LC TROUBLESHOOTING

Column Care and Feeding

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With proper care, your column will have a long and useful life.

As with our own bodies, the useful life of a liquid chromatography (LC) column can be greatly extended by regularly practicing a few healthy habits. Even when we use only the best solvents and extensive sample-cleanup procedures, unwanted materials get onto the column and cause column failure through several mechanisms. High molecular weight materials such as proteins can physically coat the column surface, changing the surface chemistry and the total available surface for separation. In extreme cases, these proteins can actually block the packing's pores. Other contaminants can bind to active sites on the column and compete with sample compounds for the column's separation capabilities. Other materials can attack the column chemically, dissolving the silica support material or cleaving the bonded phase from the packing. And, of course, particulate matter entering the system with the sample can block the frit at the head of the column, causing split or tailing peaks and high back pressure.

There is no way to prevent contaminants from reaching the column, but three tools can minimize their impact. These tools are sample cleanup, the use of guard columns, and column flushing.

SAMPLE CLEANUP

Obviously, the best way to eliminate problems caused by contaminants on the column is to bar them from the system. The objective of sample cleanup is twofold: First, sample cleanup isolates the desired sample components from some portion of the sample matrix and potentially interfering substances; second, it changes the sample into a form that is more compatible with the LC system. Sample isolation often involves such steps as dissolution, liquid–liquid extraction, chromatography (solid-phase extraction), filtration, evaporation, and derivatization. When sample preparation is finished, the sample should be dissolved in a solvent compatible with the LC system (that is, a solvent that is generally miscible with other solvents used in LC, is no stronger than the mobile phase, and whose pH is within the limits of the column).

Although sample cleanup improves the quality of the injected sample, it also has some negative aspects. Sample loss, materials costs, and time (labor costs) are the primary drawbacks of extensive sample preparation.

In an industrial laboratory, the total cost of analysis for a typical sample can be $50–100 or more. Much of this expense can be attributed to sample cleanup costs. When developing a method, it is important to examine the impact of sample cleanup in economic terms. For example, if extensive cleanup enables you to run 300 samples before the $300 column fails, the column's cost per sample is just $1. If, on the other hand, minimal cleanup (for instance, dissolution and filtration) allows you to run only 50 samples before failure, the column's cost per sample is $6. If the extensive cleanup costs >$5/sample more than the simplified cleanup, then you'll be money ahead using the second method. Always check the contribution of sample cleanup to the total cost of the assay; this enables intelligent economic decisions when designing sample-cleanup procedures.

GUARD COLUMNS

Guard columns can greatly extend the life of analytical columns. These short (generally 1–5 cm) columns trap foreign material in several ways, preventing it from reaching the main column. Trapping mechanisms include sorption of high molecular weight contaminants, chemical filtering of undesirable materials, and removal of particulate matter.

Contaminant sorption: When changing the frit at the head of an LC column, you see that the packing material at the top of the column is often green, yellow, brown, or some color other than the natural white of silica. These stains are pigmented and strongly retained materials that get trapped at the column inlet. These materials often are proteins, plant pigments, or other high molecular weight compounds from the sample matrix. The compounds adhere to the column and can be eluted only under very strong solvent conditions, if at all. As the contaminants build up, they coat the surface of the column packing and reduce the active surface available for separating desirable compounds. Gradually, changes in retention and selectivity (peak spacing) will appear. Extensive sample cleanup can reduce (but probably not eliminate) the amount of these materials in the injected sample. By placing a disposable guard column upstream from the analytical column, these strongly retained contaminants are prevented from reaching the more-expensive analytical column. The key to using a guard column successfully is to throw it away before the trapped contaminants bleed onto the analytical column.

Chemical protection: Fortunately, the silica-based bonded-phase columns predominant in LC today are very rugged. However, they are chemical systems that by nature are susceptible to chemical attack. In particular, mobile-phase pH values outside the 2.5–7.0 range can accelerate the dissolution of the silica or the cleavage of the bond holding the bonded phase to the silica surface. Other chemical interactions also cause deterioration of column performance.

A well-chosen guard column is packed with the same type of packing material as the analytical column, so it intercepts the same aggressive chemicals and deteriorates instead of the analytical column. Two conditions are necessary for the guard column to provide adequate chemical protection: First, the level of contaminants in the mobile phase and sample has to be low enough that the guard column can neutralize their effects; second, the guard column must be replaced before it can no longer trap these contaminants effectively.

Particulate trap: Another way the guard column protects the analytical column is by filtering out particulate matter before it reaches the main column. The guard column has the same porosity frit as the analytical column (typically 2 μm), so it will trap the particulates that would otherwise block the main column's frit. When the back pressure increases or when double or split peaks appear, you can replace the guard column rather than replacing the frit on the analytical column. Any time you remove an analytical column's frit, you risk damaging the packing bed. Because of the low cost and relatively short life of the guard column, changing its frit is seldom worthwhile.

To get the best performance out of the guard column, use an in-line filter between the injector and the guard column. This filter, fitted with a 0.5-μm porosity frit, will trap any material that would normally block the guard column. The in-line filter is faster to change and less expensive than the guard column, so

its use means even more cost savings. As soon as the pressure rises above an acceptable level, shut off the system, replace the in-line filter's frit, and restart.

Replacing the guard column: Remember that the guard column is meant to be a disposable part used to protect the expensive analytical column. For this reason, don't try to use it up to its limit — replacing it a bit early will be safer in the long run. When a guard column is properly installed in the LC system, its effect on the chromatography should be neutral. Although you might expect an increase in the overall plate number of the system because of an increase in total column length, this small increase tends to be canceled out by the added fittings and tubing that are required to plumb the system.

An easy way to tell if the guard column is no longer good is to measure the resolution (or the height of the valley between two closely eluted peaks) with and without the guard column in the system. If the resolution is greater with the guard column removed, it is time to replace it. When developing a method, carefully keep track of how many samples are run through the guard column before it fails. That data will serve as a source of information to set up a guard column replacement program based on the number of samples run or number of days of use. A good rule of thumb is to replace the guard column when ~75–80% of its useful life has passed, which provides an extra measure of safety and adds very little to the overall analysis cost.

COLUMN FLUSHING

No matter what sample preparation you do or how regularly you replace the guard column, contaminants will build up on the analytical column. These contaminants often are sample components that require a stronger solvent for reasonable elution times. The appearance of very broad peaks or an undulating baseline can signal that it is time to flush the column. As with so many other areas of LC operation, an ounce of prevention is worth a pound of cure. Two flushing procedures that I find useful for reversed-phase columns are strong and organic solvent flushes.

Strong solvent flush: Contaminants that build up on the column often can be removed by flushing the column with the mobile phase's strong solvent, usually acetonitrile or methanol. First, flush any buffers from the system by washing the column with buffer-free mobile phase (replace the buffer component with water). For example, if you use 80:20 acetonitrile–buffer as a mobile phase, flush with 80:20 acetonitrile–water first. It is important to avoid using straight acetonitrile (or methanol) first because there is a good chance it will precipitate the buffer in the system. After flushing with approximately five column volumes of buffer, switch to 100% strong solvent (acetonitrile or methanol) and flush with another 10 column volumes. You can leave this solvent in the system or return to running a method. Many users program their instruments to perform this flush automatically at the end of each day before shutoff. This practice is a good way to prevent excessive contaminant buildup.

Note that whenever you flush or equilibrate a column, it is the volume rather than the time of flushing that is important. Therefore, if the pressure is reasonable, you can double the flow rate and cut the flushing time in half. To estimate the volume of standard 4.6-mm i.d. columns, use the following equation:

$$V_m \approx 0.1\,L$$

where V_m is the volume of mobile phase in the column in milliliters, and L is the column length in centimeters. Thus, a 25 cm × 4.6 mm column will have a volume of ~2.5 mL. Flushing with 10 column volumes would take about 25 min at 1 mL/min or 50 min at 0.5 mL/min.

Organic solvent flush: Occasionally, the strong solvent of the mobile phase is insufficient to remove contaminants from the column. In this case you need to use an even stronger solvent. I like to use methylene chloride or another sufficiently strong organic solvent. After flushing with 10 column volumes of strong solvent, flush with 10 volumes of methylene chloride and then with another 10 volumes of the strong solvent. Be sure to use miscible solvents as you change from one solvent to another — methylene chloride is immiscible with aqueous mobile phases. If you inadvertently use immiscible solvents in your LC system, you must flush the system with isopropanol or another mutually miscible solvent.

For special cases, use other techniques to remove strongly retained materials from the column. Chelators such as EDTA can help remove metals, chaotropes such as guanidine can help remove proteins, and other chemicals specific to your sample can help clean up a dirty column. Don't be afraid to experiment a bit — if the column is too contaminated to use, you can't make it any worse by trying a new flushing solvent. On the other hand, columns cannot be expected to last forever, so replace them when they begin to deteriorate. Although columns are expensive, they are a small part of the total cost of analysis.

SUMMARY

For the longest useful life of your LC column, clean up the sample before it is injected, use a guard column, and flush the analytical column regularly. Remember to balance the cost of the guard column and analytical column against other costs involved in the analysis. Heroic measures to extend column life may actually be counterproductive from an economic standpoint.

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