(U)HPLC Troubleshooting guide

Your handbook for tackling common issues and maintaining productivity

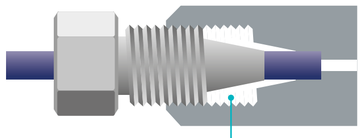
General / Good practice

1. **External factors can affect performance:** Temperature or light-sensitive samples. Make sure that the application was running trouble-free before and that no changes have been made to the system.

2. **A proper wash routine**, aligned with the sample method, helps prevent unnecessary carryover, reduces wear and tear on the autosampler flow path, and can extend the analytical column's lifespan.

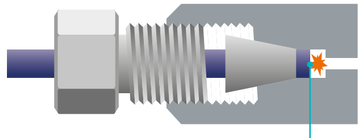
3. Connections: **Use only the supplied nuts and ferrules** to ensure good connections without leakage: The required tubing length (L) varies by connection brand; incorrect length can cause faulty peaks and carry-over. For a leak-tight connection, the ferrule must be compressed properly into the valve. If L is too long, the ferrule won't seal, potentially damaging the valve.

Part of the tubing may end up in de valve internals. Valve internals may be damaged.



Ferrule does not seal the connection

If **L** is too short, this may result in leakage or deadvolume at the end of the ferrule (a 'mixing chamber').



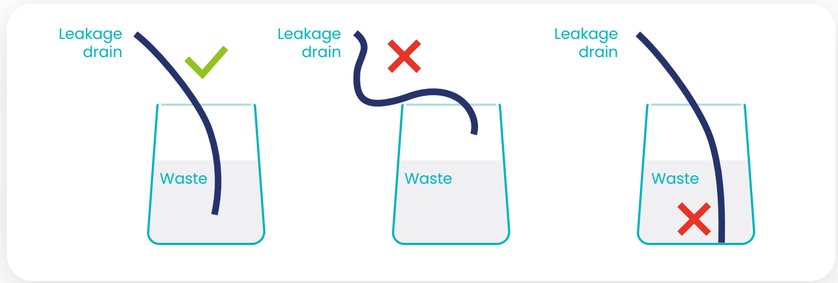
Too short

Also reference to how valves can influence the LC system:
A comparison of high-end injection valves for (U)HPLC autosamplers (sparkholland.com)

4. **Always use original connections** specifically designated for your instrument. Avoid using fittings that have been used with other instruments to prevent potential valve damage and contamination, which could lead to inaccurate analysis results.

5. Application quality controls: **routinely use quality control samples** at low, average, and high levels to monitor system performance. Ensure results fall within acceptable ranges and assess precision daily. Data outside this range should not be reported until performance is confirmed. Also, use blanks to check carryover and data integrity, and apply a reliable internal standard. Do not trust results if the system errors.

6. Waste connection: **Ensure the drain tubing slopes downward** continuously from the waste outlet to the waste container. If not properly sloped, blockages can occur, preventing waste liquid from flowing out, potentially leading to buildup within the instrument and activating leak sensors, see picture:



7. **Avoid refilling mobile phase and wash solvents** to prevent contamination of the analytical flow path and improper solvent mixtures.

8. UHPLC systems require the **use of higher-grade solvents**, such as UHPLC or LCMS grade, in conjunction with the analytical column.

9. Solvent incompatibilities: When **using solvents** in an LC system, ensure they are **compatible with all system components**. Be particularly aware of the stainless-steel flow path, as incompatible solvents—such as those containing EDTA, highly acidic solvents (pH < 1), halogenated solvents, or additives that form radicals, acids, or peroxides—can cause damage.

10. **Biocompatibility**: in an HPLC flow path it is crucial for applications with sensitive biological samples, like protein analysis and biomarker research. Using non-reactive materials, such as PEEK or titanium, prevents sample degradation and ensures accurate results in bioanalysis and clinical diagnostics. For more information reference: Biocompatibility in (U)HPLC Technology - Spark Holland and Automated sample prep systems improve biosensor platforms.

11. **Place solvent bottles on flat, stable surface** that is at the same height, or at a higher level than the pump. The solvents should also be placed in a tray that also can serve as a receptacle for possible leakage. Mobile phase and wash solvent lines should be filtered (0.2 µm filter) and covered.

Daily tasks

- Regularly check for leaks in the system
- Keep a log of maintenance activities
- Prime the pump and autosampler to clear any air bubbles present
- Check solvent levels, both mobile phase, rear seal wash and wash solvents
- Replace aqueous and organic solvents frequently

Weekly tasks

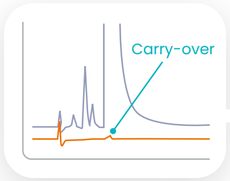
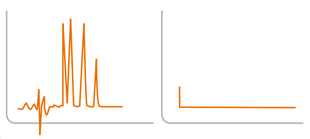
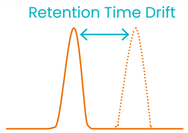
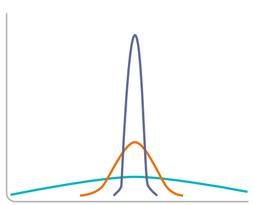
- Change rear seal wash
- Inspect solvent filters
- Check column condition including backpressure
- Monitor baseline stability

Preventative Maintenance

- Preventative maintenance on an HPLC system ensures reliable performance, reduces downtime, and extends equipment lifespan. Regular checks prevent costly repairs, maintain data accuracy, and improve system efficiency, helping labs avoid disruptions and maintain high-quality results.
- Increase system longevity
- Reduces downtime
- Enhances data quality
- Lowers repair costs
- Improves safety

Reference service videos to aide qualified service engineers on YouTube.

Oven Troubleshooting

Observation	Potential Cause	Solution
Leak (Activated leak sensor)	Wear on rotor seal in the diverter valve or columns selection valve.	Replacement of the rotor seal.
	Poor connection to valve or column.	Reconnect or replace nut/ferrule of the connection.
 **Carry-over**	If oven is suspected can be caused by column or valves.	Oven can be taken out of the flow path, do several injection to see if problem persists.
 **Missing peaks**	Usually caused by a leak.	Check connections in column oven, e.g. Column, diverter valve or column selection valve.
 **Unstable retention times**	Temperature control not used.	Use a column oven if there are variations in ambient temperature, unstable conditions can cause shifting retention times.
High backpressure	Too high LC flow rate for column dimensions.	Using a column oven with higher temperatures can lower the backpressure.
 **Peak broadening**	Thermal mismatch and viscosity changes of the solvent.	Using a solvent preheater can warm the solvent to the same temperature as the column. This is present in both the Mistral and Scirocco column ovens. The Scirocco has both an option for an active and passive option.

Mistral™ oven

Scirocco™ oven

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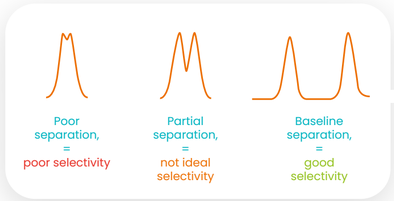
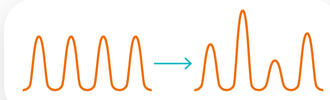
Oven Troubleshooting





Observation	Potential Cause	Solution
Separation problems	Separation of compounds not consistent or not adequate for detection	Temperature control is essential for separating thermally sensitive analytes, like proteins and peptides, and for preserving temperature-sensitive stationary phases, thereby enhancing column integrity and lifespan.
Bad reproducibility	If oven is suspected can be caused by column or valves.	Oven can be taken out of the flow path, do several injection to see if problem persists.
Column issues	Excessive backpressure caused by sample particulates or buffer precipitation.	Always consult the column manufacturer's instructions, which provide guidance on column handling, cleaning and troubleshooting procedures. Moreover, the column can be excluded from the flow path completely or exchanged with a different or new one to see if the column is causing the issue.
	Peak tailing/asymmetry caused by column contamination, degradation or interaction with active sites.	
	Peak fronting caused by overloading the column.	
	Separation problems caused by dead volumes, mobile phase incompatibility.	
	Contamination caused by sample, mobile phase buffers or salts that can appear as ghost peaks or carryover.	
	Retention time drift caused by column degradation.	
	Reduced column lifetime caused by poor maintenance, incompatible solvents or extreme pH conditions.	

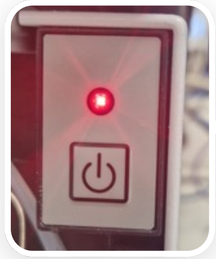
Good practice

- Optimize the temperature based on the analytes, higher temperatures could improve separation, while lower temperatures helps thermally sensitive compounds.
- Allow sufficient time to equilibrate the column set temperature.
- Use stable temperature settings by avoiding frequent temperature changes.
- Only change the column selection valve position if there is no pressure on system to protect the column.

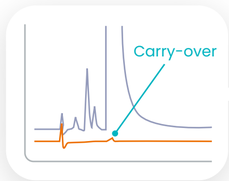
Continue to Autosamplers

Autosampler Troubleshooting

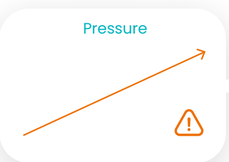
(General for all injection routines)



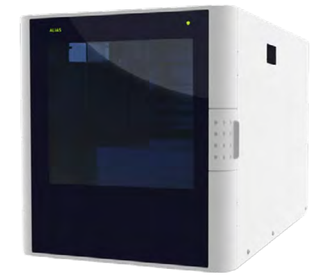
Observation	Potential Cause	Solution
Leak (Activated leak sensor)	Waste tubing wrongly fitted	Refit or reposition the tubing.
	Leak in syringe, syringe valve or injection valve.	Replace syringe or refit the connection.
	Loose connection with a fitting or syringe.	Reconnect or replace nut/ferrule of the connection.
	Wear on rotor seal.	Replace the rotor seal.
	Overpressure on the injection valve.	Observe maximum pressure allowance for different valves.



Carry-over	Contaminated needle seat.	Run various primes/washes to clean the flow path, otherwise replace needle seat.
	Dead volumes from improper exchange of parts in flow path.	Inspect connections and if necessary, redo connections with new ferrules and nuts (refer to good practice example for proper connections).
	Bends in the wash lines causing less wash than expected.	Replace wash lines.
	With double needle system, overfilled vials.	Avoid adding too much sample to vial so piercing needle does not touch it.
	Contaminated mobile phase.	Check mobile phase, make fresh.



Abnormal pressure increase during injection	Blockage in needle tubing or in needle seat assembly.	Flush the needle or needle seat assembly with the LC pump or replace part where blockage occurred if not cleared.



Alias™ autosampler

Integrity™ autosampler

Nexas™ autosampler

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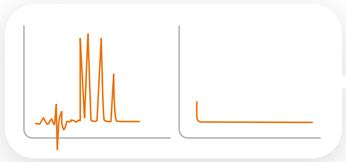
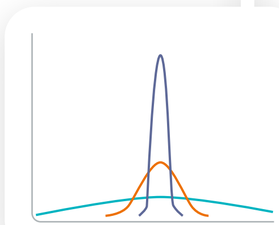
Autosampler Troubleshooting

(General for all injection routines)



Observation	Potential Cause	Solution
Missing peaks	Air in flow path.	Prime system and used degassed wash solvents.
	Insufficient sample in vial.	Add more sample or adjust needle height in LC method.
	Blockage in flow path.	Check flow path for leaks and replace or refit connections or blocked parts.
	Empty wash solvents.	Refill/check wash solvents.
Peak widening/ distortion	Incompatible wash solvent or transfer solvent.	Depending on injection routine this step needs optimization to ensure good analytical results including peak shape.
	Injection volume too large.	Reduce the injection volume or the solvent strength of the injection solvent.



Dispersion	Flow path not properly considered along with injection type	Planning of the flow path is key to minimizing the affect of dispersion. Please refer to the white paper on injection routines: The comparison of LC autosampler injection designs - Spark Holland. Also article on Dispersion.
Peak broadening	Thermal mismatch and viscosity changes of the solvent.	Can alter plug shape and cause peak widening; this can often be mitigated by reducing injection volume or reconstituting the sample in the initial mobile phase.

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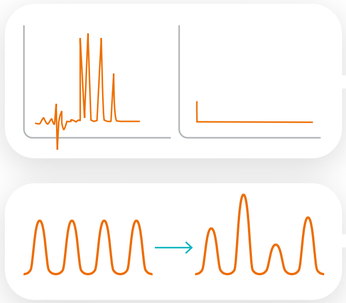
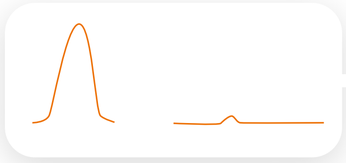
Autosampler Troubleshooting

(General for all injection routines)

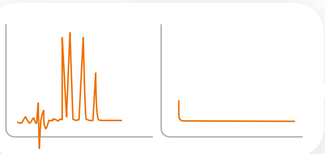
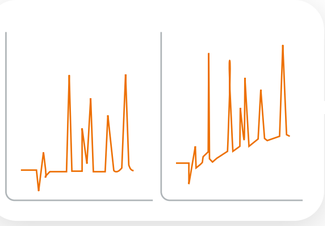
Observation	Potential Cause	Solution
 **Bad reproducibility**	Sample evaporation from not properly sealed/covered well plates or vials.	Limit number of injections from a vial to 3, and seal well plates properly.
	Sample degradation.	Use the cooling function on the autosampler.
	Leak or air in flow path.	Refit/replace connections or syringe. If air does not exit the syringe, prime with isopropanol.
	Diminishing performance.	Preventative maintenance could be needed.
	Incompatible syringe and sample loop size.	Make sure syringe and sample loop size correlate to injection volume. Too big syringe volume and too small desired injection volume can affect reproducibility: best to use a syringe volume that matches the required injection volume range.
 **Sensitivity Issues**	Sample degradation.	Use climate control in the autosampler.
	Adsorption to surfaces.	Switch from glass to plastic vials or to specially coated glass vials. Consider biocompatible flow path: Biocompatibility in (U)HPLC Technology - Spark Holland.
	Air bubbles in flow path.	Air can lead to inaccurate injection volumes, so by priming and purging lines to remove air is needed.
 **Missing vial error**	Incorrect vial or plate type selected or wrong configuration.	Ensure the correct vial position and tray type are selected and verify that the instrument configuration is accurate.

Good practice

- Sample Stability: Always verify that any issue isn't due to sample instability.
- Start day with priming of the autosampler to remove any air in the system.
- At the start of every run table ensure that there is enough wash solvents.
- Proper vial/well plate filling, fill vials to an appropriate level to prevent needle from injecting air or overfilling to contaminate piercing needle.
- Properly label vials to avoid any mix-ups and verify positions in the tray.

Continue to Pumps

Pump Troubleshooting

Observation	Potential Cause	Solution
 **Leak**	Loose connections.	Check flow path and tighten connection, e.g. check valves. Use only the supplied nuts and ferrules to ensure proper connections.
	Blockage in flow path.	Replace clogged lines and/or replace the solvent filters.
	Waste tubing wrongly fitted.	Refit or reposition the tubing.
 **Missing peaks**	Insufficient solvent in mobile phase lines.	Check solvent bottles and fill solvent lines if need be.
 **Unstable pressure**	Air bubbles inside pump head.	Prime and purge the pump.
	Solvent filter clogged.	Clean or replace solvent filters.
	Inlet/outlet check valve malfunctioning.	Pump isopropanol through the lines to clean check valves, if not cleared remove and replace check valves.
	Leak.	Inspect and tighten all connections, if necessary, nuts and ferrules may need to be replaced.
 **Flow rate unstable**	Air inside the pump head.	Prime and purge the pump.
	Solvent filter clogged.	Clean or replace solvent filters.
	Change in flow resistance.	Check for small leaks

SPH1299™ V2 pump

SPH1299Q™ V2 pump

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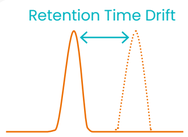
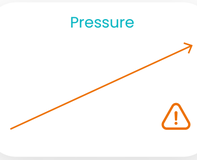
Pump Troubleshooting

Observation	Potential Cause	Solution
 **Air bubbles in flow lines**	Solvent filter is clogged.	Clean or replace the filter.
	Lack of solvent.	Refill solvent reservoir.
	Leak.	Tighten or replace fittings.
 **Unstable retention times**	Insufficient equilibration time with gradient run or modification in isocratic mobile phase.	Be sure column is equilibrated enough, usually 10 column volumes should pass through a column after a sample run.
	Kink or bend in the solvent lines.	Replace damaged lines.
 **Pressure increase**	Blockage.	Check flow path for leak, replace parts or try to flush out.
	Buffer precipitation from mobile phase.	Verify the compatibility of buffer-organic mixtures.

Good practice

- Setup a maintenance schedule that includes regular replacement of various parts in the pump.
- Be sure the pressure does not exceed the maximum system pressure.
- It is best to increase/decrease the pressure slowly in steps.
- The seal wash is always used so make sure there is always sufficient seal wash solvent.
- Use only UHPLC grade solvents.
- If buffer or salt is used, make sure to not leave them in the flow lines for long period of time as they can form crystals or residues that clog the solvent lines, damage pistons or the degasser.
- When the pump is not used for a long period of time, thoroughly clean with isopropanol.
- To keep the pump in good condition, flush tubing before starting or after finishing daily operations.

End