Column Care and Use Instructions

AUTHORIZED DISTRIBUTOR

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HPLC Columns

M170c

Important – Please Read!

Do Not Operate Columns Above Maximum Operating Pressure.
HPLC Columns are typically used in systems capable of generating substantial pressures. Appropriate safeguards must be followed to insure safe operation during use. Grace® HPLC columns are designed for safe use at the following **maximum operating pressures:**

16,000psi for VisionHT™ columns and,
5,000 psi for all other silica based Grace® HPLC columns,

unless otherwise noted in the certificate of analysis provided with the enclosed product. Users are cautioned to:

– **NOT** exceed the maximum operating pressure; and

– **ENSURE** that appropriate pressure relief devices are installed in systems using the columns to safeguard against overpressure arising due to circumstances that can cause pressure to elevate.

Certain column configurations and packings may require adjustment to the maximum operating pressure limitations. Such adjustments are noted on the certificate of analysis provided with the column.

Frequently check columns and column fittings for leaks. Solvent leakage at the column fittings may occur during operation if the column fittings are not properly fitted or tightened. Do not overtighten column fittings, and check for signs of leakage at the fittings, particularly following installation of the column.

Always wear proper personal protective equipment, such as safety glasses or goggles and gloves, when operating or repairing HPLC equipment.

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Each Grace® HPLC Column is carefully packed and tested to assure the highest quality product. These columns are guaranteed to be free from manufacturing defects for a full 90 days commencing on the date of receipt. For this reason, if you plan to store the column for use at a later date, **test the column to verify performance immediately upon receipt.** Guidelines are included in the section on "Testing The Column".

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RECEIVING THE COLUMN

Unpacking the Column

Each column is packaged in foam to reduce shipping vibrations, and accompanied by a test chromatogram which details the test conditions and the results obtained for your column. This information is useful in initial verification of column performance and also for troubleshooting as the column ages. The test chromatogram also contains information regarding the shipping solvent, upper pressure limit, lot number of packing material, pH range, column catalog number and serial number. The serial number is used to access information such as the original column performance, packing method, and date of manufacture.

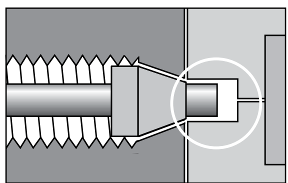
Installing the Column

Extra-column dead volume can make high efficiency columns look inefficient. To minimize extra-column dead volume, your system should be equipped with short pieces of small bore tubing (0.005"-0.010"i.d.), and zero dead volume fittings. Keep in mind that as the i.d. of the HPLC column decreases, extra-column effects become more detrimental and obvious. The stop depth on the connecting tubing must match the stop depth in the fitting. A mismatch will cause either dead volume or a leak (**Figure 1**).

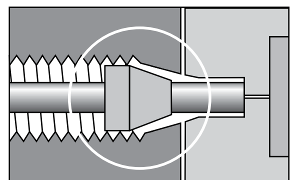
Purge the pump and fluid lines with the mobile phase to ensure contaminants and bubbles are removed from the system. Once this is completed, connect the column.

HPLC pressure requirements dictate that either stainless steel, PEEK, or carbon-reinforced PEEK fittings be used throughout the system. PEEK fittings are more convenient and easier to use. Often these are designed with a knurled nut which requires only hand tightening to obtain a leak-free seal. When loosened, the ferrule returns to its original shape allowing it to reposition itself to the appropriate stop depth each time a connection is made. PEEK ferrules and fittings can be re-used many times and can be placed into ports with different stop depths without causing leaks or extra dead volume.

Unlike PEEK ferrules, stainless steel ferrules can only be used on stainless steel tubing and will swage onto the tubing with the first connection. This ferrule-tubing connection now has a fixed stop depth and must be used only on the type of port on which it was swaged.



Dead Volume
Tubing stop depth is shorter than fitting stop depth creating dead volume and reducing chromatographic efficiency.



Leak
Tubing stop depth is longer than fitting stop depth. The ferrule does not engage the seat, causing a leak.

Figure 1 – *Problems caused by a mismatch in stop depth*

Testing the Column

Column performance may be verified by testing the column under the same conditions which produced the original quality control chromatogram. However, chromatographic systems can vary, therefore your results may differ from those obtained in our laboratories. Variations in pumping and mixing systems may lead to differences in operating pressure and retention times. Detector characteristics such as bandwidth and light path may alter the size and ratio of peak heights.

Inject the test mix, and calculate column efficiency and peak symmetry (**Figure 2**). Bear in mind that efficiency measurements reflect the performance of all system components, not just the column. Band-spreading due to the injector, connecting tubing, and flow cell, all contribute to the total peak width.

HPLC columns are tested on equipment designed to minimize extra-column effects. This is not done for the purpose of generating inflated or unrealistic plate counts, but to assure that our quality control procedures reflect column characteristics rather than combined column/system performance. Generally, changes of less than 20% are attributable to instrument differences.

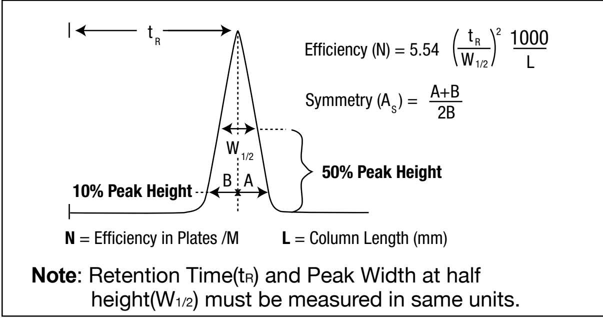


Figure 2 - *Calculation of Efficiency and Peak Symmetry*

TABLE 1 - TEST MIXES	
Description	**Part No.**
Reversed-Phase Test Mix (1mL) Contains: Uracil, Phenol, N,N-Diethyl-m-Toluamide, and Toluene	**1895**
Normal-Phase Test Mix (1mL) Contains: Toluene, Diethyl Phthalate, and Dimethyl Phthalate	**1096**

CARE & USE OF GRACE® HPLC COLUMNS

Mobile Phase Recommendations

For maximum column life, consider the following warnings when choosing and preparing mobile phase:

1. **Never pump immiscible solvents sequentially through the column.**

   Be sure that the solvent to be pumped into the column and the solvent currently in the column are miscible in all portions. If they are not, it is necessary to pump one or more miscible intermediate solvents through the column to avoid high pressure.

   For example, if you wish to use a normal-phase column tested and shipped in 95:5 Hexane:Ethanol in a reversed-phase application, an intermediate solvent such as isopropanol should be pumped through the column prior to the mobile phase. Isopropanol is miscible with both the Hexane/Ethanol mixture as well as the polar solvents typically used in reversed-phase methods. (See insert for miscibility chart)

2. **Use HPLC grade solvents in the mobile phase.**

   When preparing mobile phase, only the highest purity solvents should be used. Filter and degas all solvents prior to use.

3. **Beware of buffer precipitation upon the addition of organic solvents.**

   Buffers and ion pairing salts have limited solubility in organic solvent. Therefore, beware of precipitation as the percentage of organic content in your mobile phase increases. For example, if a separation is currently performed with a mobile phase of 60:40 acetonitrile:buffer, and you want to increase the acetonitrile to 70%, first try mixing the new ratio of 70:30 acetonitrile:buffer in a beaker. If any cloudiness appears, the added organic content has caused the buffer salt to precipitate from solution. To remedy the situation, use a lower molarity buffer or less acetonitrile.

4. **Do not use mobile phases that chemically attack the bonded phase or silica.**

   Under most circumstances, the bonded phases used in HPLC are extremely stable. Generally, a siloxane linkage is used to bond the functional group to the silica particle. Hydrolysis of this bond is possible under strongly acidic conditions, therefore the pH of the mobile phase should be kept above 2.0. If a pH below 2.0 is used, expect shorter column life. Amino bonded phases (NH2) are particularly reactive. Avoid using mobile phases that contain aldehydes and ketones as these will complex with the amino function.

   The silica used in HPLC is to some extent soluble in water. The degree of solubility varies dramatically with pH, being particularly soluble under alkaline conditions. Most silica-based HPLC packings can be used up to a pH of 7.5. This rule should be used with discretion since the ability of the mobile phase to dissolve the silica varies with the concentration and nature of the buffer, the operating temperature, and the type of bonded phase.

   The nature of the bonded phase determines the solubility of the packing material by affecting how easily the silica is wetted. Reversed-phase materials tend to protect the silica surface by

providing a waxy hydrophobic layer around the silica particle, in effect decreasing contact between the mobile phase and the silica. The more hydrophobic the bonded phase, the greater the protection. Therefore a C18 phase will provide better protection than a C3 phase.

5. **Use guards and saturation columns where appropriate.**

   Build-up of contaminants from samples, and mobile phases, or solvation of the silica matrix will eventually destroy any column, no matter how well it is manufactured. **We strongly recommend the use of guard columns and, where appropriate, silica saturation columns to minimize damage.**

Backpressure

Flow rate and viscosity of the mobile phase will affect column pressure. Ideally, viscosities should be kept below 0.5cP. Keep in mind that as the mobile phase composition varies during gradient runs, viscosity and pressure will also change.

TABLE 2 - TYPICAL FLOW RATES

Column Internal Diameter	Flow rate (For Equivalent Linear Velocity)
1.0mm	0.05mL/min
2.1mm	0.2mL/min
4.6mm	1.0mL/min
10mm	5.0mL/min
22mm	23.0mL/min

Column Equilibration

Equilibration time varies with the type of bonded phase and the nature of the mobile phase. Reversed-phase columns equilibrate in as little as 20 column volumes of mobile phase. Normal-phase columns require longer equilibration times (at least 50 column volumes). To ensure good reproducibility and faster equilibration of normal-phase columns, a small, constant percentage of water can be added to the mobile phase.

Column Storage

HPLC columns need to be stored in an appropriate solvent and capped tightly. They should be placed in a cool area, free from vibration.

Prior to storage, remove buffers and ion pairing agents by flushing with 10 to 20 column volumes of mobile phase with the salts removed. For example, an application requiring methanol:buffer (40:60) should be flushed with methanol:water (40:60).

Flushing reversed-phase columns with 100% water is not recommended as the water will repel the hydrophobic functional groups causing them to “lay flat” and trap the buffer salts. **Table 3** lists appropriate storage solvents for different types of HPLC columns.

For more information about performing bioseparations with Vydac® columns, please request "The Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC."

TABLE 3 - STORAGE SOLVENTS	
Reversed-phase* C1, C2, C3, C6, C8, C18, Phenyl	Methanol:Water (50:50)
Normal-phase Silica, CN, NH_2, PAC, NO_2, N $(CH_3)_2$, Alumina, Diol	Hexane
Normal-phase w/Aqueous Mobile Phases* Silica, CN, NH_2, PAC, NO_2, N $(CH_3)_2$, Carbohydrate, Alumina, Diol	Methanol
Ion Exchange*† SAX, SCX, WAX, WCX	Methanol:Water (10:90)
Hydrophilic Interaction HILIC	Methanol:Water (90:10)

*If buffers are used, first flush with 30-60mL of mobile phase in which the buffer has been replaced with water.

†Before reuse, flush with 30-50mL water.

TROUBLESHOOTING GUIDE

When less than optimal performance occurs, use a process of elimination to decide which HPLC component is causing the problem. If the column is at fault, use this troubleshooting guide to remedy the situation.

TROUBLESHOOTING CHART	
Problem: High Back Pressure	
CAUSES:	**SOLUTIONS:**
1. Plugged frit, prefilter.	**A.** Backflush column. **B.** Replace frit, prefilter.
2. Contaminated column.	**A.** Regenerate column (see insert).
3. Precipitation of buffer due to high organic content of mobile phase.	**A.** Flush with 90:10 water: organic solvent at low flow rate. Reduce organic content of mobile phase.
Problem: Tailing or Double Peaks	
CAUSES:	**SOLUTIONS:**
1. Poor sample solubility in the mobile phase.	**A.** Modify mobile phase.
2. Channel in packing bed.	**A.** Reverse flow through column.
3. Particulates on inlet frit.	**A.** Backflush column. **B.** Replace frit.
Problem: Shifting Retention Times	
CAUSES – ALL COLUMNS	**SOLUTIONS:**
1. Contaminated column.	**A.** Regenerate column (see insert).
CAUSES – NORMAL-PHASE:	**SOLUTIONS:**
1. Accidental moisture in mobile phase or packing.	**A.** Add small constant percentage (≤ 2%) water into mobile phase.
2. Complexation of amino phases by aldehydes or ketones.	**A.** Replace column.
CAUSES – REVERSED-PHASE:	**SOLUTIONS:**
1. Depletion of bonded phase.	**A.** Replace column.
CAUSES – ION EXCHANGE:	**SOLUTIONS:**
1. Depletion of bonded phase.	**A.** Replace column.

For HPLC Columns and Media, Scalability Matters

Small Molecule to Large Molecule

Whether you are working on small molecule separations for a new pharmaceutical or trying to purify large proteins, Grace manufactures silica and customizes it with functional phases from our extensive library of surface chemistries. We have particle sizes from 1.5-3000µm and pore diameters from 30-2000Å both of which can be modified to suit your needs. Functional phases range from universal normal and reversed phases, to highly specific affinity ligands.

Capillary to Process Scale

Additionally, Grace is able to offer our stationary phases packed in columns to accommodate a full range of sample sizes, from our 75µm capillary columns to prep and process columns that can handle production scale volumes. Our patented dynamic axial compression column helps you maintain well-packed columns that extend column lifetimes.

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