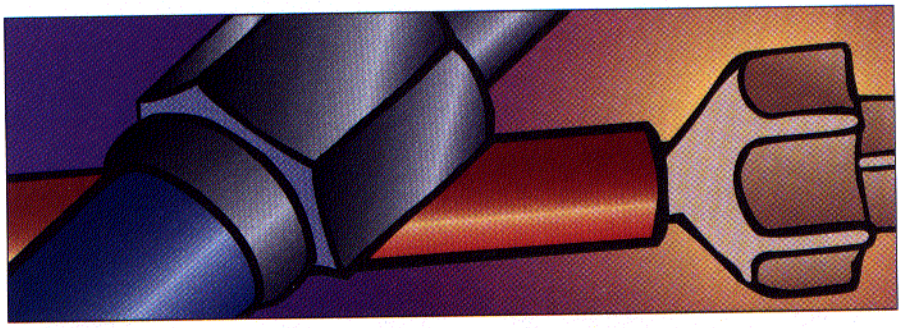
LC Troubleshooting



Readers' Questions

Carryover, Mobile-Phase Temperature, and Column Care

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Basic column care usually is independent of sample type.

This month's "LC Troubleshooting" column addresses questions submitted by several readers. The topics include how to isolate the source of carryover in liquid chromatography (LC) methods, mobile-phase temperature effects, and the care and cleaning of columns. Often simple practices — such as those described below — can make the difference between a reliable LC method and one that is problematic. (Because of space limitations, I can address only a small portion of readers' questions in this column; consult the on-line "Chromatography Forum" at http://www.lcgcmag.com for additional questions and answers.)

PROTEIN CARRYOVER

Q: When making blank injections using a C4 column and acetonitrile–water gradients with 0.1% trifluoroacetic acid in both solvents, my co-workers and I see carryover of a few percent with the most hydrophobic proteins from the previous injection. The carryover peaks have exactly the same retention times as they did in the previous run. How do I correct this problem?

A: First you need to determine if the source of the carryover is the autosampler or the column. You can find the source by injecting a large sample followed by a blank gradient in which no injection is made. Consult the autosampler operator's manual if you are not sure how to perform a no-injection run. The autosamplers in my laboratory require users to set the injection volume to −1 in the control sequence. Other systems may require users to set the injection volume to zero or use some other setting. If the peak is still present, the source is sample left on the column from the previous injection. More commonly the peak will disappear, which indicates that the carryover source is the autosampler.

Most of the time, you'll find that the carryover originates in the autosampler. Sample may be sticking to the tubing or another surface, or it may be physically trapped in a poorly assembled fitting or other reservoir. Often a more aggressive flush solvent will cure this problem. Most autosamplers do not inject the flush solvent, so you don't have to worry about chromatographic compatibility as long as the flush solvent is fully miscible with the mobile phase.

As a general rule, it is wise to use a wash solvent that is at least as strong as the mobile phase. In your case, if the gradient runs to 80% acetonitrile, you could use 80% acetonitrile as your wash solvent. If you chose water instead, it is easy to see that hydrophobic materials might find the autosampler surfaces more attractive than the wash solvent, so washing would be ineffective. I like to avoid using additives such as buffers in the wash solvent unless they are necessary for sample solubility, because the solvent could evaporate and leave a solid buffer residue. Isopropanol works well as an autosampler flush solvent to remove most organic materials that stick to the system.

The autosamplers in my laboratory use a hydraulic system in which the autosampler flush piston also is used to meter sample to the injection valve. For this reason it is important that the flush solvent is degassed. Air bubbles allow hydraulic cushioning that results in lower precision.

Another common source of carryover in autosamplers is an error in a fitting assembly. If the tubing is not fully seated in the fitting, it creates a small space that can act as a sample reservoir. For $\frac{1}{16}$-in. o.d. tubing, a 1-mm gap creates approximately 2 µL of extra volume in the fitting. When first assembling a fitting, be sure to push the tubing to the bottom of the fitting port before tightening the nut. Stainless steel fittings rarely slip under normal LC operating pressures, but the popular polyetherether ketone (PEEK) fittings can slip under excessive pressure. So if a blockage in the system occurs that causes the pressure to exceed 4000–5000 psi, it is wise to check PEEK fittings to be sure no slippage has occurred. Just turn off the pump, loosen the fitting, push the tubing in, and retighten it. The potential for slippage with PEEK fittings leads me to limit their use to system components that are connected and disconnected frequently, such as the column, detector, and in-line filters. I prefer stainless steel fittings and tubing for sections of the LC system that rarely are modified, such as pump-to-autosampler connections and internal autosampler connections.

Finally, if you have tried these adjustments and still observe carryover, you might try adding a little organic solvent to the sample solvent or change to a PEEK sample loop to reduce unwanted interactions between the sample and the tubing surfaces.

MOBILE-PHASE TEMPERATURE

Q: How does the mobile-phase temperature affect the separation? I have seen LC systems that control the column temperature, but the temperature of the mobile-phase reservoirs seems to be ignored.

A: I have never seen anyone use temperature control for the mobile-phase reservoirs, although I'm sure it is done in some situations. The important parameter to control is the column temperature in a separation. For reversed-phase separations, retention can vary by 1–3%/°C. Even with an air-conditioned laboratory, temperature swings of several degrees can occur at different times of day (day versus night), days of the week (weekday versus weekend), and between seasons. Although

small changes in temperature may be unimportant for some separations, temperature control can be critical for other separations. For this reason, it is wise to use column temperature control in all LC systems.

If a column is operated at temperatures much higher than 35 °C, it is important to preheat the solvent so that cold incoming mobile phase does not cool the column below the desired temperature. One simple way to heat the mobile phase is to use a coil of stainless steel tubing in the oven just before the column to transfer heat to the incoming mobile phase. For temperatures as high as 50 °C, 100 cm of 1/16-in. o.d. tubing will suffice; if the column will be operated at temperatures as high as 75 °C, use a 150-cm piece of tubing. The tubing should be in intimate contact with the heat source; for example, it could be clamped to the heating block in a block heater.

You may think that heating the mobile phase in the reservoir would be an effective way to control the temperature of the mobile phase and, thus, the column. The problem with this approach is that by the time the mobile phase passes through the pump, autosampler, and all the connecting tubing, it will have either gained or lost heat in the process, so the relationship between the reservoir temperature and the column temperature is indirect at best.

CLEANING A COLUMN

Q: After I finish analyzing pesticides on my C18 column, I usually need to store the column for a week or longer before I use it again. The mobile phase is methanol–buffer. Should I wash the column with water or methanol first? What storage conditions should I use?

A: The question of which solvent to wash out of a column first is a common one, but the procedure is quite simple. Because of the propensity of buffers and salts to precipitate in LC systems, it is best to remove them first. You might think that washing with water would be the best way to remove buffers, but this approach actually can inhibit buffer removal because of the collapse of the stationary phase (see April's "LC Troubleshooting" [1] for more information). Instead, the best approach is to use unbuffered mobile phase as a first step.

Replace the buffer portion of the mobile phase with water. If you are using on-line mixing with buffer in reservoir A and methanol in reservoir B, just replace reservoir A with a bottle of HPLC-grade water. If the mobile phase is hand-mixed, make an equivalent methanol–water mixture. Approximately five column volumes of flushing with this mixture usually is sufficient to remove most of the buffer. (The 4.6-mm i.d. columns most chromatographers use have a volume of roughly 0.1 mL/cm, so a 15-cm column would have a volume of approximately 1.5 mL.) Next switch to 100% strong solvent — methanol in the present case. Flush with at least 10 column volumes of methanol to remove strongly retained materials.

It seldom matters what sample is analyzed — 100% organic solvent usually is the best way to remove any solute from a reversed-phase column. You can store the column in methanol; just remove the column from the system and cap it tightly. Generally it is okay to store reversed-phase columns in 100% organic solvent. If you are unsure, consult the instructions insert that came with the column for specific directions. If you inadvertently flush the column with water and cause phase collapse, washing with >50% organic solvent for 30 min or so should be sufficient to restore the stationary phase to its normal orientation.

The new reversed-phase materials that incorporate a polar function near the base of the stationary phase provide an exception to the no-water guideline. These phases sometimes are called amide-modified phases or a similar designation. They usually have a nitrogen-containing polar function three carbons out from the base of the phase. In addition to giving the phase interesting selectivity properties, this chemical modification allows the column to perform well with 100% water or buffer as the mobile phase. Some popular brands are Agilent Technologies' Zorbax Bonus-RP (Wilmington, Delaware); Supelco's Discovery-Amide (Bellefonte, Pennsylvania), and Waters Corp.'s Symmetry Shield (Milford, Massachusetts). Consult your column supplier for the amide-type column in its column series.

EXTENDING COLUMN LIFE

Q: For budgetary purposes, we must use each column for many different analyses. Are there some simple guidelines that will help extend the useful life of our columns? Are there special precautions when using ion-pairing reagents?

A: Although it may not be obvious at first, it is almost always less expensive in the long run to dedicate a column to each type of analysis. For example, if you have 10 different methods, you will use fewer columns during a year's time if you dedicate a column to each method rather than using just one column for all the methods and replacing it when it wears out. The reason for extended life with a dedicated column is that residual peaks from one analysis may interfere with another and that the column may age in different ways, depending on the sample history and mobile-phase conditions. The net result of dedicated column use is fewer columns purchased and higher quality data. However, this approach is not always practical because of budget constraints or a very small sample load for one or more methods. This situation seems to be your case.

The trick to maximizing column life is quite simple: keep the column clean. Follow the guidelines given in the answer to the previous question. Remove the buffer and flush with strong solvent to remove strongly retained materials. Store the column with the column plugs tight so that mobile phase does not evaporate. It is a good idea to monitor the performance of the column with a system-suitability sample for each method. It may be that the column will fail system suitability for one method but still perform satisfactorily for another method, thus allowing you to make a few more runs before discarding the column.

I strongly advise against using a column for ion-pairing as well as traditional reversed-phase separations. The ion-pairing reagents are difficult, if not impossible, to remove completely, so the column chemistry may be different after using ion-pairing conditions. Dedicating a column to ion-pairing separations would be prudent. The best way to remove ion-pairing reagents is a bit different from removing standard reversed-phase solvents. Start by flushing with 50% methanol and 50% 200 mM phosphate buffer (pH 6). This cleaning solvent will readily displace most of the ion-pairing reagent. Then switch to methanol–water and finally straight methanol.

Before you resign yourself to using one column for multiple methods, it would be wise to do a quick economic analysis of the situation and see if your proposed strategy is justified. For example, most pharmaceutical companies are willing to pay $50 or more per sample for analysis by outside laboratories, suggesting that this is a reasonable estimate of the cost per sample. If a $500 column were used for only 500 samples (a rather low number), it would contribute $1/sample, or approximately 2% of the cost of analysis. Doubling the column life would make only a minor change in the overall costs. A solid-phase extraction cartridge costs roughly $2, and when manual or automated sample preparation is added, these steps can cost many times the column costs. So in this case, it would be much wiser to work on reducing sample preparation costs rather than trying to save money by using the same analytical column for several methods.

CONCLUSIONS

Most column or system-related problems with reversed-phase LC procedures have the same solutions regardless of the specific sample type. It makes the life of practical chromatographers much easier to know that once they have mastered the basic techniques for care of the LC system and column, they can apply these same techniques to a wide variety of samples.

REFERENCE

(1) R.G. Wolcott and J.W. Dolan, *LC•GC* **17**(4), 316–321 (1999).

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