LC columns

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

Applies to columns bonded with aromatic phases such as Phenyl, Phenyl X, Phenyl Hexyl, PFP and Biphenyl

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.

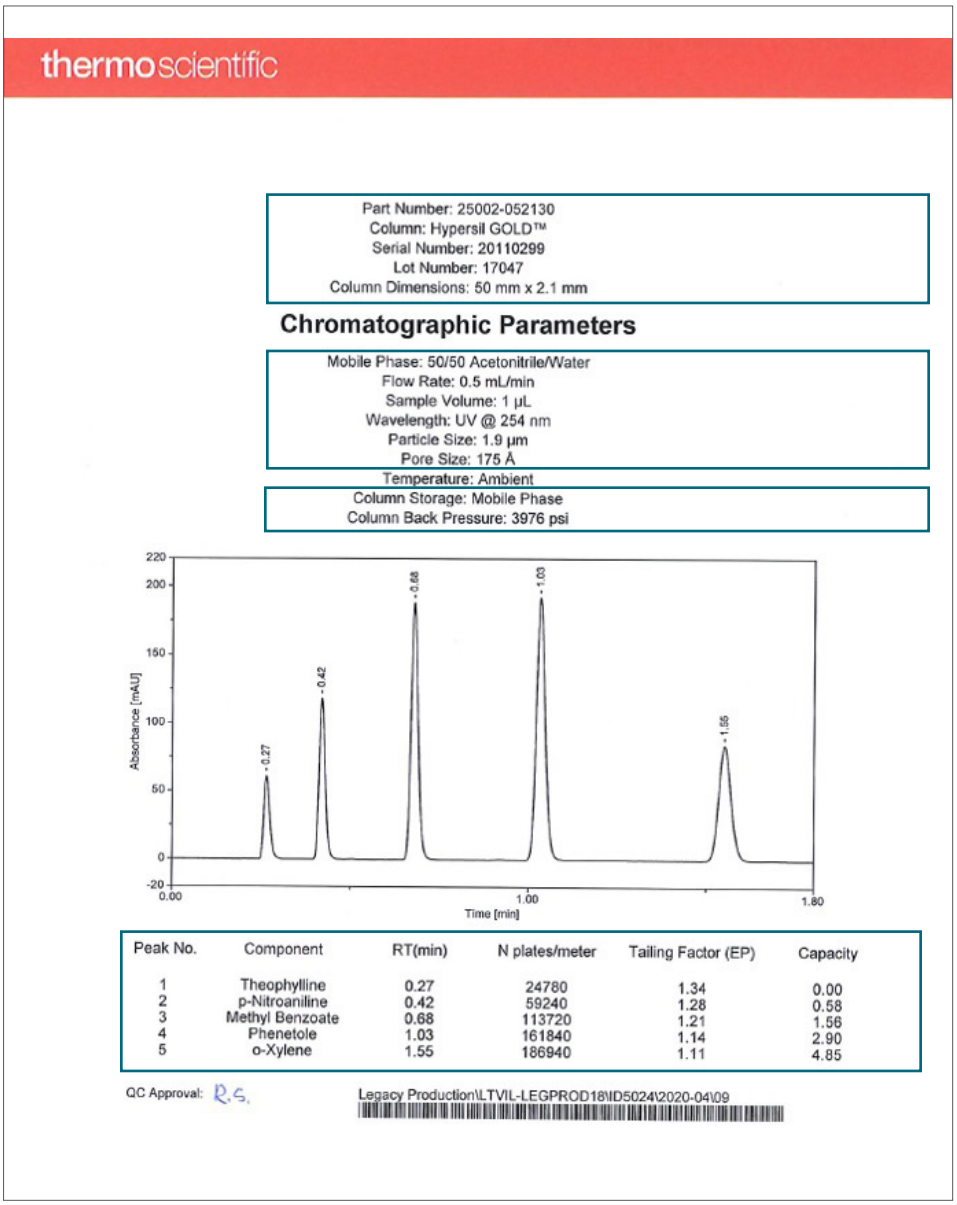
For UHPLC columns operating at high pressure > 400 bar, it can take the column 20 – 30 minutes of extra time to come to thermal steady-state after the column oven is ready. Continue equilibration until the pressure and detector baselines are stable.

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 lifetimes 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

Condition the column with the strongest solvent you will normally use (*e.g.* your highest per centage mix of organic in your gradient). Monitor the pressure and detector baselines until they are stable. Please note that viscosity of organic solvent may depend on not only the solvent you use, but also the per centage of solvent mixed with water. Methanol has higher viscosity with water at a level of 40% than at 10% or 90%, thus you can expect your pressure to rise and fall throughout the method. Assuring you pressure is well within the limits of your column and instrumentation, you add flexibility for this rise in pressure.

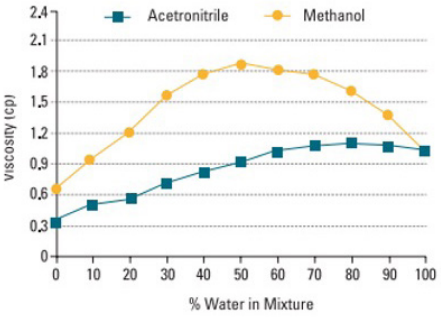


Figure 1: Mobile phase viscosity changes with composition

Equilibrate the column with the initial mobile phase conditions until the detector and pressure are stable.

For aromatic columns (phenyl, biphenyl, PFP, etc.) if you want to switch from acetonitrile to methanol, flush the LC system and column thoroughly to remove residual acetonitrile. This is to ensure the best retention time stability. Some Thermo Scientific™ Phenyl phases, such as Biphenyl, are shipped with methanol as a storage solvent. In such cases, we strongly recommend not switching solvents to CAN. Please check your respective column CoA or QAR.

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 > 70% organic solvent. The CoA or QAR will give a suitable storage solution.

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 organic run 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.

Particulate matter: If particle size of the column is > 2 µm reverse the direction of the column and perform a back flush of the column at half normal velocity. Flush directly to waste. For sub 2 µm columns do not backflush.

Hydrophobic contaminants

Cleaning procedure:

1. Start with 90% H_2O/10% organic solvent.
2. Over 5 minutes raise the gradient to 10% H_2O/90% organic solvent.
3. At 5 minutes return to 90% H_2O/10% organic solvent.
4. Repeat for the duration of minimum 30 minutes.

For difficult contaminants, use elevated temperature or lower percentages of stronger solvents such as isopropanol or Fisher Scientific™ ChromaCare™ solution (Fisher cat. no. MB1241). Be aware of backpressure changes while doing cleaning procedures due to viscosity of solvents and clogging.

Proteins: Reverse the column and apply repeated fast gradients from 5% to 90% acetonitrile or ChromaCare solution.

Metal ions: Use a chelating agent such as 20 mM EDTA, pyrophosphate or oxalate at pH ~4 and ~30% organic solvent. Monitor the UV baseline. If repeated contamination, system passivation may be required.

Ion-pairing agents: Anionic IP-agents can usually be removed by an extended flush of the LC and column with 80% organic solvent with 0.1% Formic Acid. Quaternary amine IP-agents will not be effectively removed.

De-wetting, phase collapse or air: Backflush, if possible, with degassed isopropanol for 30 min. Be aware of backpressure, especially during solvent switches.

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 for aromatic phenyl-type columns would be Methanol, used as a strong organic solvent with buffer or clean water as the weak solvent.

Selecting the right mobile phase is particularly true for phenyl phases as selectivity will vary much more depending on the use of either acetonitrile or methanol. Analyte molecules interact with the stationary phase by so-called “pi-pi” interactions. Acetonitrile includes a triple bond between a carbon and a nitrogen atom. These bonds include pi-bonding orbitals that will interact with the pi-orbitals of the aromatic stationary phase. As this pi-pi selection is important for the selectivity and how the stationary phase ability to retain and separate the various analytes, disrupting this pi-interaction can result in less resolution. Thus, when utilizing phenyl phases, it is always recommended to use Methanol, or at least a per centage of Methanol, in the mobile phase.

To develop a new method, it is recommended to do a generic screening gradient, usually from 5% organic solvent to 95% organic solvent. 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.

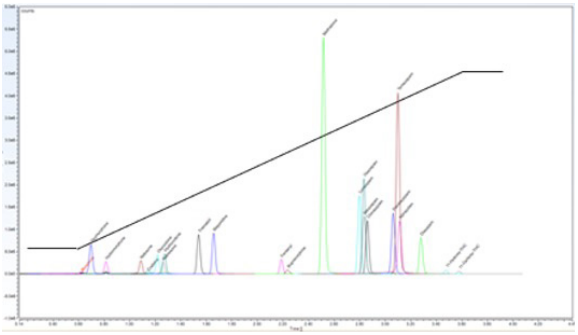


Figure 2: Generic screening

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

For an isocratic method. Start your screening at 30% organic and increase or decrease incrementally until you see resolution is improving.

Buffer selection

By controlling the pH of the mobile phase buffers control the retention of analytes and improve peak shape. Perhaps the most common HPLC buffer is some form of phosphoric acid. 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. At pH 4, phosphate is a poor buffer and would change rapidly toward one of its pKa values if a more acidic or basic sample were introduced. As a rule, one should work within ±1 pH unit of the buffer pKa value for good pH control of the mobile phase. Adequate buffer concentrations for HPLC tend to be in the 10-100 millimolar level depending on the size and nature of the sample, as well as the column packing material. Stationary phases based on highly pure silica with robust bonding are often more compatible with dilute buffers than traditional packings. When control at a lower pH (2-3) is desired, phosphate, or stronger organic acids such as trifluoroacetic acid (TFA) or formic acid are commonly used. If control at pH 4-5 is desired, an organic acid buffer such as acetate or citrate should be considered in place of phosphate. Even slight changes in pH, either from measuring errors, mixing complications with the pump, or atmospheric water adsorption into the mobile phase, can alter any method if not properly buffered. Care should be taken when choosing a buffer and organic modifier mixture to ensure that a solution of the two does not produce a solid salt which could cause blockages and system contamination.

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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

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