TROUBLESHOOTING GUIDE – HPLC

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 theses 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 give rise to system and chromatographic problems. This guide will deal with each one in the following sections:

- visual inspection
- pressure
- baseline irregularities
- changes in chromatography
- qualitative results
- quantitative results

I- 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…

II- 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 high or low pressures to the norm. This reference point should be the pressure generated in the system when everything if functioning correctly.

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 positioned above and adsorbent material to soak up all released solvents.

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

II-1. High pressure

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

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 "col. back pressure" 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.		

II-2. Low pressure

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.

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		

II-3. Fluctuating pressure

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

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 Solvent properties.
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		

III- Baseline irregularities

Baseline irregularities can be non-cyclic or cyclic. They can originate from electrical interferences, detector faults, solvent impurities, column contamination, etc.

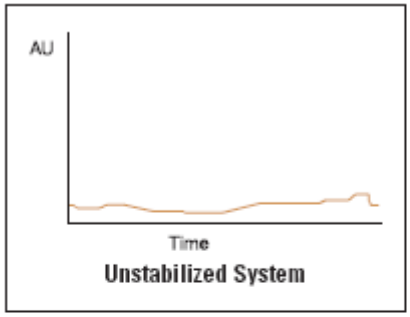
To isolate the source of a baseline irregularity, it is important to determine whether the problem lies with the fluid path, detector or electrical connections. This can be achieved by following the simple steps below:

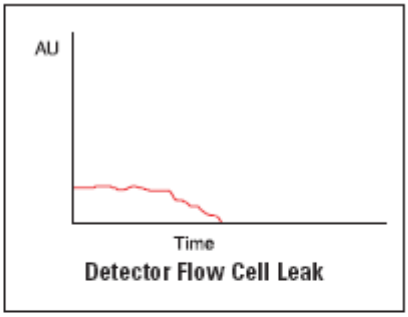
1. Turn off the instrument pump - fluid flow must be zero
2. Monitor the baseline for 5 to 10 min. note if there is any improvement in the baseline's appearance. If yes, then the problem lies within the instrument fluid path. If no, the problem is either electrical or detector related.
3. Disconnect the detector electrical cables from the A/D interface with 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…). If the noise continues, then the problem is within the data-handling device.

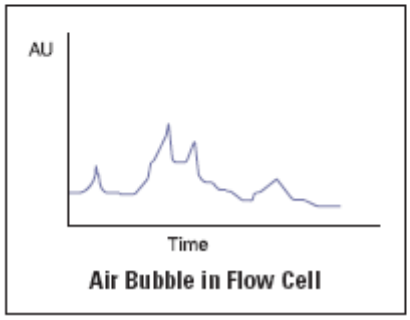
Data-handling device troubleshooting is beyond the scope of this guide: Contact your instrument provider for this service.

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

III-1. Non-cyclic noise – Fluid path problems





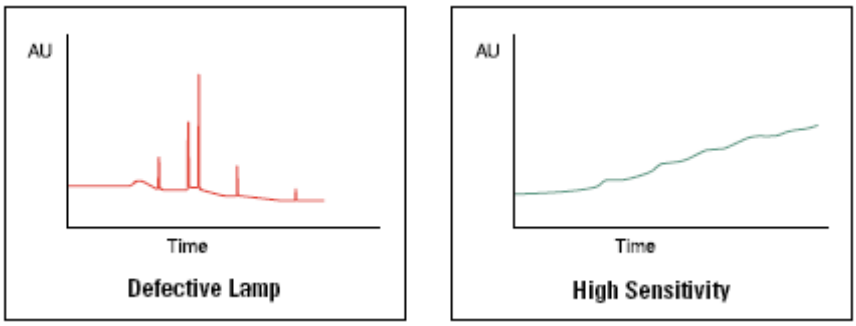


The most common cause of non-cyclic baseline noise related problems is air in the system. To overcome this, all solvents should be thoroughly degassed prior to use, all lines should be purged with solvent and the pump, should be thoroughly primed.

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

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 Column cleaning. 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.

III-2. Non-cyclic noise – Detector electronics problems

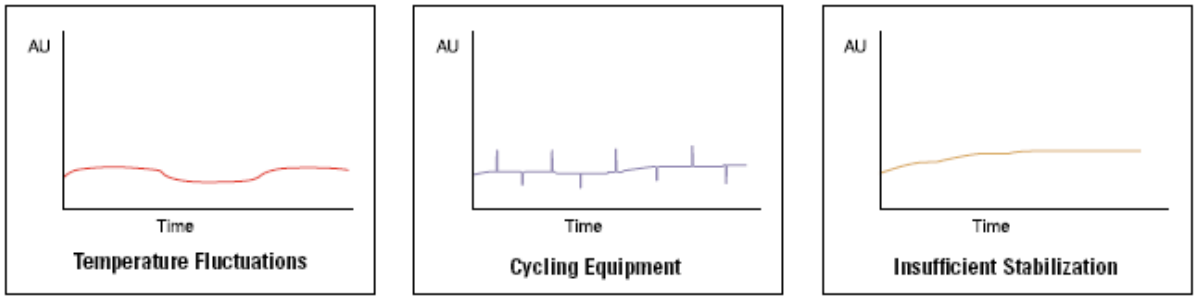


The most common cause of problems related to electronic baseline noise is the detector. Usually, if the detector is allowed insufficient time to equilibrate before an injection is performed, then the resultant chromatogram will contain spurious peaks and there will also be some evidence of baseline drift.

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 position prior to the routine maintenance.

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.

III-3. Cyclic noise – Detector related problems and others



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.

IV- Changes in chromatography

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

The evaluation stage is best preformed 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.

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.

IV-1. Retention time changes from injection to injection

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.

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 II-3. 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.*

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

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 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.

We recommend the pre-mixing of all solvents used in isocratic methods.

IV-2. Continually increasing or decreasing retention times

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 Column cleaning. 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.

IV-3. Increasing/decreasing to a new constant retention time

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

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 IV-1.
Column Contamination	Refer to the table in section IV-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.

IV.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 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, fitting, etc.

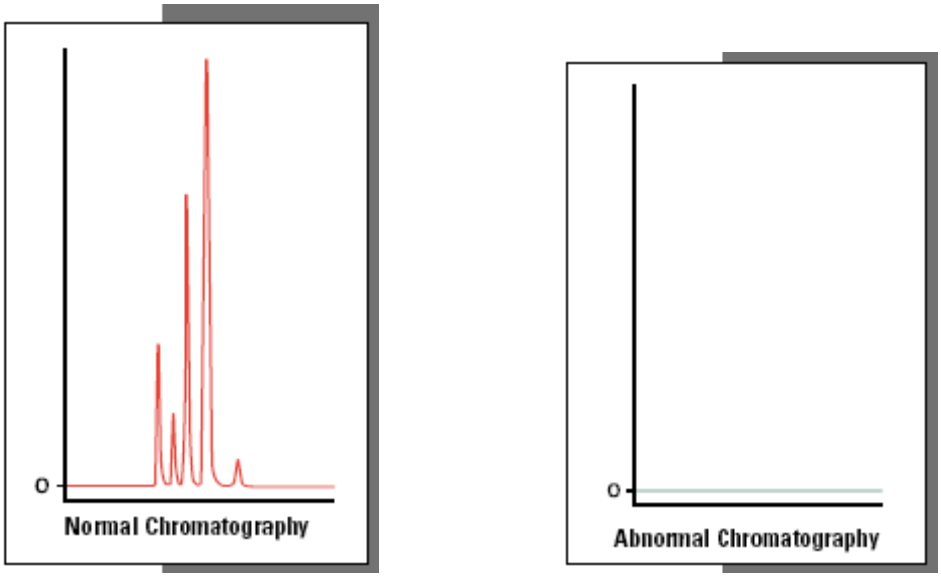
If single peaks are affected, then it suggests that there might be a specific chemistry problem. The method in use should be examined for areas where the chemistry may not be correct.

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

Isocratic methods, where early eluting peaks are abnormal and 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 peaks are abnormal, then extra-column band broadening or other changes in the system are the most likely causes.

Each table in this section will show an example of the type of chromatography being investigated, it is cause and any corrective action that can be taken.

No peaks

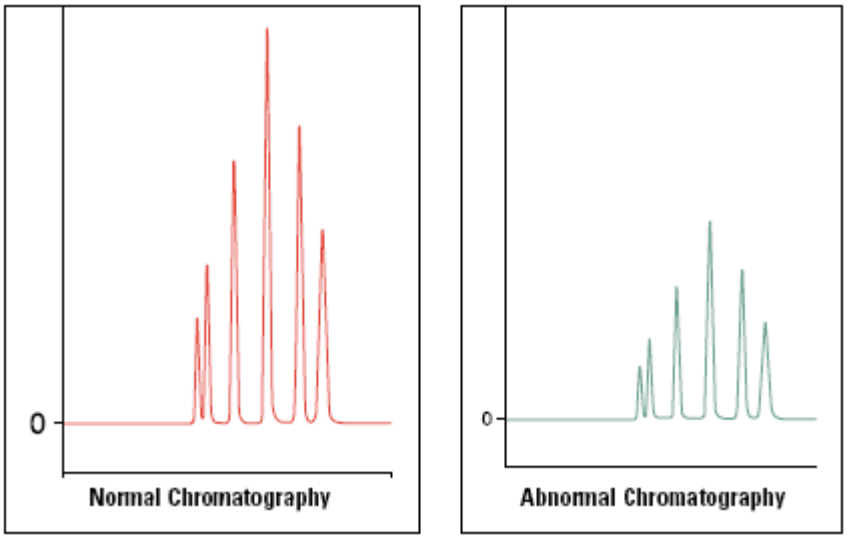


Lack of chromatogram peaks is often due to either the wrong sample being injected, the detector not being switched on or a blockage between the injector and detector lines.

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.

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.*

Smaller than expected peaks



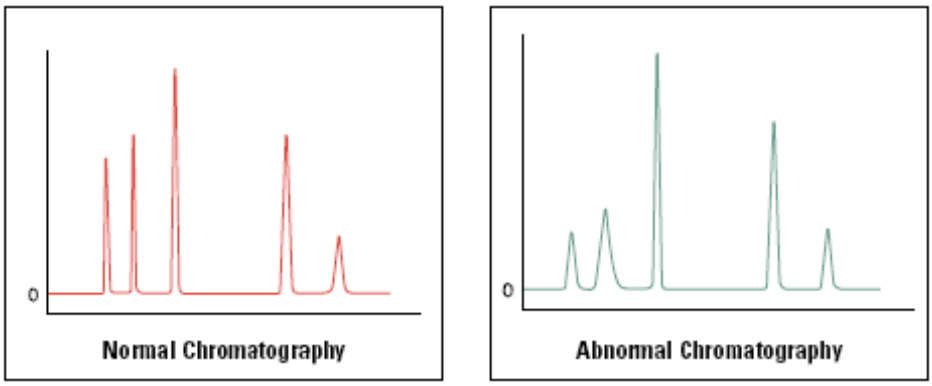
Smaller than expected peaks are often due to either the wrong sample being injected, an incorrect sample volume being injected, or a blockage between the syringe needle and detector. Problems with the syringe plunger sticking in the barrel can occur if the sample contains particulates.

Note:

Viscous sample 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 Chromophore detection wavelengths.
Sample Too Viscous	Dilute the sample or decrease the rate at which the syringe draws the sample.

Early eluting peaks broad

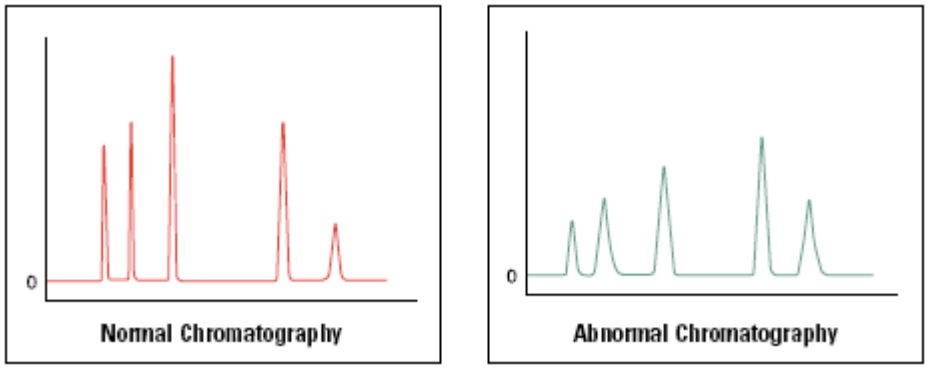


Broad early eluting peaks are most commonly associated with sample overload or incorrect system plumbing.

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 Band spreading 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.*

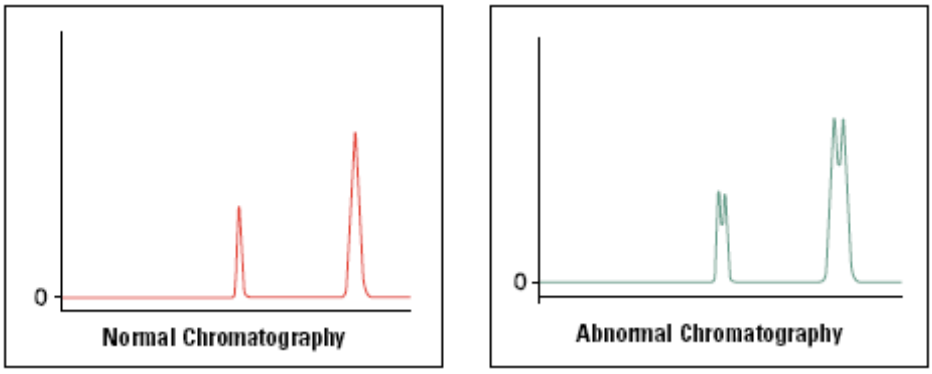
All peaks broad

Broad peaks 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 Column cleaning. 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 Column cleaning.
	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.

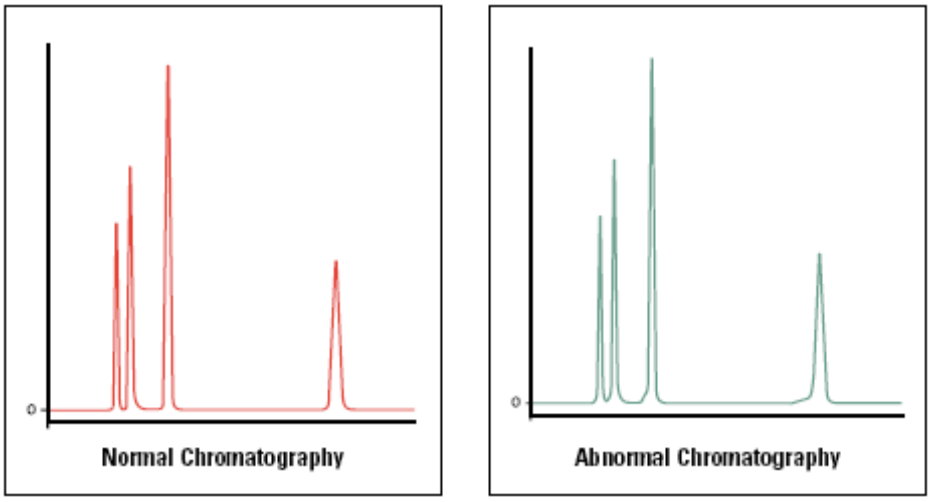
All peaks doubling



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

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 Column cleaning. 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.

Fronting peaks

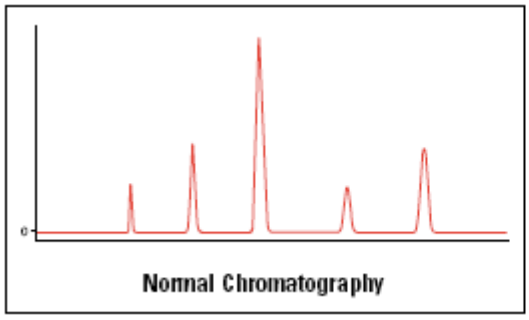


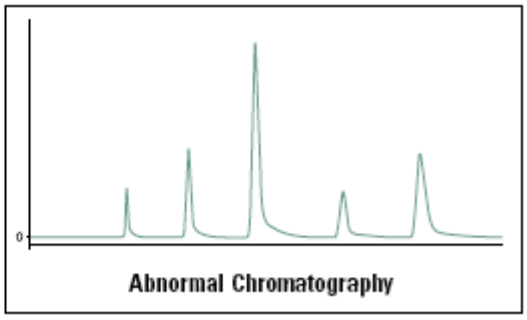
Fronting peaks are very often due to large injection volumes of a sample that is dissolved in solvents that are incompatible with the mobile phase being used. The next most common cause of peak fronting is a voided or contaminated guard or column.

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 Column cleaning. . 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.

Tailing peaks

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

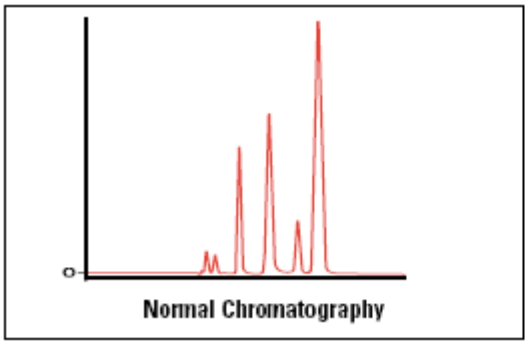


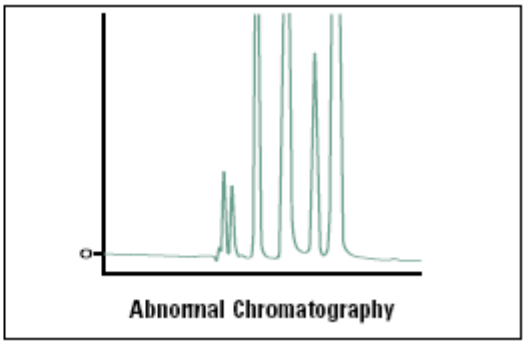


Possible Cause	Corrective Action
Column Problems	Column contamination: Clean the column following the guidelines in section Column cleaning. If the problem remains after cleaning, replace the column.
	Column degradation: Replace the column with one where the performance is known. Perform a series of 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 Column cleaning.
	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

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



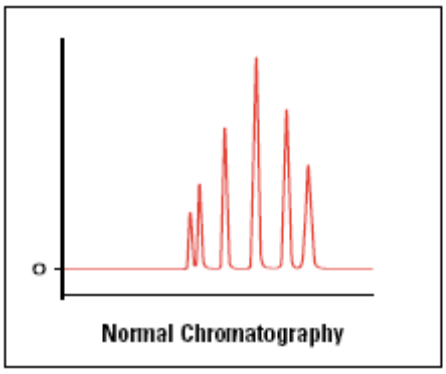


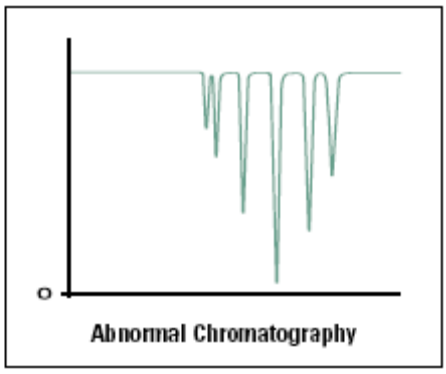
Possible Cause	Corrective Action
Injection Problem	Ensure that the volume of sample injected is correct and that the sample is of the correct strength.
	Dilute the sample or perform a smaller injection.
Detector Setting Error	Check the wavelength, zero and sensitivity settings. Adjust if necessary.
Recorder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

Negative peaks

Negative peaks are most often caused by difference 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.





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.

V- 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 misidentification.

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

V-1. Missing peaks

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

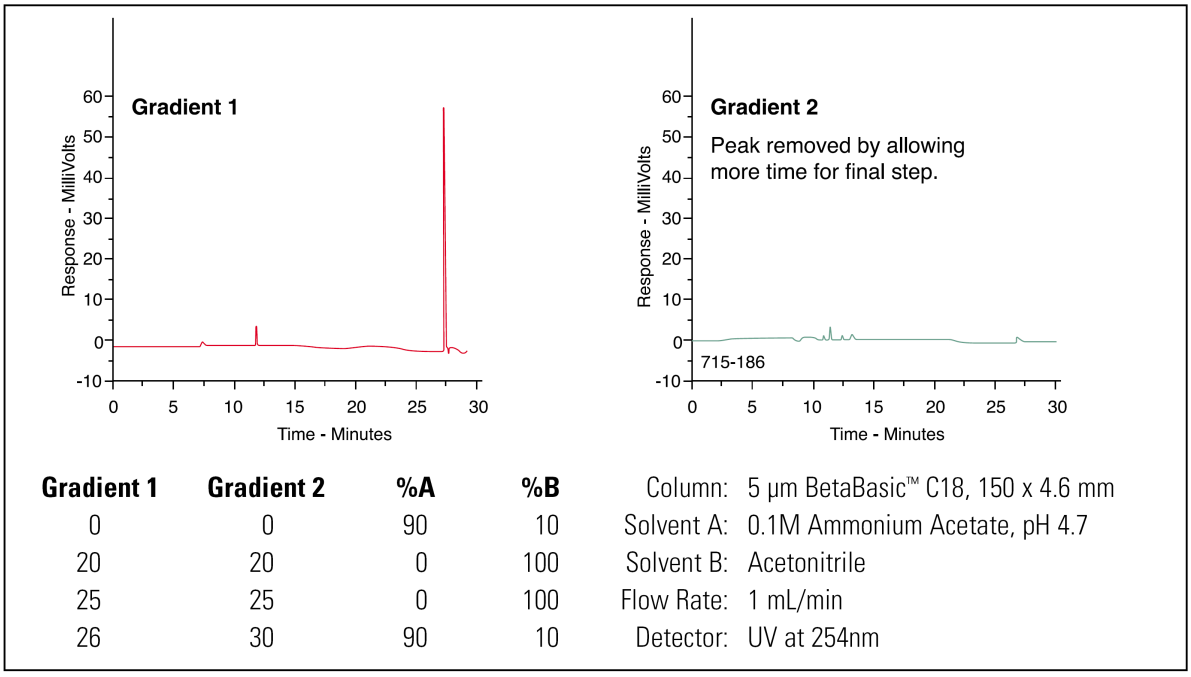
Equally likely though is a loss of resolution due to column/solvent inconsistencies.

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 II-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 Column cleaning. 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 IV-4.

V-2. Extra peaks

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 peaks are in the sample alone, perform a blank injection of sample solvent. The peaks should be absent.

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 Baseline irregularities.
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 Column cleaning. 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.



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

V-3. Peaks misidentified

Peaks 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.

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 IV-1 to IV-3.

VI- 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, is the result generated is correct, 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.

VI-1. Loss of accuracy

Loss of accuracy is most often related to the sample.

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.

VI-2. Loss of precision

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 IV.
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.*