LC TROUBLESHOOTING

Changing System Conditions

John W. Dolan

Sometimes making "logical" changes in our methods produces unexpected results.

This month's column addresses three readers' problems with changed system conditions that failed to produce the expected results. The first case shows that although changing gradient-elution conditions is simple if we follow a few rules, making certain "logical" changes can make things worse instead of better. In the second case, a reader attempted to clear a blocked frit using an accepted technique, which solved the problem in one instance but not in another; the result was a confused operator. Finally, another reader reported that using a mobile-phase additive to improve the baseline resulted instead in a noisier baseline.

GRADIENT PROBLEMS

Q: I'm running a gradient method using a 25-cm C18 column. Solvent A is 15:85 (v/v) acetonitrile–25 mM phosphate buffer (pH 3.0), and solvent B is 80:20 (v/v) acetonitrile–buffer. The program is a linear gradient from 0 to 100% B in 20 min at 1.5 mL/min. The pressure was too high, so I lowered the flow rate to 1.0 mL/min. This solved the pressure problem, but the separation was no longer satisfactory. I thought lowering the flow rate increased the column efficiency, so I expected the separation to improve. What happened?

JWD: Your assumption about column efficiency (theoretical plate number, N) is correct: Lowering the flow rate generally results in higher plate numbers. However, changing the flow rate produces other effects in the gradient that you must compensate for to maintain the same separation.

Equation 1 expresses the relationship between the important variables in gradient elution:

$$\overline{k} \approx c t_G F/(\Delta\%B\ V_m) \qquad [1]$$

Here, $\overline{k}$ is the average capacity factor during a gradient-elution run, c is a constant, t_G is the gradient time, F is the flow rate, $\Delta\%B$ is the gradient range, and V_m is the column volume. To maintain the same selectivity or relative peak spacing, you must keep $\overline{k}$ constant. Another way to think of this is that the gradient slope must be constant. Thus, if you decrease F from 1.5 to 1.0 mL/min, you need to adjust one of the other parameters to compensate for the flow change. The easiest variable to change is t_G. According to equation 1, a change in t_G from 20 to 30 min ($t_G F = 20 \times 1.5 = 30 \times 1.0$) preserves the value of $\overline{k}$.

You can use equation 1 to simplify the adjustment of other gradient parameters, such as the gradient range. For example, if all of the peaks are eluted by the time the program has reached 80% B, you can shorten the gradient so that you don't waste the last few minutes of the program. To reduce $\Delta\%B$ by 20% and keep $\overline{k}$ constant, you need to make a proportional change in the gradient time (or flow rate). Thus, you can obtain the same separation using a gradient from 0 to 80% B in 16 min as you obtained using a gradient from 0 to 100% B in 20 min ($t_G/\Delta\%B = 16/80 = 20/100$).

Alternatively, if you obtain excess resolution using the 25-cm column, you can switch to a 15-cm column. In this case, you need to change the gradient time to 12 min ($t_G/V_m = 20/25 = 12/15$). (You're using the same column diameter, so you can substitute column length for V_m because the ratio of column length to column volume stays the same.) Adjusting the gradient is easy if you keep the relationships in equation 1 in mind.

COLUMN PRESSURE PROBLEMS

Q: I have a column pressure problem that I cannot solve. After using the column for about half its normal life, I observed that the pressure was ~50% above normal. Because I do not filter my samples before analyzing them, I suspected that the frit at the column head had become partially blocked. To correct this problem, I normally reverse the column and continue running samples. In this instance, when I reversed the column, the pressure returned to normal as expected, and I continued to use the column in the reverse direction. When the pressure rose again a few days later, I repeated the reversal procedure, but this time the pressure did not return to normal (the column was now in its original orientation). After flushing the column for ~30 min, I again reversed the column (to the reverse position), and the column pressure returned to normal. These results don't make sense to me. Can I do something to minimize this problem? Because of the inconvenience and expense, I'd prefer not to filter the samples, which usually are free of particulates.

JWD: Two common techniques exist for removing particulate matter from the column head. One is to reverse-flush the column, as you have done. Alternatively, the frit at the column head can be replaced. Each method seems to work about one-third of the time, and the choice of methods is largely a matter of personal preference.

I suspect that your problem is similar to one that I once encountered when an injection-valve rotor in my system became severely worn. Using a microscope, I could see fibrous material partially lodged in the frit. When the frit was on the upstream end of the column, the fibers' tail lay across the frit, blocking many of the pores. Reversing the column pushed the tail away from the frit and freed the blocked pores, but the other end remained lodged in the frit. The result was the same pattern you observed: high pressure in the normal flow direction and normal pressure in the reverse flow direction. The particulates acted like a flapper valve, restricting flow in one direction and allowing flow in the opposite direction. I solved the problem by replacing the frit. In your case, I suspect that the original inlet frit was blocked in this manner, and the other frit was blocked with particulate matter that could be washed off during reverse-flushing.

Reverse-flushing can be very effective for removing particulate matter, but it also speeds the removal of very strongly retained material from the column head. To flush the column, reverse it and leave the outlet (formerly the inlet) disconnected to prevent contaminants from being washed into the detector. Flush the column with 10–20 column volumes of mobile phase (a total of 50 mL is sufficient). During this procedure, the pressure should return to normal and all particulate matter should be flushed to waste. Now reconnect the detector and continue to run in the reverse direction. To remove strongly retained materials, reverse the column as described above and flush it with a strong solvent. The advantage of this technique is that strongly retained materials have to travel only 1 cm or so to the column exit instead of through the entire column. After this procedure, you may want to return the column to the normal flow direction so that strongly re-

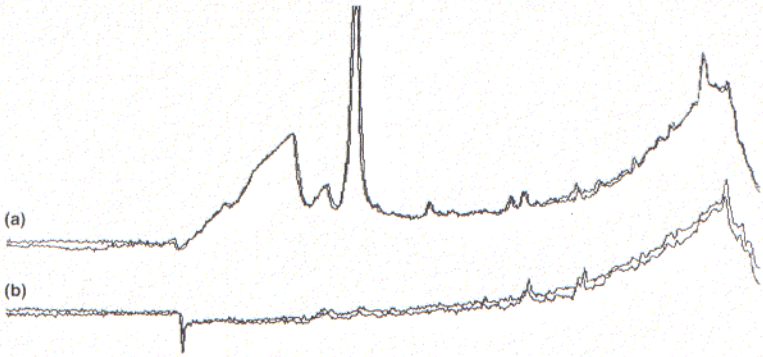

FIGURE 1: Blank chromatograms generated using a mobile-phase gradient (a) with and (b) without 5 mM triethylamine. Mobile phase A: 20 mM phosphoric acid (pH 2.5); mobile phase B: acetonitrile; gradient: 0–70% B in 65 min; detection: UV, 220 nm.

tained materials contaminate only one end of the column.

You can reduce the likelihood of frit blockage at the column head in three ways. The most obvious technique is to prevent the particulate matter from reaching the column in the first place by filtering all samples or at least those that are cloudy or contain visible particulates. In your case, however, filtering is an unacceptable solution because of other factors. One alternative is to use an in-line filter just downstream from the injector. Generally, it is best to use one of these filters whenever you analyze samples that may contain particulate matter. The filter's 0.5-μm frit becomes blocked sooner than the 2-μm frit at the column head. The filter is designed to introduce an insignificant amount of extra-column band broadening. When you notice a pressure rise, you can change the frit in a couple of minutes without disturbing the column. Using an in-line filter is a simple, effective way to trap particulate matter from an occasional dirty sample, but if particulate matter is frequently present, use sample filtration instead.

Finally, I recommend using a guard column whenever you analyze "real" samples, especially those from biological or environmental matrices. Guard columns act as chemical filters that trap strongly retained materials before they reach the analytical column, and they contain a 2-μm frit that traps larger particles. As with an in-line filter, don't use a guard column to avoid filtering samples that contain particulate matter. You can use an in-line filter and a guard column in series (filter first) for added protection.

A final word on reverse-flushing: If reversing the column fails to reduce the pressure, you probably should replace the blocked frit. Reinstall the column so the blocked frit is upstream and then turn on the pump for a few minutes before replacing the frit. This technique should settle the column packing and prevent the packing material from extruding from the column when the endfitting is removed.

TRIETHYLAMINE PROBLEMS

Q: I run reversed-phase gradients with acetonitrile–buffer mobile phases using UV detection at 210–230 nm. When I use a mobile phase consisting of 20 mM phosphoric acid adjusted to pH 2.5 with ammonia, I obtain acceptable baselines. However, when I add 5–10 mM levels of triethylamine before adjusting the pH, the baseline shows greatly increased noise (Figure 1). I have used several brands of triethylamine and even tried distilling it, but these adjustments do not improve the baseline. What can I do to improve the baseline?

JWD: First, purchase HPLC-grade triethylamine. Many chemical suppliers sell triethylamine that has been specially purified for use with low-wavelength UV detection for liquid chromatography. We routinely use HPLC-grade triethylamine at 210 nm without excessive noise or contaminant peaks. You can purchase triethylamine in ampules or ~100-mL bottles. For our purposes, the 100-mL size is more cost-effective. We flood the bottle with nitrogen to eliminate as much air as possible before resealing the bottle. You can use other cleanup techniques to further purify triethylamine in the laboratory, but I doubt that in-lab purification is as cost-effective as purchasing HPLC-grade triethylamine.

Another way to improve your method is to increase the triethylamine concentration. I never work with mobile-phase additives at concentrations lower than ~20–25 mM unless I have a good reason. Below these levels, retention times can be affected by small changes in additive concentration.

"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. *Direct correspondence about this column to "LC Troubleshooting,"* LC•GC, *P.O. Box 10460, Eugene, OR 97440, USA.*