6. Column Protection

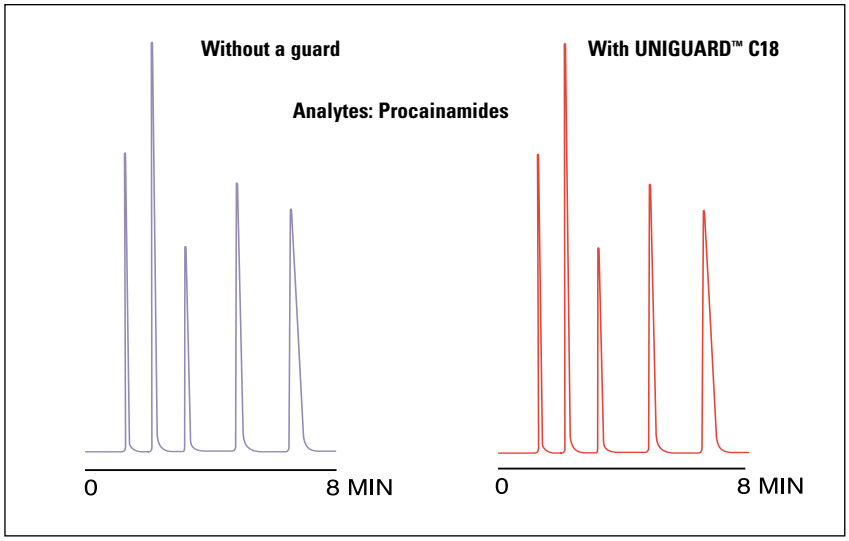
4.6.1 Guard Columns and Cartridges



It is advisable to protect your analytical column from sample and system debris and contaminants to maintain the column performance and efficiency. Guard columns or cartridges are one of the most cost effective and efficient ways of trapping these unwanted system components. We recommend a 10 mm length guard for moderate to heavy contamination.

Note: *Guards are designed to be disposable, so once contaminated, they should be replaced rather than regenerated. Dolan and Snyder[1] recommend that the guard is replaced every 50 – 100 injections to ensure that there is no loss in column performance.*

The performance of an analytical column should not be affected by adding a guard unit to the system. As an example, the following chromatograms of procainamides were generated both with and without a C18 guard unit. As you can see, there is no change in peak separation, shape or analysis time.



The choice of guard often causes concern. The simple rule of thumb is to choose a guard that is packed with the same particle size and type of material as the analytical column. The guard and column diameters should be as close as possible to eliminate solvent flow and chromatographic discrepancies. A reference table is provided on the left for your convenience.

Specialist guard units are also available for LC/MS and use with finger tight fittings. Please contact your Thermo HPLC columns representative for more information.

Diameter (mm)	Column Guard
1	1
2 and 2.1	2
3	3
3.9 and 4	4
4.6	4 or 4.6
7.8	7.8
10	10

4.6.2 Pre-Column Filters

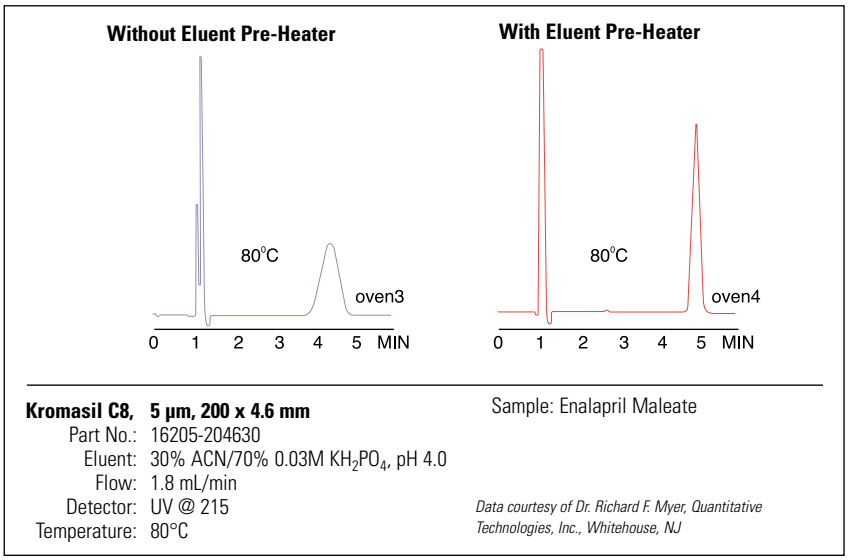
These units are positioned between the solvent inlet filter and the column inlet. They are designed to trap particulates from the fluid path. They will not trap dissolved substances. Once again, these units are designed to be disposable and should be replaced regularly. There are a number of designs of filters available. For further information on these designs or for advice on your application, please contact your Thermo representative.

4.7 Column Operating Temperature

Solvent Heater Unit

It is vitally important that columns are maintained at a constant temperature while in use. Fluctuations in temperature can cause peak drift and other undesirable effects in your chromatography. For this reason, we recommend the use of a thermostatically regulated temperature control unit to house your column during use such as HOT POCKET™ or COOL POCKET™ temperature controllers. An ideal temperature control unit will both cool and heat the column efficiently and is not subject to changes in the ambient room temperature.

In applications where the column will be maintained at temperatures above or below ambient, it is also worth considering the use of a solvent heater (shown on the left). The effect of solvent heating prior to analysis is shown below.



Kromasil C8, 5 µm, 200 x 4.6 mm
Part No.: 16205-204630
Eluent: 30% ACN/70% 0.03M KH_2PO_4, pH 4.0
Flow: 1.8 mL/min
Detector: UV @ 215
Temperature: 80°C

Sample: Enalapril Maleate

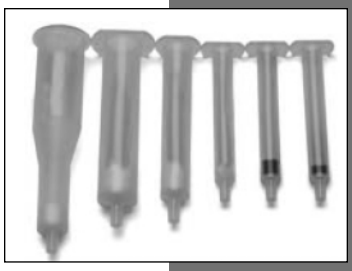
Data courtesy of Dr. Richard F. Myer, Quantitative Technologies, Inc., Whitehouse, NJ

The recommended operating temperatures for columns is very much dependent on the analytical conditions and the packing inside the column. Typically, carbon and polymer based columns can be used at far higher temperatures than can a silica based column. This is because elevated temperatures increase the risk of chemical attack on the bonding and base silica by the solvent. For example, at 60°C and with a buffer at pH 2, a silica based amide column packing can start to hydrolyze. This would drastically affect the chromatography available from the column.

The choice of particle size is also a contributing factor when considering operating temperature. Particles below 5 µm in diameter are more prone to bed collapse and loss of efficiency at highly elevated temperatures.

Temperature can be a powerful method development tool if it is used correctly. Lower temperatures can be used to help increase retention, selectivity and resolution. Higher temperatures can be used to help decrease retention, selectivity and resolution.

4.8 Sample Preparation



Sample preparation is about more than just the dissolution of a solid in a liquid. Samples may require other techniques such as filtration, extraction or derivitization as well as accurate weighing and/or dissolution.

Samples require filtration if they contain suspended solids. This can be performed on-line using a pre-column filter or as the sample is introduced to the vial.

Important: *If using a membrane filter make sure that it is compatible with all the solvent used.*

Samples that require extraction usually contain the analyte of interest at low levels. The most common forms of extraction are liquid-liquid, solid-liquid, and solid phase extraction. The latter of these three is perhaps the quickest and easiest to perform. The question most often asked is when do I perform an extraction? The simple answer is,

(i) whenever the matrix, that is the substance that your analyte is contained in, is liable to contaminate your HPLC system or block it with particulate matter or

(ii) whenever the analyte is at such low levels that pre-concentration is required. Solid phase extraction is not difficult to perform and requires very little specialist equipment.

Sample derivatization can occur before or after the column. It can also be performed manually or automatically. It is generally required for detection purposes, for example, the UV detection of analytes without chromophores. There are many different derivitization techniques and care should be taken to choose one that is suitable for your application and that does not produce side products that will cause chromatographic problems later in the analysis.

Important: *Sample solvents should match the proposed mobile phase as closely as possible. This will avoid baseline errors and spurious peaks seen as the injection solvent passes through the detector.*

5. Reference Data

5.1 Chromatographic Performance Tests

5.1.1 Resolution (R)

Resolution is defined as the distance between two adjacent peak apexes, divided by the average base width of both peaks. It is represented by the equation:

$$R = \frac{(T_2 - T_1)}{0.5\,(W_1 + W_2)}$$

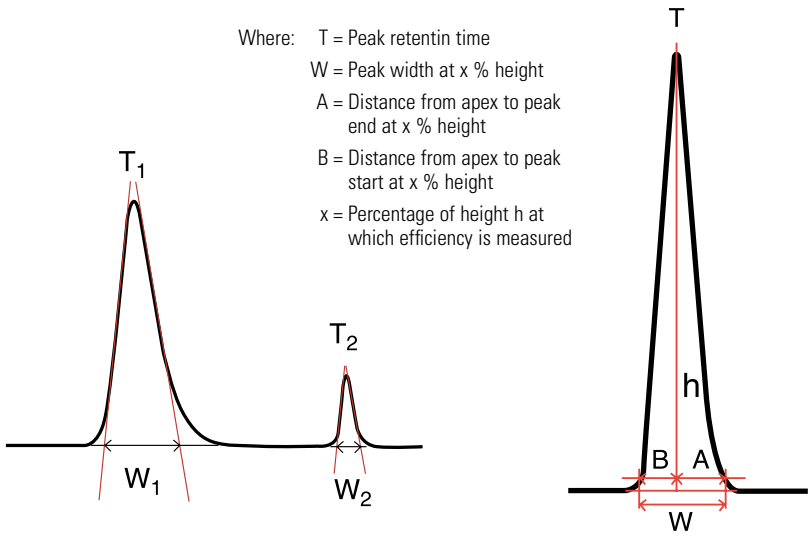
Where T_2 and T_1 are measured in seconds and are the peak apex retention times and W_1 and W_2 are the baseline widths of the peaks, also measured in seconds.

Resolution is dependant on three other variables, the column efficiency N, the capacity factor k' and the selectivity α.

Decreasing N decreases the resolution because peak width increases. Increasing N increases resolution because peak width decreases.

Decreasing k' sharpens the peaks but decreases resolution. Increasing k' broadens the peaks but improves resolution.

Increasing a increases resolution. One peak moves relative to the other. Likewise, decreasing α decreases resolution.



5.1.2 Column Efficiency (N)

Column efficiency, or the theoretical plate count, is a measure of peak band spreading. The lower the level of band spreading, the higher the column efficiency and vice versa.

Important: *The column efficiency figure quoted on the supplied certificate of analysis is actually the efficiency for the column AND HPLC system. If the efficiency calculation is repeated on a different instrument when the column is new, it is very likely that there will be a difference between the certificated value of N and your new calculated value of N. This difference is not due to the column but the instrument.*

There are a number of different methods used to calculate column efficiency. Some take into account peaks that are unsymmetrical, others do not. For consistency, the method you use should always be the same.

In order of sensitivity to peak shape (most sensitive first), the calculations for measuring N are as follows:

a. Asymmetry Based

$$N = \frac{41.7\ (T/W)^2}{(1.25 + (A/B)}$$

Where x = 10%

b. 5-Sigma

$N = 25\ (T/W)^2$

Where x = 4.4%

c. 4-Sigma

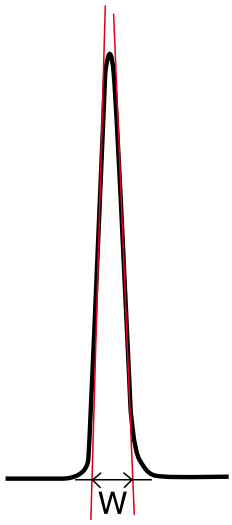
$N = 16\ (T/W)^2$

Where x = 13.4%

d. Tangent

$N = 16\ (T/W)^2$

Refer to diagram on right for W



d. Tangent

e. 3-Sigma

$N = 9\ (T/W)^2$

Where x = 32.4%

f. Half-height

$N = 5.54\ (T/W)^2$

Where x = 50%

g. 2-Sigma (inflection)

$N = 4(T/W)^2$

Where x = 60.7%

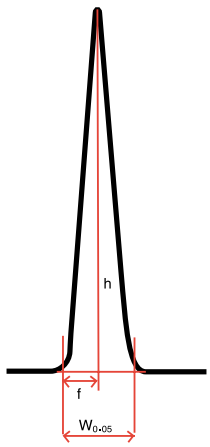
5.1.3 Tailing Factor (T)

Tailing factor is a measure of the symmetry of a peak. Ideally, peaks should be Gaussian in shape (totally symmetrical).
A peak's tailing factor is measured using the following equation:

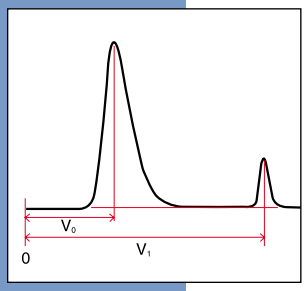
$T = W_{0.05}/2f$

Where $W_{0.05}$ = peak width at 5% height

f = distance from peak front to apex point at the baseline



5.1.4 Capacity Factor (k)



Capacity factor is a measure of the retention of an analyte relative to the column void volume, V_0. It is measured using the following equation:

$$k = \frac{V_1 - V_0}{V_0}$$

Where V_0 = Column void volume
V_1 = Retention volume of peak

Important: *The column void volume can be measured by injecting a compound that will be unretained by the column packing. Typical compounds used include uracil (RP) and toluene (NP). Contact your column supplier for advice if you are unsure about the retention characteristics of your chosen void marker.*

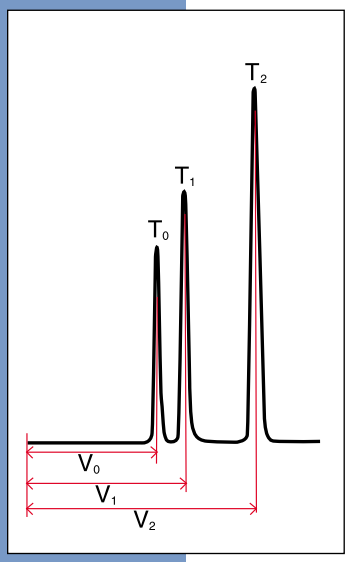
Capacity factor is affected by changes in mobile phase, operating temperature, analyte retention characteristics and changes to the surface chemistry of the column.

Changes in capacity factor that occur both with standard and sample mixes are likely to be due to changes in the column, temperature or mobile phase composition.

Changes in capacity factor that occur only in the sample mix and not the standard mix are most likely to be due to the composition of the sample.

Note: *Capacity factor will change by up to 10% for a 5°C rise in column temperature.*

5.1.5 Selectivity (α)



Selectivity is a measure of the relative retention of two adjacent peaks in a chromatogram. It can be calculated using capacity factors or retention volumes:

$$\alpha = \frac{k_2}{k_1} \quad \frac{V_2 - V_0}{V_1 - V_0}$$

Where $k_1 = V_1$ capacity factor
$k_2 = V_2$ capacity factor
V_0 = void volume
V_1 = peak 1 retention volume
V_2 = peak 2 retention volume

Selectivity can be affected by changes in mobile phase composition, temperature and column chemistry.

Changes in selectivity that occur both with standard and sample mixes are most likely to be due to changes in the column, temperature or mobile phase composition.

Changes in selectivity that occur only in the sample mix and not the standard mix are most likely to be due to the composition of the sample.

5.1.6 Band Spreading or Band Broadening

Broad peaks, often accompanied by a change in retention time, indicate band spreading. It can occur within the HPLC column, but is more often due to incorrect system plumbing. The following procedure describes a method for measuring the band spreading due to the HPLC system. Column effects can be measured using efficiency calculations.

1. Remove the HPLC column from the system and replace with a zero dead volume union.
2. Configure the HPLC system with the following parameters;

   Flow Rate: 1 mL/min
   Detector Sensitivity: 0.5 to 1.0 AUFS
   Detector Time Constant: 0.2 or less
   Chart Speed (if req'd): 20 cm/min
3. Perform a ten-fold dilution of the column efficiency test solution. Inject 5 µL of this diluted mix.
4. Adjust the detector sensitivity until the peak height is approximately 75% of the full-scale readout.
5. Measure the peak width at 4.4% peak height (5-sigma column efficiency method).
6. Convert the peak width to mL using the following conversion:

   Band Spread (µL) = Peak Width x (1/20) x 1 x 1000

   Where Peak width is measured at 4.4% peak height and expressed in cm; (1/20) represents the chart speed, min/cm; 1 represents the flow rate, mL/min and 1000 represents the volume correction factor, µL/mL.

   Important: *100 mL ± 30 µL is a typical system band spreading value. Larger values may indicate a problem in the detector, injector, tubing or fittings.*

   Ensure that your HPLC system does not have built-in extra dead volume, as this will also increase the band spreading value.
7. For high values of band spreading, troubleshoot your HPLC system then repeat the determination of band spreading. If the value decreases to acceptable levels, then the problem is resolved. A partial decrease will require further investigation.

   If the problem persists, contact your instrument supplier for technical advice.

The table below shows details of the volume of solvent per unit length contained in tubing of varying volumes. For ease of use, both metric and imperial measurements are shown.

Tubing Diameter (mm)	Tubing Volume (µL/cm)	Tubing Diameter (inch)	Tubing Volume (µL/inch)
0.12	0.127	0.005	0.323
0.17	0.249	0.007	0.632
0.25	0.507	0.010	1.288
0.51	2.026	0.020	5.146
1.02	8.103	0.040	20.581

5.2 System Calculations

5.2.1 Column Backpressure and Typical Operating Flow Rates

Column operating backpressure is affected by column length, internal diameter, media particle size, temperature, solvent properties and solvent flow rate. It can also be affected by the use of gradients, where the pressure may vary with solvent composition.

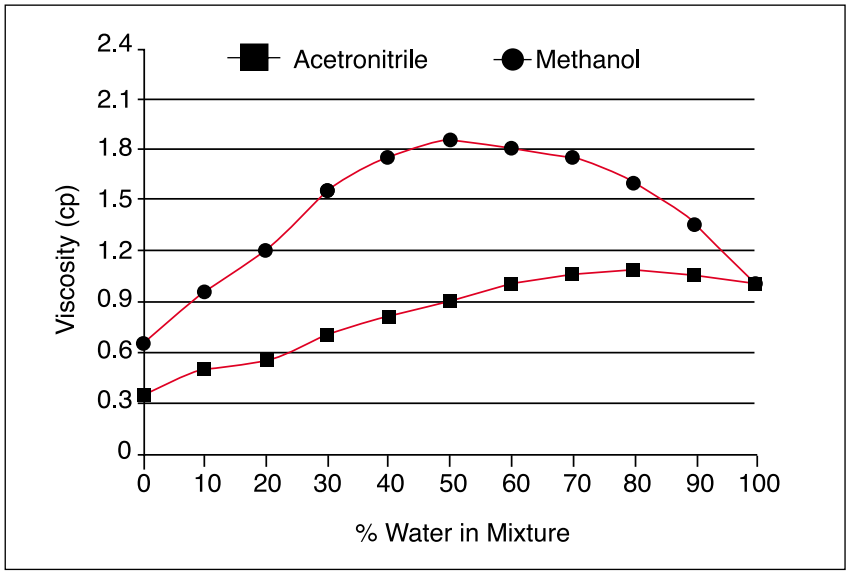
Typical operating backpressure for columns or cartridges can be calculated quite easily. The initial calculation uses water as the mobile phase. The backpressure obtained is then multiplied by the viscosity of the actual mobile phase. The calculation is:

$$\text{Pressure at 1 mL/min water} \quad \frac{2.1 \times f \times L}{dp^2 \times d^2} \times \eta$$

Where Pressure is measured in atmospheres

f = 1000 for columns (4.6 mm ID columns)
L = Column length (cm)
dp = Particle diameter (mm)
d = Column diameter (mm)
η = Mobile phase viscosity (centipoises)

Mobile phase viscosity varies with composition. As an example, the following plot shows how water viscosity varies with the addition of methanol or acetonitrile.



Note: *Although HPLC columns are manufactured under extremely high pressure, it is recommended that you do not exceed the maximum pressure recommended by your instrument manufacturer.*

The typical solvent flow rate through a column is very dependent on it's internal diameter and the particle size of the column packing material. The flow rates shown in the following table are intended as a guide only and, allowing for experimental differences, it is possible to use a range of flow rates around those quoted.

Internal Diameter (mm)	Media Particle Size (µm)	Typical Flow Rate (mL/min)
1.0	5	0.1
2.0, 2.1	5	0.2
3.0	5	0.5
4.0, 4.6	3	0.5
	5	1.0
	10	2.0
10.0	5	5.0
21.2	10	21.0

5.2.2 Scaling Up or Scaling Down

Flow rate and column load scaling are only required when changing column internal diameters. Scaling up is performed when moving from a small to a large diameter column, for example, analytical to preparative chromatography. Scaling down is performed when moving from a large to a small diameter column, for example, analytical to capillary chomatography. The scaling allows peak retention times to remain relatively constant between different dimension columns.

Assuming column length is a constant, the scale factor can be calculated using the following formula:

$$\text{Scale Factor} = \frac{(\text{Column A internal diameter})^2}{(\text{Column B internal diameter})^2}$$

As an example, the calculated scale factors based on a 4.6 mm internal diameter column are:

New Column ID (mm)	Flow Rate Scale Factor
1.0	x 0.05
2.1	x 0.2
3.0	x 0.4
4.0	x 0.8
10.0	x 4.7
21.2	x 21.2

5.3 Solvent Properties

The following tables list a series of commonly used HPLC solvents and their most pertinent physical properties, including viscosity and miscibility number. The miscibility numbers can be used to predict the miscibility of solvents.

If the smaller miscibility number is subtracted from the larger and the difference is 15 units or less, then the two liquids are soluble in all proportions at 15°C.

If the smaller miscibility number is subtracted from the larger and the difference is 16 units, then the two liquids have a critical solution temperature between 25 and 75°C with 50°C as the optimum temperature.

If the smaller miscibility number is subtracted from the larger and the difference is 17 or greater, then the two liquids are immiscible, or their critical temperature is greater than 75°C.

There is also a quick reference miscibility table in Appendix B of this guide.

Solvent	Polarity Index	Viscosity (cp) at 20°C	Boiling Point (°C) at 1 atm	Miscibility Number	Refractive Index	UV Cut Off (nm)
Acetic Acid	6.2	1.26	117.9	14	1.372	230*
Acetone	5.4	0.32	56.3	15, 17	1.359	330
Acetonitrile	6.2	0.37	81.6	11, 17	1.344	190
Benzene	3.0	0.65	80.1	21	1.501	280
Chloroform	3.4	0.57	61.2	19	1.443	245
Cyclohexane	0.0	0.98	80.7	28	1.427	200
Dimethyl Sulphoxide	6.5	2.24	189.0	9	1.477	268
p-Dioxane	4.8	1.54	101.3	17	1.422	215
Ethanol	5.2	1.20	78.3	14	1.361	210
Ethyl Acetate	4.3	0.47	77.1	19	1.370	256
Formamide	7.3	3.76	210.5	3	1.446	≈260
Hexane	0.0	0.31	68.7	29	1.372	195
Methanol	6.6	0.60	64.7	12	1.329	205
Methyl Ethyl Ketone	4.5	0.43	80.0	17	1.381	330
1-Propanol	4.3	2.30	97.2	15	1.380	210
2-Propanol	4.3	2.35	117.7	15	1.380	205
i-Propyl Ether	2.2	0.33	68.3	26	1.368	220
Tetrahydrofuran	4.2	0.55	66.0	17	1.408	230
Toluene	2.3	0.59	101.6	23	1.496	285
Water	9.0	1.00	100.0	–	1.330	190
p-Xylene	2.4	0.70	138.0	24	≈1.50	290

** Value refers to a 1% solution in water*

Solvents that have a double miscibility number are immiscible with other solvents at extremes of the lipophilicity scale. The lower of the two numbers relates to solvents with high lipophilicity and the second to solvents with low lipophilicity.

Solvents with double miscibility numbers can, in some circumstances, be immiscible with each other.

Important: *molecular interaction (hydrogen bonding for example) between liquids can change the expected level of miscibility.*

The following tables list a series of commonly used HPLC buffers, their alternative name, where applicable, and their pK_a values at 20°C.

5.4 Buffer Properties

Buffer transparency is a variable that should be measured prior to buffer use, as it will vary with salt concentration.

Buffer	Buffer Range	pK_a at 200C
ACES/N-(2-Acetamido)-2-aminoethanesulfonic Acid	6.4 – 7.4	6.9
Acetamidoglycine/N-(2-Acetamido) Glycine		7.72
Acetic Acid/Ammonium (K- & Na-) Acetate	3.8 – 5.8	4.8
ADA/N-(2-Acetamido)-iminodiacetic Acid	6.4 – 7.4	6.6
Mono- & Di- Ammonium (K- & Na-) Carbonate	5.4 – 7.4 9.3 – 11.3	6.4 10.3
Ammonium Hydroxide (Chloride)/Ammonia	8.2 – 10.2	9.2
BES /N,N-Bis(2-hydroxyethyl)-2-aminoethane-sulfonic Acid	6.6 – 7.6	7.15
Bicine/N,N-Bis(hydroxyethyl) glycine	7.8 – 8.8	8.35
BIS-TRIS Propane/1,3-Bis[tris (hydroxymethyl)methylamino]propane	5.8 – 7.8	6.8
Borate	8.2 – 10.2	9.24
CAPS	9.7 – 11.1	10.40
CHES	9.0 – 10.1	9.55
Cholamine Chloride/(2-Aminoethyl) trimethylammonium Chloride Hydrochloride	–	7.1
Citric Acid/Tri potassium citrate	2.1 – 6.4	3.1 4.7 5.4
Diethylamine Hydrochloride/Diethylamine	9.5 – 11.5	10.5
Formic Acid/Ammonium (K- & Na-) Formate	2.8 – 4.8	3.8
Glycinomide/Glycinamide Hydrochloride	–	8.2
Glycine Hydrochloride/Glycine	8.8 – 10.8	9.8
Glycylglycine	–	8.4
HEPES/N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid	7.0 – 8.0	7.55
HEPPS	7.6 – 8.6	8.00
MES/2-(N-Morpholino)-ethanesulfonic Acid	5.8 – 6.5	6.15
1-Methylpiperidine Hydrochloride/1-Methylpiperidine	9.1 – 11.1	10.1
MOPS	6.5 – 7.9	7.20
Phosphoric Acid; Mono & Di potassium phosphate	<3.1 6.2 – 8.2 11.3 – 13.3	2.1 7.2 12.3
PIPES/Piperazine-N,N'-Bis (2-ethanesulphonic Acid)	6.4 – 7.2	6.8
TAPS/N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic Acid	7.7 – 9.1	8.4
TES/N-Tris(hydroxymethyl)methyl-2-amino-ethanesulfonic Acid	7.0 – 8.0	7.5
Trifluoroacetic Acid	1.5 – 2.5	>>2
Tricine/N-Tris(hydroxymethyl)methylglycine	7.6 – 8.8	8.15
Triethylamine Hydrochloride/Triethylamine	10.0 – 12.0	11.0
TRIS/Tris(hydroxymethyl)aminomethane	7.3 – 9.3	8.3

Buffer choice will be very dependent on the analyte and the instrumentation used. Ideally, LC/MS applications should use a volatile buffer as this will not form a contaminating deposit on the cone and source. Inorganic acids, involatile buffers and ion-pair reagents should all be avoided. Typical LC/MS buffers include:

- **Ammonium acetate/formate/hydrogen carbonate (<50mM)**
- **Formic/acetic acid (0.01 – 1% v/v)**
- **Trifluoroacetic acid (<0.1% v/v)**
- **Trialkylamine and aqueous ammonia type bases**
- **TRIS**
- **BIS-TRIS propane**

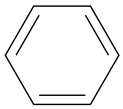
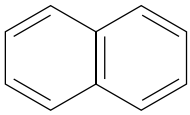
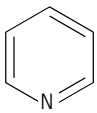
Electrolyte additives are often added to LC/MS buffers to improve peak shape. These additives should also be volatile. Care should be taken when choosing a buffer and additive mixture to ensure that a solution of the two does not produce a solid salt which could cause system contamination.

Buffers should always be flushed from the analytical column and instrument after use to avoid salts being deposited on delicate frits etc.

Note: *There are LC/MS instruments available, for example the Finnigan™ Surveyor™ MSQ™ LC/MS, which incorporate a self-cleaning mechanism to reduce the build up of inorganic buffers etc. during routine use. Care should still be taken not to purposefully over-contaminate the instrument source as this will lead to operating difficulties.*

5.5 Chromophore Detection Wavelengths

Chromophores are light absorbing groups. Their behaviour is used to allow the detection of analytes. They have one or more detection wavelengths, each of which has a molar adsorbtivity associated with it. The information contained in the following table is intended as a guide to common chromophores. It is not an exhaustive list.

Chromophore		**λmax (nm)**	**εmax (L/m/cm)**
Acetylide	-C≡C-	175 – 180	6000
Aldehyde	-CHO	210	Strong
		280 – 300	11 – 18
Amine	$-NH_2$	195	2800
Azidin	>C=N-	190	5000
Azo	-N=N-	285 – 400	3 – 25
Benzene		184	46,700
		202	6,900
		255	170
Carboxyl	-COOH	200 – 210	50 – 70
Ester	-COOR	205	50
Ether	-O-	185	1000
Ethylene	-C=C-	190	8000
Ketone	>C=O	195	1000
		270 – 285	18 – 30
Napthalene		220	112,000
		275	175
		312	5,600
Nitrate	$-ONO_2$	270	12
	$-(C=C)-_2$ acyclic	210 – 230	21,000
	$-(C=C)_3$	260	35,000
	C=C-C=C	219	6,500
	C=C-C=N	220	23,000
	C=C-C=O	210 – 250	10,000 – 20,000
	$C=C-NO_2$	300 – 350	Weak
Nitrile	-C≡N	160	
	-ONO	220 – 230	1000 – 2000
		300 – 400	10
Nitro	$-NO_2$	210	Strong
Nitroso	-N=O	302	100
Oxime	-NOH	190	5000
Pyridine		174	80,000
		195	6,000
		251	1,700
Sulfone	$-SO_2-$	180	
Sulfoxide	>S-O	210	1500
Thioether	-S-	194	4600
		215	1600
Thiol	-SH	195	1400

5.6 Column Cleaning and Regeneration

In all instances, the volume of solvent used is 40 – 60 column volumes unless otherwise stated.

The column efficiency, capacity factor etc. should be measured at the start and end of the clean-up procedure to ensure that it has been performed successfully and has improved the column performance.

Ensure that no buffers/samples are present on the column and that the solvent used prior to the clean up is miscible with the first wash solvent.

After the clean up, ensure that the test mobile phase is compatible with the last solvent in the column.

5.6.1 Normal Phase Media

1. Flush with tetrahydrofuran
2. Flush with methanol
3. Flush with tetrahydrofuran
4. Flush with methylene chloride
5. Flush with benzene-free n-hexane

5.6.2 Reversed Phase Media

1. Flush with HPLC grade water; inject 4 aliquots of 200 µL DMSO during this flush
2. Flush with methanol
3. Flush with chloroform
4. Flush with methanol

5.6.3 Anion Exchange Media

1. Flush with HPLC grade water
2. Flush with methanol
3. Flush with chloroform

5.6.4 Cation Exchange Media

1. Flush with HPLC grade water; inject 4 aliquots of 200 µL DMSO during this flush
2. Flush with tetrahydrofuran

5.6.5 Protein Size Exclusion Media

There are two wash/regeneration procedures associated with the removal of contaminants from protein size exclusion media

Weakly retained proteins

1. Flush with 30 mL 0.1 M pH3.0 phosphate buffer

Strongly retained proteins

1. Flush for 60 minutes using a 100% water to 100% acetonitrile gradient

5.6.7 Porous Graphitic Carbon

There are four wash or regeneration procedures associated with porous graphitic carbon. The one(s) used will depend on the analytes and solvents that have been used with the column

Acid/Base Regeneration

Suitable for ionized species analyzed in strongly aqueous mobile phases.

1. Invert the column
2. Flush at 1 mL/min with 50 mL tetrahydrofuran/water (1:1) containing 0.1% trifluoroacetic acid
3. Flush at 1 mL/min with 50 mL tetrahydrofuran/water (1:1) containing 0.1% triethylamine or sodium hydroxide
4. Flush at 1 mL/min with 50 mL tetrahydrofuran/water (1:1) containing 0.1% trifluoroacetic acid
5. Flush with methanol/water (95:5) to re-equilibrate
6. Re-invert the column

Author: R. Plumb – Glaxo, UK

Strong Organic Regeneration

Suitable for applications involving polar and / or ionized species analyzed in aqueous mobile phases.

1. Flush at 1 mL/min with 50 mL acetone
2. Flush at 1 mL/min with 120 mL dibutylether
3. Flush at 1 mL/min with 50 mL acetone
4. Flush with aqueous mobile phase until equilibrated

Normal Phase Regeneration

Suitable for applications running predominantly in normal phase mobile phases.

1. Flush at 1 mL/min with 50 mL dichloromethane
2. Flush at 1 mL/min with 50 mL methanol
3. Flush at 1 mL/min with 50 mL water
4. Flush at 1 mL/min with 50 mL 0.1M hydrochloric acid
5. Flush at 1 mL/min with 50 mL water
6. Flush at 1 mL/min with 50 mL methanol
7. Flush at 1 mL/min with 50 mL dichloromethane
8. Flush with mobile phase until equilibrated

Author: A. Karlsson – Uppsala, Sweden

Removal of Trifluoroacetic Acid

Suitable for applications running mobile phases containing trifluoroacetic acid

1. Flush the column with acetonitrile that has been heated to 75°C. The column should also be maintained at this temperature

5.6.8 Polymeric Media with Metallic Counter Ions

There are three types of regeneration available for polymeric columns with metal counter ion. Details of each procedure are listed in the following table.

Column Type	Metal Contamination	Organic Contamination	Column Cleaning
Hydrogen Counter Ion	Pump in reverse flow mode at 0.1 mL/min with 0.1M H_2SO_4 @ 25°C for 4 to 16 hrs	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN: H_2O @ 25°C for 4 hrs	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN: 0.01N H_2SO_4 @ 65°C for 4 hrs
Calcium Counter Ion	Pump in reverse flow mode at 0.1 mL/min with 0.1M $Ca(NO_3)_2$ @ pH 6.3 and 85°C for 4 to 16 hrs	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN:H_2O @ 25°C for 4 hrs	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN:H_2O @ 25°C for 4 hrs
Sodium Counter Ion	Pump in reverse flow mode at 0.1 mL/min with 0.1M $NaNO_3$ @ 85°C for 4 to 16 hrs	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN:H_2O @ 25°C for 4 hrs	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN:H_2O @ 25°C for 4 hrs
Silver Counter Ion	**No Regeneration Procedure Available**	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN: H_2O @ 25°C for 4 hrs	Pump in reverse flow mode at 0.1 mL/min with 25:80 ACN:H_2O @ 25°C for 4 hrs
Lead Counter Ion	Pump in reverse flow mode at 0.1 mL/min with 0.1M $Pb(NO_3)_2$ @ pH 5.3 and 85°C for 4 to 16 hrs	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN: H_2O @ 25°C for 4 hrs	Pump in reverse flow mode at 0.1 mL/min with 20:80 ACN: H_2O @ 25°C for 4 hrs

5.7 Chemical Compatibility of Polymeric Tubing with Solvents

The first table in this section contains information on the compatibility of a wide range of solvents at 20°C with PEEK™, polyethylene, polypropylene, PVDF, Teflon® and Tefzel®.

The second table contains information on the compatibility of PEEK with solvents at elevated temperature.

Note: *PEEK tubing will swell in the presence of DMSO, methylene chloride and THF.*

Solvent Compatibility with Polymeric Tubing

Solvent	PEEK	Polyethylene	Polypropylene	PVDF	Teflon	Tefzel
Acetaldehyde	1					
Acetic Acid, 20%	1	1	1	1	1	1
Acetic Acid, 80%	1	1	1	2	1	
Acetic Acid, glacial	1	1	1	1	1	1
Acetone	1	2	1	3	1	1
Acetonitrile	1				1	1
Ammonia, 10%	1	2	1	1	1	1
Ammonia, liquid	2					
Ammonium Hydroxide	1	1	1	1	1	1
Aromatic Hydrocarbons	1	2	3			
Benzene	1	2	3	1	1	1
Butanol	1	1	1	1	1	1
Chloroform	1	2	2	1	1	1
Cyclohexane	1	2	3	1	1	1
Cyclohexanone	1	3	3	3	1	1
Diethylamine	1	3	1	3	1	1
Diethylether	1					
Dioxane	1					1
Ethanol	1	2	1		1	1
Ethylacetate	1	2	1	3	1	1
Hexane/Heptane	1	2	2	1	1	1
Hydrochloric Acid, 100%	1		2	1	1	1
Hydrochloric Acid, 20%	1	1	2	1	1	1
Isopropanol	1	1	1		1	
Isopropyl Ether		1	2	3	1	
Ketones, general	1	2	2	2	2	
Methanol	1	1	1	1	1	1
Methyl Dichloride				3	3	
Nitric Acid, 100%	3	2	3	3	1	1
Nitric Acid, 20%	1	2	1	1	1	1
Perchloric Acid	1	2	2	1	1	
Phosphoric Acid, 100%	1	2	1	2	1	1
Phosphoric Acid, 20%	1	1	1	2	1	1
Sodium Hydroxide, 80%	1	2	1	3	1	
Sodium Hydroxide, 20%	1	1	1	1	1	1
Sulphuric Acid, 100%	3	2	2	1	1	1
Sulphuric Acid, 40%	1	1	1	1	1	1
THF	1	2	2	2	1	1
Toluene	1	2	2	1	1	1
Triethylamine			3	1	1	1

KEY

1 = Compatible, no adverse effect.

2 = Application dependant.

3 = Not compatible/ recommended.No figure denotes compatibility not calculated

KEY

1 = Compatible, no adverse effect.
2 = Application dependant.
3 = Not compatible/ recommended.No figure denotes compatibility not calculated

Solvent Compatibility with PEEK Tubing at Elevated Temperature

Solvent	Temperature (°C)	PEEK Compatibility
Acetic Acid	200	2
Ammonia, Liquid	200	1
Hydrogen Sulphide, gas	200	1
Methane, gas	200	1
Methylethylketone	200	3
Phosphoric Acid, 50%	200	1
Sodium Hydroxide, 20%	200	1
Sulfuric Acid, 50%	200	2
Sulfur Dioxide, gas	200	1

5.8 USP Specifications for HPLC Columns

The USP specifications are listed below with the appropriate Thermo columns listed for your convenience. In some cases, there is more than one column listed. When in doubt, it is recommended that you consult the original complete method as stated in the USP, or contact our technical support team for additional information or help in choosing the correct column.

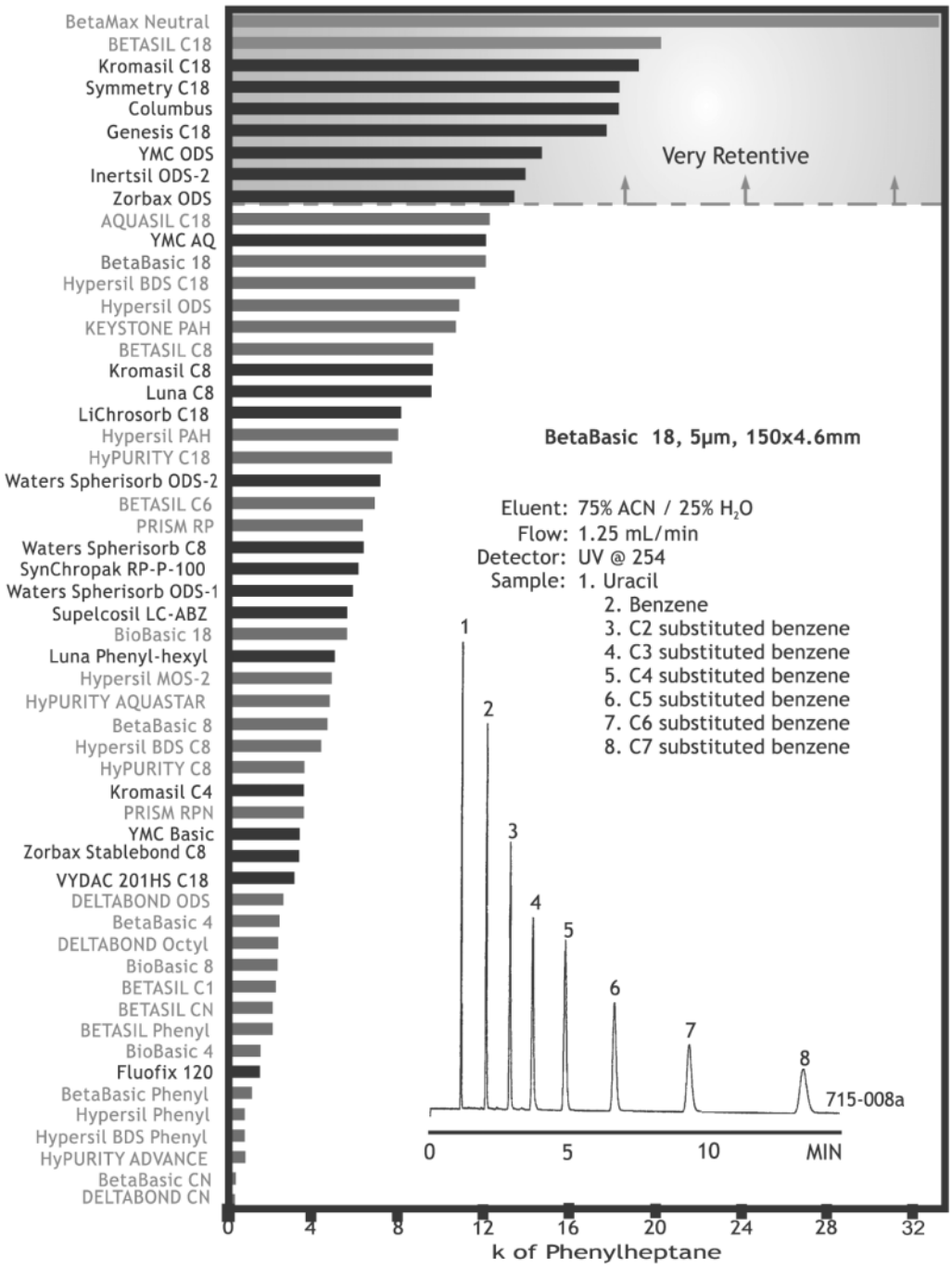
Phase	Particle Size	Particle Shape	End-Capped	Comments
L1 – Octadecyl silane (C18) chemically bonded to porous or ceramic microparticles, 3-10 µm in diameter				
Hypersil GOLD	3, 5 and 8 µm	spherical	yes	use when peak shape is critical
HyPURITY C18	3, 5 and 8 µm	spherical	yes	use for acids, bases, neutrals or chelators
HyPURITY AQUASTAR	3, 5 and 8 µm	spherical	-	wettable packing for enhanced polar retention
BioBasic 18	5 and 10 µm	spherical	yes	use for peptides, proteins and other biomolecules
BetaBasic 18	3 and 5 µm	spherical	yes	appropriate for most applications
AQUASIL C18	3 and 5 µm	spherical	-	use with highly aqueous eluents
Hypersil BDS C18	3 and 5 µm	spherical	yes	use for basic compounds
BETASIL C18	3, 5 and 10 µm	spherical	yes	100Å for high retention
BetaMax Neutral	5 µm	spherical	yes	use with at least 10-20% organic eluents
Hypersil ODS	3, 5 and 10 µm	spherical	yes	best for acids and neutrals
Hypersil ODS-2	5 µm	spherical	yes	alternative to Waters Spherisorb ODS-2 columns
L2 – Octadecyl silane chemically bonded to a silica gel of controlled surface porosity bonded to a solid spherical core, 30-50 µm in diameter				
Pellicular ODS	50 µm	spherical	no	
L3 – Porous silica microparticles, 3-10 µm in diameter				
BETASIL Silica	3 and 5 µm	spherical	–	available in 60Å, 100Å, 200Å and 300Å
Hypersil Silica	3, 5 and 10 µm	spherical	–	general purpose
L4 – Silica gel of controlled surface porosity bonded to a solid spherical core, 30-50 µm in diameter				
Pellicular Silica	50 µm	spherical	–	
L5 – Alumina of controlled surface porosity bonded to a solid spherical core, 30-50 µm in diameter				
L6 – Strong cation exchange packing; sulfonated fluorocarbon polymer coated on a solid spherical core, 30-50 µm in diameter				
L7 – Octyl silane (C8) chemically bonded to porous or ceramic microparticles, 3-10 µm in diameter				
HyPURITY C8	3 and 5 µm	spherical	yes	use for acids, bases, neutrals or chelators
BioBasic 8	5 µm	spherical	yes	use for peptides, proteins and other biomolecules
Hypersil BDS C8	3 and 5 µm	spherical	yes	use for basic compounds
BetaBasic 8	3 and 5 µm	spherical	yes	150Å appropriate for most applications
BETASIL C8	3 and 5 µm	spherical	yes	100Å appropriate for most applications
Hypersil MOS	3, 5 and 10 µm	spherical	no	best for acids and neutrals
Hypersil MOS-2	3, 5 and 10 µm	spherical	yes	best for acids and neutrals
L8 – An essentially monomolecular layer of aminopropylsilane chemically bonded to totally porous silica gel support, 10 µm in diameter				
Hypersil APS-2	3, 5 and 10 µm	spherical	no	best for reversed phase applications
L9 – 10 µm irregular or spherical, totally porous silica gel having a chemically bonded, strongly acidic cation exchange coating (SCX)				
Partisil SCX	10 µm	irregular	no	80Å pore size
L10 – Nitrile groups (CN) chemically bonded to porous silica particles, 3-10 µm in diameter				
HyPURITY Cyano	5 µm	spherical	yes	use for acids, bases, neutrals or chelators
BioBasic CN	5 µm	spherical	yes	use for peptides, proteins and other biomolecules
BetaBasic CN	3 and 5 µm	spherical	yes	150Å appropriate for most applications
BetaMax Base	5 µm	spherical	yes	very retentive
BETASIL CN	3 and 5 µm	spherical	yes	100Å appropriate for most applications
Hypersil BDS Cyano	3 and 5 µm	spherical	yes	use for basic compounds
Hypersil CPS	3, 5 and 10 µm	spherical	no	use for acids and neutrals
Hypersil CPS-2	5 and 10 µm	spherical	yes	better for reversed phase applications

Phase	Particle Size	Particle Shape	End-Capped	Comments
L11 – Phenyl groups chemically bonded to porous silica microparticles, 5-10 µm in diameter				
BioBasic Phenyl	5 µm	spherical	yes	use for peptides, proteins and other biomolecules
Hypersil BDS Phenyl	3 and 5 µm	spherical	yes	use for basic compounds
BetaBasic Phenyl	3 and 5 µm	spherical	yes	150Å appropriate for most applications
BETASIL Phenyl	3 and 5 µm	spherical	yes	100Å appropriate for most applications
Hypersil Phenyl	3, 5 and 10 µm	spherical	no	use for acids and neutrals
Hypersil Phenyl-2	5 and 10 µm	spherical	yes	use for acids, neutrals and slightly basic compounds
L12 – A strong anion exchange packing made by chemically bonding a quaternary amine to a solid silica spherical core, 30-50 µm in diameter				
L13 – Trimethylsilane chemically bonded to porous silica particles, 3-10 µm in diameter				
BETASIL C1	5 µm	spherical	yes	100Å appropriate for most applications
Hypersil SAS	3, 5 and 10 µm	spherical	no	use for acids and neutrals
L14 – Silica gel 10 µm in diameter having a chemically bonded, strongly basic quaternary ammonium anion exchange (SAX) coating				
Partisil SAX	10 µm	irregular	no	80Å pore size
L15 – Hexylsilane (C6) chemically bonded to totally porous silica particles, 3-10 µm in diameter				
BETASIL C6	3 and 5 µm	spherical	yes	100Å appropriate for most applications
L16 – Dimethylsilane (C1) chemically bonded to porous silica particles, 5-10 µm in diameter				
L17 – Strong cation exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form, 7-11 µm in diameter				
HyperREZ XP Carbohydrate H+	8 µm	spherical	-	use for organic acids, alcohols, glycoproteins, etc.
L18 – Amino and cyano groups chemically bonded to porous silica particles, 3-10 µm in diameter				
L19 – Strong cation exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the calcium form, about 9 µm diameter				
HyperREZ XP Carbohydrate Ca^{++}	8 µm	spherical	-	use for saccharides, alcohols, food adulteration, etc.
L20 – Dihydroxypropane groups chemically bonded to porous silica particles, 5-10 µm in diameter				
BETASIL Diol 100	5 µm	spherical	yes	100Å appropriate for most applications
L21 – A rigid spherical styrene-divinylbenzene copolymer, 5-10 µm in diameter				
HyperREZ XP RP100	5 µm	spherical	yes	100Å for compounds needing pH extremes and high temperature
HyperREZ XP RP300	5 µm	spherical	yes	300Å for peptides and small proteins
L22 – A cation exchange resin made of porous polystyrene gel with sulfonic acid groups, about 10 µm in size				
HyperREZ XP SCX	8 µm	spherical	yes	1000Å for macromolecules
L23 – An anion exchange resin made of porous polymethacrylate or polyacrylate gel with quaternary ammonium groups, about 10 µm in diameter				
L24 – A semi-rigid hydrophilic gel consisting of vinyl polymers with numerous hydroxyl groups on the matrix surface, 32-63 µm in diameter				
L25 – Packing having the capacity to separate compounds with a MW range from 100 to 5000 daltons (as determined by polyethylene oxide), applied to neutral, anionic and cationic water-soluble polymers. A polymethacrylate resin base, cross-linked with polyhydroxylated ether, (surface contained some residual carboxyl groups) was found suitable				
L26 – Butyl silane (C4) chemically bonded to totally porous silica particles, 5-10 µm in diameter				
HyPURITY C4	5 µm	spherical	yes	use for acids, bases, neutrals or chelators
BioBasic 4	5 µm	spherical	yes	use for peptides, proteins and other biomolecules
BetaBasic 4	3 and 5 µm	spherical	yes	150Å appropriate for most applications
Hypersil C4	3, 5 and 10 µm	spherical	-	use for acids and neutrals
L27 – Porous silica particles, 30-50 µm in diameter				
HyperPrep Silica	30 µm	spherical	yes	

Phase	Particle Size	Particle Shape	End-Capped	Comments
L28 – A multifunctional support which consists of a high purity, 100Å, spherical silica substrate that has been bonded with anionic exchanger, amine functionality, in addition to a conventional reversed phase C8 functionality				
L29 – Gamma alumina, reversed phase, low carbon percentage by weight, alumina-based polybutadiene spherical particles, 5 µm in diameter with a pore diameter of 80Å				
L30 – Ethyl silane chemically bonded to totally porous silica particle, 3-10 µm in diameter				
L31 – A strong anion exchange resin-quaternary amine bonded on latex particles attached to a core of 8.5 µm macroporous particles having a pore size of 2000Å and consisting of ethylvinylbenzene cross-linked with 55% divinylbenzene				
L32 – A chiral ligand-exchange packing-L-proline copper complex covalently bonded to irregularly shaped silica particles, 5 to 10 µm in diameter				
L33 – Packing having the capacity to separate proteins by molecular size over a range of 4,000 to 400,000 daltons. It is spherical, silica-based, and processed to provide pH stability				
BioBasic SEC	5 µm	spherical	-	60Å, 120Å, 300Å, and 1000Å for aqueous size exclusion
L34 – Strong cation exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the lead form, about 9 µm in diameter				
HyperREZ XP Carbohydrate Pb++	8 µm	spherical	-	use for saccharides, food adulteration, etc.
L35 – A zirconium-stabilized spherical silica packing with a hydrophilic (diol-type) molecular monolayer bonded phase having a pore size of 150Å				
L36 – A 3,5-dinitrobenzoyl derivative of L-phenylglycine covalently bonded to 5 µm aminopropyl silica				
L37 – Packing having the capacity to separate proteins by molecular size over a range of 2000 to 40,000 daltons. It is a polymethacrylate gel				
L38 – A methacrylate-based size exclusion packing for water soluble samples				
L39 – A hydrophilic polyhydroxymethacrylate gel of totally porous spherical resin				
L40 – Cellulose tris-3,5-dimethylphenylcarbamate coated porous silica particles, 5-20 µm in diameter				
L41 – Immobilized a1-acid glycoprotein on spherical silica particles, 5 µm in diameter				
L42 – Octylsilane and octadecylsilane groups chemically bonded to porous silica particle, 5 µm in diameter				
L43 – Pentafluorophenyl groups chemically bonded to silica particles, 5-10 µm in diameter				
Fluophase PFP	5 µm	spherical	yes	use for halogenated compounds and shape selectivity
L44 – A multifunctional support, which consists of a high purity, 60Å, spherical silica substrate that has been bonded with a cationic exchanger, sulfonic acid functionality in addition to a conventional reversed phase C8 funtionality				
L45 – Beta cyclodextrin bonded to porous silica particles, 5-10 µm in diameter				
L46 – Polystyrene/divinylbenzene substrate agglomerated with quaternary amine functionalized latex beads, 10 µm in diameter				
L47 – High capacity anion exchange microporous substrate, fully functionalized with trimethylamine groups, 8 µm in diameter				
L48 – Sulphonated, cross-linked polystyrene with an outer layer of submicron porous anion exchange microbeads, 15 µm in diameter				
L49 – A reversed phase packing made by coating a thin layer of polybutadiene onto spherical porous zirconia particles, 3-10 µm in diameter				
L50 – Multifunction resin with reversed phase retention and strong anion exchange functionalities. The resin consists of ethylvinylbenzene, 55% cross-linked with divinylbenzene copolymer, 3-15 µm in diameter, and a surface area not less than 350m2/g. Substrate is coated with quaternary ammonium functionalized latex particles consisting of styrene cross-linked with divinylbenzene				
L51 – Amylose tris-3,5-dimethylphenylcarbamate-coated, porous, spherical, silica particles, 5-10 µm in diameter				
L52 – A strong cation exchange resin made of porous silica with sulphopropyl groups, 5-10 µm in diameter				
BioBasic SCX	5 µm	spherical	-	300Å strong cation exchanger for peptides and proteins

Appendix A

Column Selection Guide: Thermo Column Hydrophobicity Chart



Reversed Phase Chemistries

Thermo reversed phase materials are based on high purity silicas giving excellent chromatography of acids, bases, and neutral compounds. Our premium columns include HyPURITY and BioBasic families in a wide range of chemistries, an excellent choice for all of your chromatography needs. Choose any of our families for method development, LC/MS, and your most demanding separations. All of our reversed phase materials offer superb reproducibility and reliable performance for routine and QC analyses. Our experienced Technical Support staff are always able to help you select the appropriate column for your application.

Premium Columns

Hypersil™ GOLD Columns: ***The key to outstanding peak shape***

- Exceptional peak symmetry and resolution
- Based on next-generation ultra pure silica
- Outstanding pH stability
- Ideal first choice for new method development

HyPURITY™ Columns: ***The choice for superior chromatography***

- Ultra-pure, highly stable silica for long column lifetime, reliability and performance
- Excellent peak shapes for acids, bases, chelators and neutral compounds
- 190Å pore size for small molecules, peptides, and protein digests
- Family of phases for a variety of selectivities
- Ideal for LC/MS applications
- Excellent alternative to other highly pure silica columns

BioBasic™ Columns: ***Better chromatography of biomolecules***

- 300Å pore size for better protein and peptide separations
- Excellent reproducibility, efficiency, and column lifetimes
- Range of stationary phases to optimize selectivity, including ion exchange and size exclusion
- Ideal for proteomics and LC/MS of biomolecules
- Excellent alternative to Vydac®, YMC-Pack™ and Zorbax® wide pore columns

Performance Columns

BetaBasic™ Columns: ***Stable, inert and reproducible for general HPLC***

- Superb stability at both high and low pH
- Efficient and reproducible columns
- A family of useful stationary phases
- 150Å pore size for small molecules, peptides and protein digests
- Excellent alternative to YMCbasic® columns

BETASIL™ Columns: ***High retention, efficiency and selectivity***

- Highly retentive phases with excellent peak shapes and lifetimes
- High surface area with high bonded phase coverage
- Phases with unique selectivity for reversed phase and normal phase
- Ideal for LC/MS applications, including short, fast DASH™ columns
- Excellent substitute for Inertsil® columns

BetaMax™ Columns: ***Maximum retention for maximum results***

- Ultra-high surface area and surface coverage for maximum retention
- 3 chemistries for 3 types of analytes: acid, base, and neutral
- Excellent stability for long column lifetimes
- BetaMax Neutral provides maximum C18 reversed phase retention for LC/MS applications

Classical Hypersil™ Columns

Classical Hypersil reversed phase chemistries from Thermo provide a proven record of performance for routine analyses year after year. Specified in thousands of methods world-wide, our classical Hypersil families offer exceptional reproducibility and reliability.

Hypersil BDS Columns: ***The standard base deactivated column***

- Base deactivated for reduced peak tailing and excellent peak symmetry
- Highly reproducible and efficient
- Reliable with long column lifetimes
- Excellent performance with basic, acidic and neutral compounds

Hypersil Classical Columns: ***Reliable and reproducible***

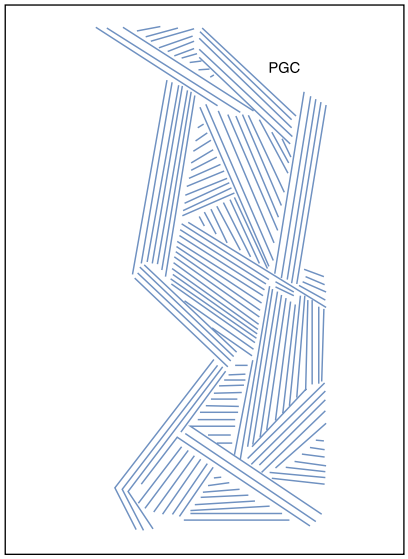
- Excellent phase used for thousands of existing methods
- High efficiency, proven reproducibility, and long column lifetimes
- Wide range of bonded phases including dedicated columns for specific applications

Unique Selectivities

Thermo offers a variety of HPLC columns with unique selectivities for both routine and challenging separations. These columns provide chromatographic solutions for many problematic analyses. Choose any of our innovative phases for method development or for standard separations. Please contact Technical Support for assistance in choosing the appropriate column for your application.

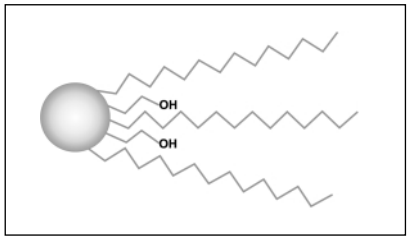
Hypercarb™ Columns: 100% porous graphitic columns solve problem separations

- Exceptional retention of polar compounds
- pH stable from 1 to 14
- Separate structurally similar compounds
- Ideal for LC/MS applications
- Superior performance compared to Waters XTerra®, ZirChrom®, and other non-silica based columns



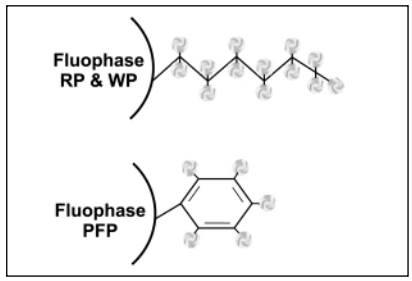
HyPURITY AQUASTAR™ Columns & AQUASIL C18 Columns: Retention of polar compounds

- Polar end-capped reversed phase columns for enhanced retention of polar compounds
- Compatible with 100% aqueous mobile phases and standard reversed phase mobile phases
- Run with reduced buffer concentrations
- C18 phase for reliability and high performance
- Ideal alternative to YMC-Pack ODS-AQ, Phenomenex® AQUA™, MetaSil™ AQ and other 2nd generation polar end-capped columns



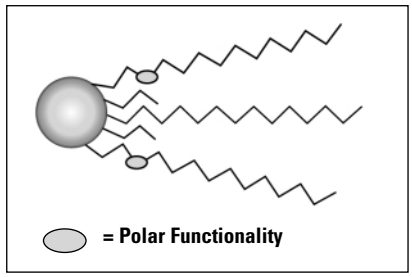
Fluophase™ Columns: Fluorinated phases for highly selective reversed phase chromatography

- Range of fluorinated phases provides unique selectivity
- Extra retention and selectivity for fluorinated and chlorinated compounds
- Excellent selectivity for polar non-halogenated compounds with nitro, hydroxyl, carboxyl and other polar groups
- Shape selective for structural isomers
- Use typical reversed phase solvents and conditions



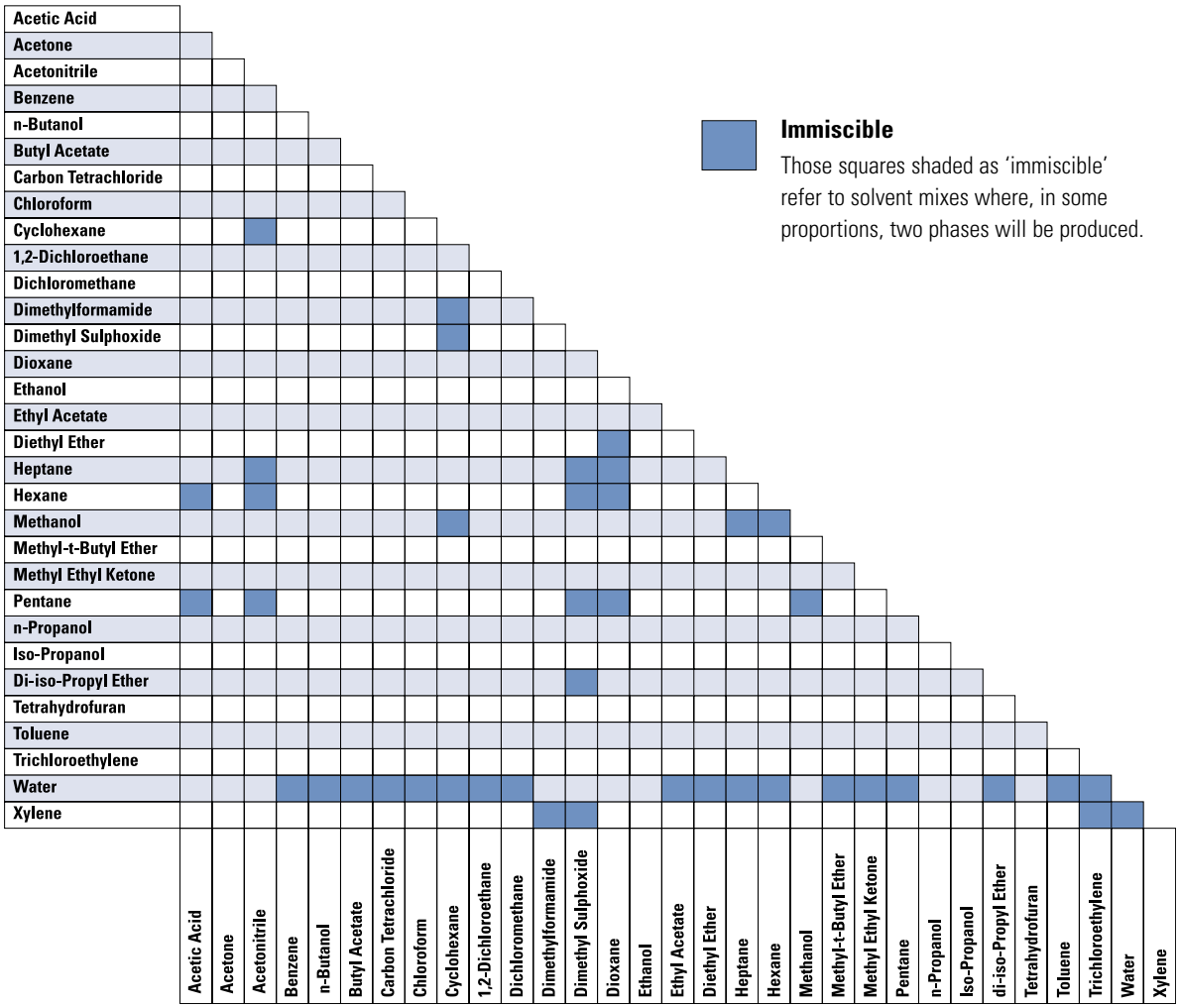
HyPURITY ADVANCE™ Columns & PRISM™ Columns: Polar embedded phase for reversed phase chromatography

- Unique reversed phase selectivity
- End-capped and non-end-capped versions of PRISM for selectivity choices
- Ideal for the separation of polar compounds
- Excellent alternative to Waters SymmetryShield™, Zorbax Bonus-RP, or Supelcosil® ABZ+Plus columns.



Appendix B

Solvent Miscibility Chart



Immiscible

Those squares shaded as 'immiscible' refer to solvent mixes where, in some proportions, two phases will be produced.

Appendix C

Example Routine Maintenance Log

Sheet Number	Issued	By
Detector Serial Number		
Pump Serial Number		
Autosampler Serial Number		
Column Heater Serial Number		
Other		

Date	Analyst	Maintenance Details

Appendix D

Example Problem Log

Sheet Number	Issued	By
Detector Serial Number		
Pump Serial Number		
Autosampler Serial Number		
Column Heater Serial Number		
Other		

Date	Analyst	Problem & Troubleshooting Outcome

Appendix E

Example Column History Log

Sheet Number **Issued** **By**

Date	Analyst	System Suitability Test Results			Comments
		Resolution	Tailing	Precision	

Appendix F

Example Instrument Use Log

Sheet Number	Issued	By
Detector Serial Number		
Pump Serial Number		
Autosampler Serial Number		
Column Heater Serial Number		
Other		

Date	Analyst	Method Details	System Clean-Up Details

References

1. Troubleshooting LC Systems, John W Dolan & Lloyd R Snyder, Humana Press 1989 ISBN 0-89603-151-9

2. Foley & Dorsey, Anal Chem 1983, 55, 730

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