HALO® COLUMN CLEANING PROCEDURES AND BEST PRACTICES

TABLE OF CONTENTS:

1. General factors that can contribute to column deterioration
2. Tips on column blockage, contamination and cleaning
3. Procedures for cleaning HALO® BIOCLASS Protein Columns
4. Column storage
5. Guidelines for low-volume columns
6. Recommended flow rates

GENERAL FACTORS THAT CAN CONTRIBUTE TO COLUMN DETERIORATION

INTRODUCTION

To maximize the lifetime of an HPLC column and ensure its top performance, it is necessary to properly maintain the column. Many different factors can contribute to loss of resolution or sensitivity, but often times the following factors lead to the vast majority of column performance issues.

Contamination

Often, the sample may contain compounds, originating from the sample matrix or low quality mobile phase solvents, which can be retained by the stationary phase. Salts, lipids, plasticizers, and polymers are some of the possible substances that can come in contact with the stationary phase during the analysis. These substances can then have deleterious effects on the column, the detector, and cause transient peaks during the analysis. If these substances are not eluted by the mobile phase, they can accumulate on the column. Over time, analytes can interact with these impurities and affect the separation mechanism, resulting in retention time shifts and peak tailing. In addition, these accumulating impurities can create a blockage, causing column back pressure to spike, damaging the pump, and can lead to the formation of a void in the column bed.
The use of a guard column is highly recommended to avoid these types of problems. Guard columns are short columns packed with similar packings as the analytical column that are installed between the injector and the analytical column. After a given period of use, they are discarded and a fresh guard column is installed to maximize the lifetime of the analytical column.

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pH Instability

Always ensure that the analysis is operated within the pH range for the column. Please consult the care and use sheet for the column to confirm the operational pH stability. When not in use, the column should be rinsed from acidic or basic buffer salt modifiers.

High Operating Pressure

Superficially porous particles (SPP) based stationary phases provide high resolution separations at lower backpressures than fully porous particles (FPP) of smaller particle size. It is good laboratory practice to design analytical methods to run at the lowest possible operating pressures to maximize instrument and column life. It is also important to avoid pressure shocks on the column. Pressure shocks are sudden increases or decreases of pressure in a short time span such as a slow turning of a manual injector valve. They may lead to the formation of a void in the column bed and contribute to peak splitting in the corresponding chromatogram.

Pressure Ratings:

Capillary columns

Particle size	Internal diameter (mm)	Pressure rating (bar)
All	0.75 and 0.1	600
All	0.2 – 0.5	400

Non-capillary columns

Particle size	Internal diameter (mm)	Pressure rating (bar)
All	1.0	600
All	1.5	600
2 µm	2.1, 3.0	1000
2.7 µm 1000Å (pore size)	2.1, 3.0	1000
2.7 µm	2.1, 3.0, 4.6	600
3.4 µm 400Å (pore size)	2.1, 3.0, 4.6	600
5.0 µm	2.1, 3.0, 4.6	600

NB : a designation of All indicates all particle sizes available

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Poor Mobile Phase Quality

It is essential to use the highest quality solvents available (HPLC or MS grade) for the mobile phase, and to maintain the mobile phase pH at the operational pH range for the column being used. Please consult the care and use sheets for the specified column and phase. Low quality solvents often have impurities that can absorb on the column and interfere with the separation.

It is recommended to filter the mobile phase before use, and the use of a guard column during the analysis will help to ensure the mobile phase is particle free.

Excessive Temperature

It is essential for maximum column life to not exceed the maximum operating temperature rating for the column. The temperature rating can be found on the care and use sheet.

Insufficient Equilibration Time

The equilibration time of a column depends on the column dimensions. In general, a column is equilibrated after flushing with 20 column volumes. To calculate the column volume, use the following equation. An example calculation follows.

1. **Void volume(µL)= (1/4)[(diameter2)(π)(Length)(Pore volume)]**
2. **π = 3.14**
3. **Pore volume = 0.50 for SPP particles**
4. ***Diameter and length should be kept in mm***

For a column with dimensions of (2.1mm x100mm):

$(2.1mm)^2$= 4.41mm

(1/4)[(4.41)(3.14)(100)(0.50)] =173 µL =0.17 mL

With a flow rate of 0.4mL/min 20 column volumes can be reached in 8.5 minutes

TIPS ON COLUMN BLOCKAGE, CONTAMINATION AND CLEANING

Sudden Pressure Increase - All Phases

A large pressure spike, or a noticeable sharp increase in column pressure, is usually indicative of a column being clogged. To remove the clog, removing the column from the instrument and running it in the reverse direction without connecting it to the detector for about 20 column volumes can be attempted to dislodge any clog or particles that may have accumulated on the frit. —***Please note it is not recommended to back flush the capillary or 2 µm particle HALO® columns***

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Column cCeaning and Regeneration

Absorption of accumulated impurities can be detrimental to the column, but in some cases these impurities can be removed. The following section will outline how this can be accomplished.

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All HALO® Reversed Phases

Below is a procedure that has been effective at regeneration of reversed phase (RP) columns. In general, regeneration procedures are similar, with the common trait that wash solvents used are increased in their solvent strength, and often conclude with a solvent that is non polar. It is critical that all solvents used are miscible in series.

Steps 1-4 are usually sufficient to thoroughly clean most columns, however if this is insufficient, steps 5 and 6 can be added. However, it is critical to wash with IPA after using a strong non polar solvent such as hexane before introducing the re-equilibration solvent system as water will not mix with hexane.

1) Methanol
2) Acetonitrile
3) 50/50 ACN/IPA
4) IPA
5) Dichloromethane
6) Hexane
7) IPA
8) Re-equilibrate to starting conditions

HALO® HILIC Stationary Phases

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HALO® HILIC

To remove strongly retained materials from the column, flush the column with very strong solvents such as 50/50 methanol and deionized water. Extreme cases may require the use of very strong solvents such as 100% of the most polar component of the mobile phase in use, usually water. To rehydrate a HILIC column after cleaning it, it may be necessary to condition it first by running a mobile phase of 70/30 ACN/H_2O at a slow flow rate through the column for 2 – 3 hours.

HALO® Penta-HILIC

To remove strongly retained materials from the column, flush the column with very strong solvents such as 10/90 methanol and deionized water. Extreme cases may require the use of very strong solvents such as 100% of the most polar component of the mobile phase in use, which is typically water.

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PROCEDURES FOR CLEANING HALO® BIOCLASS PROTEIN (400 Å AND 1000 Å) COLUMNS

1. If hydrophobic materials are suspected on the column, a series of steep gradients (1-5 minutes long) of 100% water/0.1% TFA to 100% ACN/0.1% TFA or to 0.1% TFA in 80:20 isopropanol/water or 100% isopropanol/0.1% TFA.
   a. Column temperature should be set at 50 °C.
   b. Large injections (100 µL for 4.6 mm ID column, proportionally smaller volumes for smaller ID columns) of 2,2,2-trifluoroethanol while running gradients can be coupled with this procedure.
2. If hydrophobic materials are suspected to be building up on the column, rinse the column with acidified isopropanol for 20-50 column volumes at a column temperature of 50 °C.
3. If protein aggregates are suspected to be building up on the column, follow the procedure below:
   a. Flush channel to be used with 100% water without the column.
   b. Flush column (installed) with 100% water (10-20 column volumes) to remove any organic solvent.
   c. Prepare 6 M urea + 1% (v/v) acetic acid in a volume large enough to pump the mixture through the column for 2-3 hours at the normal flow rate for the column.
   d. Flush the column and channel thoroughly with 100% water to ensure all salt removed.
   e. Flush column with 20:80 organic/water and then 100% organic to re-wet the stationary phase.

After using any of these procedures, several large injections of the target protein(s) or BSA are recommended before routine use of the column.

COLUMN STORAGE (ALL PHASES)

Long term storage

Long term storage (more than 5 days) of most silica based reversed phase columns is best in 100% acetonitrile. The exception to this is the Biphenyl phase, which should be stored in 100% methanol. After analysis flush out the buffers or salts used during the analysis with the mobile phase minus the buffers or salts, then flush with at least 10 column volumes of storage solvent before storage.

Short term storage

Short term storage (less than 5 days) of silica based reversed phase columns can be kept safely stored in most common mobile phases. However, it is recommended, after analysis flush out the buffers or salts used during the analysis with the mobile phase minus the buffers or salts before the column is stored.

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GUIDELINES FOR LOW-VOLUME COLUMNS

High performance columns with small internal volumes (shorter lengths, narrow internal diameters < 3 mm) are being increasingly used for high sensitivity and high speed separations, especially with mass spectrometers. These low-volume columns generate peaks having considerably less volume than those eluting from columns of larger dimensions (e.g., 4.6 mm x 150 mm). The efficiency of separations performed in low-volume columns is highly dependent on the HPLC system having components designed to minimize band spreading. All low-volume columns perform best when used with proper attention to the following factors:

- LC/MS –Spray tips should be of low-volume design (preferably ~2µL or less) to minimize band spreading.

- UV Detector – Flow cells should be of low-volume design (preferably ~2µL or less) to minimize band spreading. To properly sense and integrate the often very fast peaks that elute from low-volume columns, the detector response time should be set to the fastest level (~ 0.1 second) and the integration software should sample the detector signal at least 20 points per second.

- Injector – The injection system should be of a low-volume design (e.g., Rheodyne Model 8125). The volume of sample injected should be kept as small as possible. It is highly recommended that a concentration trap cartridge is used to reduce injection volume and remove unwanted salts.

- Connecting Tubing – The shortest possible lengths of connecting tubing with narrow internal diameters (at most 120µm ID) should be used to connect the column to the injector and the detector cell. The tubing must have flat ends and should bottom out inside all fittings. Zero-dead-volume fittings should always be used where required.

- Peak Retention – As retention is increased, the volume of a peak increases, decreasing the effects on band spreading caused by components of the instrument. For quantitation, retention factors (k-prime) of 2 to 10 are recommended.

- Sample Solvent – For isocratic separations, the sample should be dissolved in the mobile phase or in a solvent that is weaker (more aqueous for reversed phase; more organic for HILIC mode) than the mobile phase. For gradient separations, the sample should be dissolved in the initial mobile phase or in a solvent substantially weaker than the final mobile phase.

- Injection Volume – For isocratic separations, the volume of sample injected should be kept as small as possible (typically 2 µL or less). Sample volumes are less critical for gradient separations, especially if the sample is dissolved in a weak solvent.

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RECOMMENDED FLOW RATES FOR HALO® COLUMNS

Due to the superficially porous particle design of HALO® particles, the recommended flow rate is higher than what is typically used for fully porous particle columns. The table below lists recommended flow rates for HALO® particle sizes in various column IDs.

Particle Size (μm)	Column ID (mm)	Recommended Flow Rate (mL/min)
2	3.0	0.8
2	2.1	0.4
2	1.5	0.2
2.7	10	5-10
2.7	4.6	1.8
2.7	3	1.0
2.7	2.1	0.5
2.7	1.5	0.2
2.7	1.0	0.1
2.7	0.5	0.025
2.7	0.3	0.009
3.4	10	5-10
3.4	4.6	1.5
3.4	3.0	0.7
3.4	2.1	0.3
3.4	1.5	0.15
3.4	1.0	0.07
3.4	0.5	0.018
3.4	0.3	0.006
5	10	5-10
5	4.6	1.0
5	3.0	0.5
5	2.1	0.25
5	1.0	0.05
5	0.5	0.013
5	0.3	0.005

For additional questions, please contact us: HERE

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