LC columns

Column care guide and general method development information for Thermo Scientific HILIC columns

Applies to columns bonded with phases such as HILIC, amino, cyano and bare silica

Before you get started

Manuals, specification sheets or technical guides for your column might be available to download from **thermofisher.com**. Type the P/N or product name in the search box. Helpful literature is near the bottom of the product page. Some columns include a Quick-Start Guide in the box and/or a yellow caution tag on the column. Please read these before using the column.

Always start by investigating the Certificate of Analysis (CoA) or Quality Assurance Report (QAR) accompanying your column. This document includes a lot of valuable information. For instance, investigate what solvent the column is shipped in. If the column is filled with something incompatible with your mobile phase, flush it out with a mutually compatible intermediate solvent. Some detectors such as charged aerosol and mass spectrometers are highly sensitive to column bleed. Condition the column before connecting it to the detector.

You should always strive to reproduce the chromatogram in your CoA or QAR when you receive the column into your lab. This way you can assure that the column is operating correctly when you start you method, and if you routinely repeat the column's CoA or QAR, you can notice column degradation early on and implement preventative measures if needed.

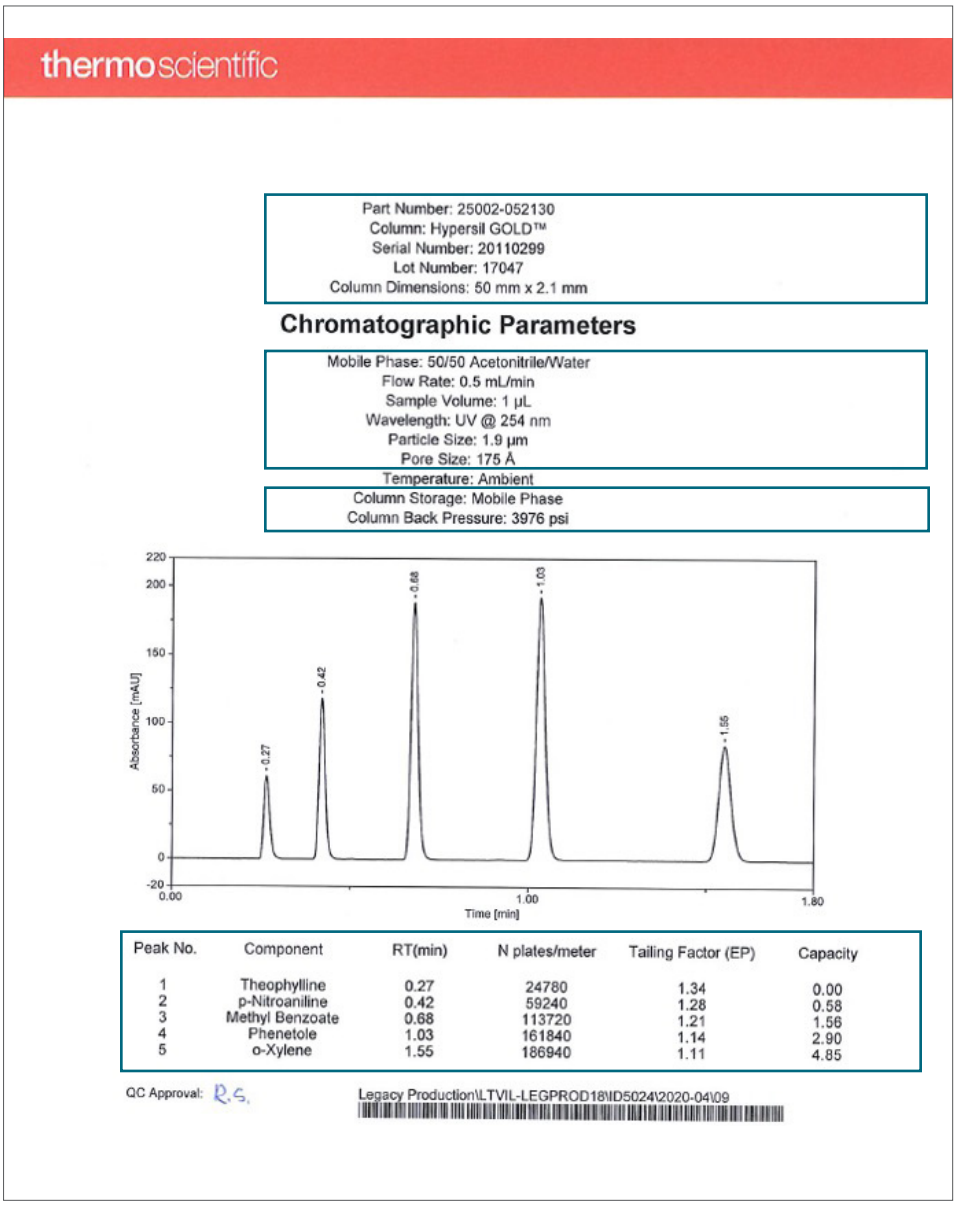
For UHPLC columns operating at high pressure > 400 bar, it can take the column 20–30 minutes of extra time to come to thermal steady-state after the column oven is ready. Continue equilibration until the pressure and detector baselines are stable.

Always check for leaks before use.

Operational limits

Respect the limits for pressure, pH, temperature and solvent compatibility. The product manual, specification sheet or technical guide is the best reference for operational limits. If there is not a manual, see the online **catalog** or product web page on **thermofisher.com**

Operating near the extremes of the pH or temperature limits can reduce column life and increase column bleed.



Stationary phase as well as lot and serial number

Test conditions

Shipping solvent

Test results

This is an example of how you would read your CoA or QAR

Operational best practices

Clean samples make for robust methods and longer lifetime of your column. Always strive to clean you samples as much as possible to assure your best results. Filter samples to 1/10 of the particle size of the column. This in general means for sub 2 µm or near 2 µm particle column—use a 0.2 µm filter. For larger particle sizes, such as 5 µm or 10 µm, you can use 0.45 µm filters. Alternatively perform other sample preparation techniques such as Solid Phase-Extraction (SPE) to clean your sample for chemical as well as particulate contaminants. Always use a guard column or an inline filter to prolong the lifetime of your column. Exchange guard cartridges or filters regularly.

When considering the use of mobile phases, use appropriately high-quality ingredients. Ideally use factory-filtered HPLC-grade (or higher) solvents. Regularly maintain your water purifier to assure best quality. Do not “top up” buffer reservoirs. Always make a fresh batch in a clean bottle. Check buffers daily for microbial growth, especially if Phosphate buffers are used. As much as practical, make solvent mixtures and buffers by weight. Check the pH before use. Filter buffers through a 0.2 µm membrane (0.1 µm for UHPLC).

Initial installation

Most HILIC columns are shipped with various proportions of acetonitrile, but can also be shipped in normal phase solvents. Take care when introducing solvents onto your column and assure miscible solvents. What solvent your column is shipped in is noted in the CoA or QAR as explained above. Columns using bare silica, urea-HILIC, amino, cyano, and diol stationary phases are compatible with either normal-phase or HILIC solvents. If your mobile phase is not miscible with the shipping solvent, use a compatible intermediate solvent to flush the column before equilibrating with mobile phase.

After ensuring your column is in correct HILIC solvents, to condition, flush with 50% acetonitrile, 50% buffer until the baseline is stable. Then flush with 90% acetonitrile, 10% buffer until the baseline is stable. Then equilibrate with initial mobile phase conditions. Please note that for a best performing HILIC method, equilibration times need to be 2 or 3 times longer than that of a traditional reversed phase method.

Storage

For short-term (< 3 days), it is acceptable to leave the column installed on the LC and filled with mobile phase at room temperature.

For long-term storage:

1. Flush the column with unbuffered mobile phase to remove non-volatile salts, acids and ion-pairing agents. Take extra care if you are using phosphate buffers to avoid precipitation.
2. Flush the column with > 90% acetonitrile. Then find optimal storage solution in the CoA or QAR.
3. Remove the column from the instrumentation and attach the solid end fitting. Store the column at room temperature

Cleaning

It is always advised to have regular cleaning of your column. If you run gradients this could include a few minutes longer run at the top of your gradient. For isocratic runs, this could be an elevated run with strong solvent in-between samples to assure that the column is cleaned for the next run. Alternatively, a cleaning procedure at the end of a sequence may also suffice, depending on the method and sample cleanliness.

However, situations may occur where you will have to clean your column more extensively. Before using any cleaning solvent outside your usual mobile phases, check that it is compatible with the column and LC system. Below follows a series of various contaminants and how to clean these from the column.

General cleaning:

1. Slowly raise increase to 90% H_2O/10% ACN hold for 45 minutes. Amide-HILIC columns show increased pressure for > 50% H_2O, so reduce the flow as needed.
2. Then, over 45 minutes change gradient to 40% A/60% B.
3. Hold this gradient step for 2 hours.
4. Over next 45 minutes bring gradient back to starting mobile phase*.

*This is typically 90% ACN

Clean out metal contamination:

1. Start by flushing with 50% buffer and 50 % acetonitrile (pH ~4 ammonium acetate or ammonium formate as appropriate).
2. Then increase to 80% buffer and 20% acetonitrile.
3. Then slowly change the buffer to a chelating buffer (buffer + 50 mM EDTA, pyrophosphate or oxalate at pH ~4). Continue at 80% buffer and 20% acetonitrile.
4. Monitor UV for stable baseline.
5. Then slowly change the buffer from a chelating buffer back to a normal buffer. Continue at 80% buffer and 20% acetonitrile until all the chelating agent has been flushed from the column and system.
6. Equilibrate to mobile phase conditions.

Mobile phase selection

Selecting the right mobile phase can be just as important as selecting the correct stationary phase. There are many considerations in making the selection. Choose mobile phases that are compatible with the column and LC equipment. Mass spectrometers and charged aerosol detectors require that all ingredients are volatile. UV detection requires that the mobile phase is transparent at the wavelengths of interest. Pay attention to the viscosity of the mobile phase so as not to exceed the pressure limit for the column or system. Use high-quality ingredients of the appropriate grade (HPLC, UHPLC, LC-MS, UHPLC-MS) for the application.

Typical mobile phases would be acetonitrile, used as a weak solvent with buffer or clean water as the strong solvent.

To develop a new method, it is recommended to do a generic screening. Because HILIC is the inverse of reversed phase, meaning the organic solvent is the weak solvent and water is the strong solvent, the gradient would run from 95% acetonitrile to 5% acetonitrile. The typical gradient time is 1 minute per centimeter of column length. *E.g.* 100 mm column is 10 cm, which would suggest a screening from 5% to 95% organic over 10 minutes.

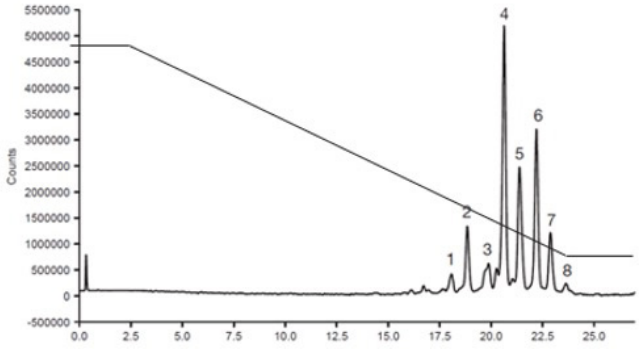


Figure 1: Screening example

After first injection evaluate where more resolution is needed or less resolution is needed and adjust the gradient accordingly.

In HILIC water is a functional part of the stationary phase. It covers the stationary phase and participates in the retention of your analyte. Thus, it is imperative that you do not reduce your percentage of water to less than 5%. Less than 5% water in the mobile phase can create reproducibility problems and loss of retention. Also, as the water layer covering the stationary phase is so important for robustness of your analysis, you should never use Methanol or any other polar organic solvent in the mobile phase as this will disrupt the water layer covering the stationary phase and cause robustness issues. Even small volumes of Methanol in injections solvent may cause challenges with the method robustness. As much as possible, try to match the sample solvent to the mobile phase. To be safe, use only ACN.

The adsorbed water layer takes time to re-equilibrate after a gradient. Allow for a longer re-equilibration time than for typical reversed-phase methods. Allow at least 3 – 5 gradient delay volumes plus 5 – 10 column void volumes for re-equilibration. If possible, develop an isocratic method.

Buffer selection

By controlling the pH of the mobile phase buffers control the retention of analytes and improve peak shape. Because HILIC is performed under low-aqueous conditions, there are fewer good options for buffers than for reversed-phase. Buffers are usually based on formic acid, acetic acid, ammonium formate or ammonium acetate. These buffers are miscible with acetonitrile and don't precipitate. Avoid phosphate salts because of the risk of precipitation. Buffer concentration is typically 10 – 100 mM in the aqueous mobile phase. Remember that a true buffer should have the ability to resist pH change when a sample is introduced at a different pH, and that buffer capacity is only 100% at the pK value of the acid or base. For example, ammonium acetate at pH 7 has very little buffering capacity, and the pH will shift toward the pKa of either acetate or ammonia if a mis-matched sample is injected.

Common buffer systems

Buffer		pK_a	Useful pH range	MS-compatible
TFA		0.30		Yes
Phosphate	pK_1	2.1	1.1 – 3.1	No
	pK_2	7.2	6.2 – 8.2	No
	pK_3	12.3	11.3 – 13.3	No
Citrate	pK_1	3.1	2.1 – 4.1	No
	pK_2	4.7	3.7 – 5.7	No
	pK_3	5.4	4.4 – 6.4	No
Formate		3.8	2.8 – 4.8	Yes
Acetate		4.8	3.8 – 5.8	Yes
Tris base (Trizma, THAM)		8.3	7.3 – 9.3	Yes
Ammonia		9.2	8.2 – 10.2	Yes
Borate		9.2	8.2 – 10.2	No
Diethylamine		10.5	9.5 – 11.5	Yes
Carbonate	pK_1	6.4	5.4 – 7.4	Yes
	pK_2	10.3	9.3 – 11.3	Yes
Triethanolamine	—	7.80	—	Yes

Expect reproducible results with sample prep, columns and vials

GC IC LC C CDS SP UNITY LAB SERVICES

Don't see what you need? We would be happy to discuss your specific requirements. Please contact your local sales representative for custom orders.

Learn more at **thermofisher.com/lccolumns**