LC TROUBLESHOOTING

Column Flushing Demystified

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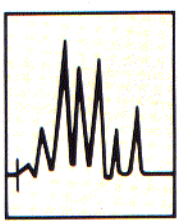

Flushing liquid chromatography (LC) columns regularly is a procedure that every lab should practice. However, based on the letters I receive and the questions I hear in short courses and seminars, it seems that many users have an incomplete understanding of procedures for column flushing or the reasons for following such procedures. Columns are flushed to remove unwanted materials from the column, but how long should this take, what solvents should be used, and what precautions should be observed? These and other questions regarding column flushing are discussed in this month's "LC Troubleshooting."

FLUSH TO PROTECT THE SYSTEM

The column is not the only part of the LC system that is protected by flushing. You should follow a regular washout procedure to protect the system against damage whenever you use buffered mobile phases. Buffers can cause two general system problems: mechanical wear and corrosion.

The primary problem of mechanical wear occurs when buffer salts are allowed to stand in an unused system. Moving parts in the LC system, particularly the pump piston assembly, injection valve, and autosampler, do not seal completely. As a result, under normal operating pressures (for example, 2000 psi), mobile phase leaks to a certain extent between moving and stationary parts. Most commonly, this occurs at the point where the pump seal contacts the piston. The mobile phase leaks under the seal, lubricating the piston so that it moves smoothly back and forth through the pump seal. When the system is turned off, the damp portion of the piston behind the pump seal dries out, and crystalline buffer deposits can result. When the pump is restarted, the buffer crystals act as an abrasive, accelerating the wear on the pump seal and possibly scratching the piston. By the time the system reaches the normal operating pressure, the crystals should have redissolved, but the damage they caused remains. (See reference 1 for a more complete discussion of this problem.) Accelerated wear of injection valve parts can occur similarly.

Stainless steel, which is one of the major construction materials for LC systems, is not at all stainless. It is common to see rust stains on stainless steel parts because of corrosive conditions within the LC system. Some mobile phase additives are more aggressive than others in terms of their ability to corrode stainless steel. This problem is one of the reasons that some manufacturers are using more durable materials (for example, Hastelloy, ceramics, and plastics) in some of the newer LC systems designed for use with corrosive mobile phases. Because corrosion is a chemical reaction, it is important to remove the corrosive agents in order to stop the reaction when the system is not in use; system flushing accomplishes this goal, as well. Passivation techniques, which reduce the reactivity of the stainless steel, will be discussed in a future column.

The presence of buffers in an unused column can result in similar corrosion problems inside stainless steel columns, or in accelerated degradation of the stationary phase or packing material. The reasons discussed below, however, are more likely to justify a regular column-flushing program.

FLUSH TO CLEAN THE COLUMN

The primary reason most workers flush the column is to remove late-eluting materials. Strongly retained sample components can cause problems of varying severity. For example, for an assay in which the last peak of interest comes out at 10 min, but the last peak in the run comes out in 20 min, the run time is effectively doubled by the unwanted peak. The problem of a late peak can be overcome in some cases by timing the injections so that the peak comes out in an unimportant portion of a future chromatogram. In other cases, it may be necessary to use a strong solvent flush between injections, either in an isocratic step fashion or by using gradient elution.

A problem that happens at least as often as well-defined late peaks is the occurrence of small peaks at very long retention times. These peaks show up as increased long-term noise or baseline undulations. This problem may not be noticed until several samples are run. The result is poorer quality baselines and, often, reduced assay precision or sensitivity because of baseline deterioration. These peaks generally require a much more rigorous washout procedure than for the previous example. This buildup of strongly retained contaminants is what most workers associate with the need for column flushing.

FIRST TRY THE STRONG SOLVENT

The easiest way to remove strongly retained material from the column is to wash the column with the strong solvent of the mobile phase. This generally is a two-step procedure that can be incorporated in the system shutdown routine at the end of each day's work.

The first step is to remove the buffer from the system. Do this by switching from buffered to nonbuffered mobile phase. For example, if the mobile phase is 63% acetonitrile (ACN)/water with a phosphate buffer and triethylamine, switch to a mobile phase that has about the same ratio of ACN to water but no additives. This is a very important step that you should not bypass, because it is possible to precipitate the buffer by switching to 100% organic immediately. Precipitation problems are somewhat system-dependent, but I have seen phosphate buffers precipitate when the mobile phase was switched to 100% acetonitrile before the buffer was removed. About five column volumes of unbuffered mobile phase are sufficient to remove the buffer at this stage. The column volume in milliliters can be estimated for standard 4.6-mm i.d. columns as $0.1L$, where L is the column length in centimeters. Thus, a 25-cm column will have about 2.5 mL of volume, so 6–7 min at 2 mL/min should remove most of the buffer.

Once the buffer is removed, the second step is to flush the column with strong solvent. In the present example, this is 100% ACN. You can run a gradient from the present (unbuffered) mobile phase to the pure strong solvent or just make a step change — it doesn't really matter.

HOW MUCH? HOW LONG?

If we remember that strongly retained materials behave chromatographically in the same manner as our sample compounds, column flushing can be demystified. Two items are important to remember: First is the "Rule of Three," and second is that volume, not time, is the important measurement.

The Rule of Three is a simplified way of remembering the effect of solvent strength changes on retention in reversed-phase LC. The rule states that retention changes by a factor of about three for each 10% change in mobile phase solvent. For example, if a band

elutes at 15 min with a 60% methanol/water mobile phase, it would be expected to elute at about 5 min with a 70% methanol mobile phase. This rule is handy to use when doing methods development, but it also gives us information on what to expect under column flushing conditions. Consider a very late peak with a 100-min retention time in the example above using 63% ACN/water. This peak would be very broad (about 4 min at the base for a 10,000-plate column) and probably would be seen only as a baseline hump. Let's use the Rule of Three to see what happens as we use stronger and stronger solvents to flush the column. Switching to 73% ACN would reduce its retention to ~33 min; 83% ACN would elute it at ~11 min; 93% ACN would drop it to ~4 min; and 100% would make the peak elute at t_0 (2.5 min). So you can see that changing to a strong solvent can wash strongly retained material off the column in short order.

So far, we've been talking about retention and flushing in terms of time, but we really should be considering volume instead because chromatographic retention is best expressed in column volume (even though it is more convenient to use time units). We can use the volume relationship to help us calculate how much solvent is needed to flush the column. If you work through several examples using the Rule of Three as we did above, you'll soon see that flushing with about 10 column volumes is likely to remove anything that is going to come off the column. In the case of a 25-cm column, this is 2.5 mL × 10, or about 25 mL of strong solvent. Most workers like to increase the flow rate during the flushing process so they can get it over with sooner. This is perfectly all right because the volume is more important than the time. Raising the flow rate also has an added bonus: Higher flow rates will give higher pressures, and more clean mobile phase will be forced past the pump seals than would be under normal operation. This will more effectively flush the surface of the piston behind the pump seal, helping to remove any contaminant buildup there. During flushing, however, you should not exceed your laboratory's guidelines for maximum system operating pressure (our lab uses 2500 psi as a target for this). Excessive pressure will increase system wear and should be avoided.

NEED A STRONGER SOLVENT?

Sometimes, flushing with the strong solvent of the mobile phase does not adequately remove strongly retained contaminants. This can be a common problem if "dirty" samples, such as biological or environmental samples, are analyzed routinely. In this case, increase the solvent strength still further. For reversed-phase methods, this means switching to a normal-phase solvent. Generally, methylene chloride is the solvent of choice. Just switch from the strong solvent (for example, methanol, acetonitrile, or tetrahydrofuran) to methylene chloride and pump 10–20 column volumes of

solvent. Then return to the strong solvent, pump another 10 column volumes, and you should be done. This process is summarized to the right. Remember that each time you switch solvents, you must be sure they are completely miscible. For example, you can't go directly from 50% methanol/water to methylene chloride because aqueous solutions won't mix with methylene chloride. If you do make a mistake and mix aqueous and normal-phase solvents, you generally can straighten things out by flushing with 2-propanol, which is miscible with both aqueous and nonpolar organic solvents.

Column Flushing Steps

1) Change to nonbuffered mobile phase (5 column volumes)

2) Flush with strong solvent of mobile phase (10–20 column volumes)

3) If Step 2 is not adequate, flush with 10–20 volumes of methylene chloride

4) Return to strong solvent (Step 2, 10 column volumes)

5) Return to nonbuffered mobile phase (Step 1, 5 volumes)

6) Return to mobile phase (10–20 volumes before first injection)

AND IF THAT DOESN'T WORK. . .

Sometimes, solvent flushing will not remove column contaminants. You usually can improve the efficacy of a column-flushing procedure by applying your knowledge of the chemistry of the sample that you are using. For example, changing the mobile phase pH will change the polarity of ionizable compounds, thus altering their retention characteristics. If, during methods development, you note which conditions give poor retention, these are the ones that should be used to speed column flushing. In cases in which metal-ion contamination is a problem, flushing with ethylenediaminetetraacetic acid (EDTA) can help clean the column. When bound proteins are a problem, you can use detergents or chaotropes (for example, 8 M urea or guanidine) to solubilize and remove the proteins.

Strongly bound materials can be washed off the column more quickly if the column is reversed and then flushed. For example, a compound that has migrated only 1 cm through the column under normal operating conditions must be flushed through the remaining 24 cm before it is washed off. If, however, the column is reversed before flushing, the compound needs to migrate only 1 cm before it is washed off. Whenever the column is reversed for flushing purposes, be sure to leave the outlet (the old inlet) disconnected so that the column is flushed directly to waste rather than through the detector cell. This will prevent any particulate matter that might wash off the frit from blocking the detector flow cell.

AN ALTERNATIVE TO FLUSHING

In many labs, the sample load is small or the samples are always clean, so flushing to remove contaminants is not necessary on a daily or even a weekly basis. In this case, it is still important to remove buffers from the system before turning it off for the night. One way to get around having to flush out the buffers each day is to leave the system running at a very low flow rate (for example 0.1 mL/min) all the time. In this manner, the seals are always wetted, and buffer deposits will not form. To start the system in the morning, just increase the flow rate and inject the first sample. If you use this procedure, it is still a good idea to use a nonbuffered wash at least once a week.

A WORD ON COLUMN STORAGE

Selecting the solvents to leave in the column during storage is related to column flushing. Generally, columns should be stored in a buffer-free mobile phase and capped tightly so that they don't dry out. With reversed-phase columns, any solvent with less than about 50% water is suitable for storage. If you have any questions about whether or not a solvent is compatible with your column, consult the manufacturer's instructions that came with the column. If you are still in doubt, store the column in the same solvent that was in it when you received it; if it is good enough for the manufacturer, it is good enough for you!

SUMMARY

System flushing may seem like just one more thing to clutter up the day and take time away from running samples, but you should consider it as a preventive maintenance task that will pay you back in more reliable system operation and less downtime in the long run. By programming the washout procedure to be executed after you leave for the day, and a similar "wash-in" procedure to occur before you arrive in the morning, you can have the best of both worlds — your LC system will be flushed thoroughly, yet the procedures will not interfere with your normal workday.

REFERENCE

(1) J.W. Dolan, *LC•GC* **7**, 224 (1989).

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Lafayette, California, USA, and is a member of the Editorial Advisory Board of LC•GC.