VYDAC® Reversed-Phase Columns Care and Use Guide

Column Installation

To secure 1/16" tubing to the column inlet and outlet, please use PEEK (NOT stainless steel) Finger-Tight™ compression fittings. Particularly, for 10 and 22mm i.d. columns, the use of stainless steel fittings may embed the ferrule in the CAP™ frit/insert, and can cause a leak.

Pressure and Temperature Limits

VYDAC® silica-based HPLC columns are stable from 0-60°C and at pressures up to 5000 psi (335 bar).

Performance Testing

Each lot of media is tested with a set of peptides and proteins. Each column is individually tested for packing efficiency using small molecules such as toluene, biphenyl, or benzoates. Efficiency test conditions and results are included with the column.

Troubleshooting Pressure Problems

HPLC columns may become contaminated by strongly adsorbed sample constituents, causing an increase in column backpressure and/or a loss of resolution. If the backpressure is high:

1. Disconnect the column from the injector and run the pumps to ensure that the backpressure is due to the column and not the HPLC system.
2. If the column backpressure is high, the column may be reversed and rinsed to try to flush contaminants from the inlet frit. Begin the reverse rinse at a low flow rate (0.5mL/min for a 4.6mm i.d. column) for 10-15 minutes and then increase the flow to 1.5-2.0mL/min (for a 4.6mm i.d. column).
3. Wash the column with 10-20 column volumes of a strong eluent (70-80% organic solvent).

Column Conditioning

Due to the nature of all reversed-phase surfaces, column performance (e.g., resolution, retention) may change slightly during the first few injections of a polypeptide. Thus, for applications of peptide and protein separations, we recommend that each column be conditioned by repeated injections of a polypeptide until the column characteristics remain constant. This typically requires injection of about 100 to 200µg of polypeptide on a 4.6mm i.d. x 250mm column. Below is an example conditioning protocol:

1. Prepare the mobile phases such as:
   Solvent A: 0.1% v/v TFA in Water
   Solvent B: 0.085% v/v TFA in Acetonitrile (ACN)
2. Prepare an individual solution of a pure protein at a concentration of 10mg/mL. Examples of proteins include ribonuclease, bovine serum albumin, or cytochrome c (available from Sigma-Aldrich). Alternatively, a routinely tested laboratory protein may be used.
3. Run a gradient from 20% to 80% B over 30 min. at 1mL/min. for a 4.6mm i.d. column.
4. Inject 10 to 20µL (equal to 100 to 200µg protein load) on a 4.6mm i.d. column. In order to help ensure that insoluble material is not injected on a column, we recommend centrifuging (16,000 x g for 10 min) or filtering the protein standard prior to injection. Centrifuging a protein standard is very helpful, since it is easy to see a pellet at the bottom of the centrifuge tube if the sample is not completely soluble.
5. Repeat injection and gradient runs two additional times to condition each column.

Column Storage

VYDAC® Reversed-Phase HPLC columns may be stored in any combination of organic solvent and water. For long term storage ion-pair reagent should be flushed from the column and the organic content should be at least 50%. The column should be sealed with the plastic plugs originally supplied.

Chemical Stability

VYDAC® Reversed-Phase HPLC columns are stable in most common organic solvents including acetonitrile, methanol, ethanol, isopropanol, dichloromethane and chloroform. When switching solvents it is important to verify that subsequent solvents are miscible with the previous solvent used. Protein/Peptide columns are very resistant to hydrolysis, can be used with eluents as low as pH 2 (such as 0.1% trifluoroacetic acid) for long periods of time, and are stable to occasional use at lower pH if columns are stored at pH higher than 2. **Silica-based Protein/Peptide Reversed-Phase columns should not be used above pH 7. USE ABOVE pH 7 IS LIKELY TO REDUCE THE COLUMN LIFETIME!** Common protein detergents such as sodium dodecylsulfate (SDS) can be used without harm to columns and may be removed by rinsing the column with acetonitrile or isopropanol. SDS is not recommended if the column will be used with a mass spectrometer. Detergents are likely to affect the resolution of the column during the run in which they are present. Oxidative eluents or sample additives should be avoided.

Recommended Elution Conditions

Peptides and proteins are typically eluted with an increasing gradient of organic solvent in the presence of an ion-pairing agent. The most common organic solvent used is acetonitrile due to its low viscosity, good UV transparency and high volatility. Ethanol or isopropanol also may be used. Generally, it is recommended that the gradient does not exceed 80-90% organic solvent for routine protein separations, in order to prevent on-column protein solubility issues. For peptides, reaching 90 to 95% ACN maximum is fine. Trifluoroacetic acid (TFA) is the most commonly used ion-pair reagent and is usually present at concentrations of 0.05 - 0.2% (v/v). However, VYDAC® LC-MS grade columns (i.e., 208MS, 214MS, 218MS, 219MS, 238MS, 238DE, 238EV) produce excellent separations with TFA concentrations as low as 0.01% (v/v). As an alternative to TFA, one of the following ion-pairing reagents may be used: acetic acid, formic acid, heptafluorobutyric acid (for basic polypeptides).

Typical flow rates and loading capacities for VYDAC® Reversed-Phase HPLC columns

Column i.d. (mm)	Typical Flow Rate (1)	Sample Capacity (2)	Maximum Practical Sample Load (3)
0.075	0.25µL/min.	0.05µg	
0.15	1µL/min.	0.2µg	
0.30	5µL/min.	1µg	
0.50	10µL/min.	2µg	
1.0	25 – 50µL/min.	0.05 – 10µg	
2.1	100 – 300µL/min.	0.2 – 50µg	
4.6	0.5 – 1.5mL/min.	1 – 200µg	10mg
10	2.5 – 7.5mL/min.	1000µg	50mg
22	10 – 30mL/min.	5mg	200mg
50	50 – 100mL/min.	25mg	1000mg
100	150 – 300mL/min.	125mg	5000mg

1. Actual flow rates can be a factor of two higher or lower depending on the method.
2. Sample capacity is the quantity of polypeptide that can be loaded onto the column without reducing resolution.
3. Maximum practical sample load is approximately the maximum quantity of sample that can be purified with reasonable yield and purity on the column.

Column Cleaning and Regeneration

VYDAC® Reversed-Phase HPLC columns may become contaminated by strongly adsorbed sample constituents causing a loss in column performance. If the recommendations given on page 1 fail to correct the problem, try one or more of the approaches listed below:

- Inject up to 12% of the column volume (CV) with 1% sodium dodecyl sulfate (SDS) solution (500µL for 4.6mm x 250mm column). Then run a 20-minute gradient from 5% to 95% ACN with 0.1% (v/v) TFA. The SDS approach is not recommended if using the column in mass spectrometry applications.
- Flush the column at half the normal flow rate using 70:30 n-propanol: water or isopropanol:water.
- If the loss in column performance appears to be due to adsorbed protein, rinse any of the polymeric-bonded columns with a mixture of one part 0.1N nitric acid and four parts isopropanol. Rinsing at a low flow rate (20% of normal) overnight is most effective. **NOTE: WASHING WITH NITRIC ACID IS NOT RECOMMENDED FOR 238TP, 238MS, 238DE, and 238EV "MONOMERIC" REVERSED-PHASE COLUMNS.**
- Use 6M Guanidine HCl in water. Filter the Guanidine prior to mixing 1:1 with isopropanol. Inject 5% of the CV (for a 4.6mm i.d. column, inject 200µL) while running 50% isopropanol in water. This achieves a "plug flow" of Guanidine, which will break loose protein contaminants from the column.
- Synthetic Peptides: The cleavage of synthetic peptides off solid phase resins generates very reactive carbonium ions that are "scavenged" by anisole and thioanisole. These scavenger-carbonium ion reactions yield large, organic soluble, aromatic molecules that can be found in the cleavage solutions. Washing the column with 100% acetonitrile or 100% methanol may not elute these molecules. Or, at times, washing the column with these organic solvents may remove some contaminants giving a waxy precipitate that are at times mistakenly assumed to be the C18 phase coming off the column. To clean: Wash with isopropanol, 0-100% over 10 column volumes, hold at 100% isopropanol or until baseline. Monitor at 260nm. Rinse with 100% dichloromethane until baseline. Remove dichloromethane with isopropanol before use. This method is very effective at removing contaminants introduced by the synthesis process.
- If lipids or very hydrophobic small molecules are causing the change in column performance, rinse the column with several column volumes (CV) of solvent in the following order: Acetonitrile (10 CV), Dichloromethane (10 CV), Hexane (10 CV), Dichloromethane (10 CV), Acetonitrile (10 CV)

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5/2011 M383

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