LC TROUBLESHOOTING

# Method Reproducibility, Leaks, and Check-Valve Problems

*John W. Dolan*

***Sometimes, precisely following the instructions may not be enough to get someone else's method to work in your laboratory.***

This month's "LC Troubleshooting" addresses a reader's question about problems encountered when trying to duplicate a method described in the literature. As is often the case, variables that may have been unimportant in the original laboratory may be a source of problems in another laboratory. This column also answers readers' questions about extremely slow leaks from fittings and pressure problems related to check-valves.

## METHOD REPRODUCIBILITY

**Q:** I have been analyzing phospholipids for some time and recently have been trying to use an assay developed in another laboratory (1). As you can see in Figure 1, the original separation looks pretty good. However, when using this method, I observe a large baseline rise at the beginning of the chromatogram and obtain an inferior separation (Figure 2). I modified the method by adding steps such as preparing fresh mobile phase daily but have not achieved a satisfactory separation. What could I be doing wrong?

**JWD:** When I read over the referenced method (1), I noticed potential problems. These problems do not imply that the original workers did poor work but that the necessary conditions are difficult to reproduce in your laboratory. We looked at a similar problem of method transfer in last month's "LC Troubleshooting" (2), in which a method worked well in one lab but not in another.

## POTENTIAL PROBLEMS

The authors of the original method used a dual-pump gradient liquid chromatography (LC) system equipped with a UV detector operating at 195 nm. The column system comprised a 25 cm × 4.6 mm diol analytical column and a 6 cm × 4.6 mm silica guard column (both using 5-μm particles). For gradient elution, solvent A was 80:20 (v/v) acetonitrile–5mM phosphate, pH 5.0, and solvent B was 5 mM phosphate, pH 5.0. A multistep gradient started with a 2-min isocratic hold at 98% solvent A and ended with a hold at 10% solvent A. Other relevant conditions are mentioned below.

**Wavelength errors:** At low detection wavelengths, such as 195 nm, almost any organic component in the sample will absorb strongly. This strong absorbance results in a much larger $t_0$ peak than in assays that are monitored at higher wavelengths. Furthermore, any small error (for example, 1–2 nm) in the wavelength setting could cause a dramatic change in the signal, especially at $t_0$. Because of this effect, verify that your detector is properly calibrated. Sample preparation problems may also contribute to the large $t_0$ peak in your chromatogram (Figure 2), so be sure to carefully check the extraction procedures for errors.

**Buffer:** As discussed in detail in an earlier "LC Troubleshooting" column (3), incorrect buffer conditions can cause problems. There are a couple of things that I don't like about the mobile phase for this method. First, the buffer concentration is marginal. Unless I have to, I don't use buffer concentrations lower than 20 mM. The recommended 5-mM buffer lies within the region in which the method can be very sensitive to variation in buffer concentration. (Although the original workers may have had valid reasons for choosing this buffer concentration, they stated none in the article.) You might check for concentration effects by increasing the buffer concentration to 10 mM and 20 mM; then, observe the results. If the results are essentially equivalent at the higher concentrations, use a higher buffer concentration.

Second, on-line mixing of acetonitrile and buffer can be a source of problems. Acetonitrile can cause buffer to precipitate, and in bulk solution this precipitation is rapidly diluted and the buffer redissolves. In the liquid chromatograph, however, the precipitate may not redissolve immediately because of the system's flow dynamics. The original method used two pumps to deliver the mobile phase components to a high-pressure mixer. Such systems may be better at mixing organic solvents and buffer because they deliver each mobile phase component on a continuous basis in the proper proportions.

In low-pressure mixing, on the other hand, discrete aliquots of pure solvents are mixed. Under such conditions, precipitation and then redissolution could occur on a very small scale. This phenomenon would be similar to what is observed when a few drops of buffer are added to a beaker of acetonitrile: the formation of a cloudy precipitate that dissolves when the solution is swirled. If you are using a low-pressure mixing system, you may be observing a similar precipitation–dissolution phenomenon. The way around this problem is to premix (by hand) the starting concentration of solvent A (80:20 acetonitrile–buffer), as described in the original article.

**Dwell volume:** A third potential problem is the system dwell volume. This is the volume between the solvent mixer and the head of the column. An earlier *LC•GC* article (4) discussed the importance of dwell volume. In a nutshell, the problem is that systems can have different dwell volumes. The authors of the original work used a high-pressure mixing system, which probably had a dwell volume of ~2 mL. If you are using a low-pressure mixing system, the dwell volume can be as large as 5–6 mL, and if you are using an autosampler, it can be as large as 10 mL. Increasing the dwell volume lengthens the isocratic hold at the beginning of the gradient (2 min in the original method). This effect can produce significantly different chromatograms for nominally identical methods on two different systems. To circumvent this problem, shorten the isocratic hold at the beginning of the run so that the sum of the dwell volume and the hold for your system is the same as that for the original method.

***Unless I have to, I don't use buffer concentrations lower than 20 mM.***

**Injection solvent:** The method calls for injecting 20 μL of sample that has been dissolved in 2:1 (v/v) chloroform–methanol. Any time you inject a sample in a solvent that is not identical with the mobile phase (in this case 22:78 [v/v] acetonitrile–buffer), you can expect the solvent peak to be larger than for an injected sample that has been dissolved in mobile phase. I would verify this result by injecting a blank (solvent only) of the chloroform–methanol mixture and one of just solvent A (buffer plus acetonitrile). Compare the $t_0$ peaks and note whether the disturbance is re-

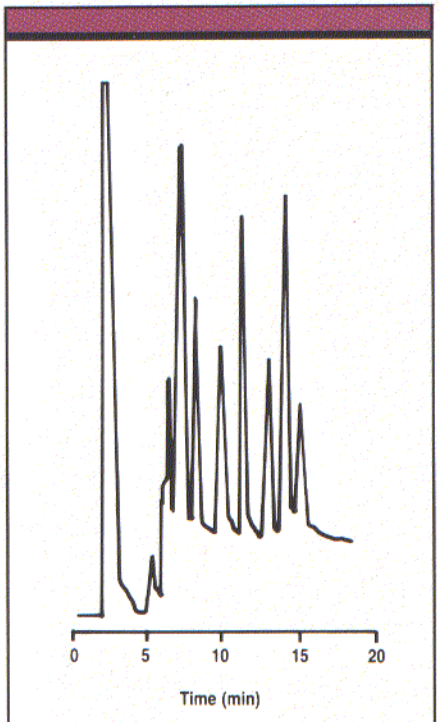


**FIGURE 1: Phospholipid separation obtained in the original laboratory. (Reprinted with permission from reference 1.)**

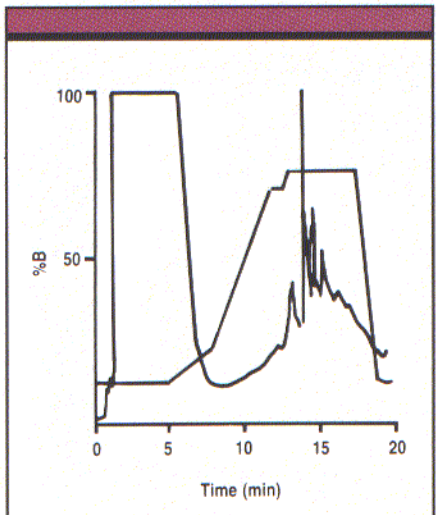


**FIGURE 2: Phospholipid separation obtained when the reader tried to duplicate the separation shown in Figure 1. The gradient is superimposed on the chromatogram.**

lated to the injection solvent. A possible solution is to use acetonitrile instead of methanol or chloroform to dissolve the sample. Also, check the miscibility of the chloroform–methanol solution with the starting mobile phase (do this with a test tube) — poor miscibility might also be causing a problem. In general, you can get by with a larger volume of a dilute solvent (for example, adding buffer or water to the sample) for injection. Diluting the sample with mobile phase might improve things as well.

**Guard column:** Finally, the guard column may be a problem. The method specifies using a silica guard column and a diol analytical column. Generally, a method performs best when the guard column and analytical column are matched. To test whether the guard column is the problem, try a few injections after removing the guard column, or substitute a diol guard column for the silica one.

## IN SUMMARY . . .

As you have discovered, one encounters many pitfalls when reproducing a method from the literature in one's own laboratory. Because some of the variables are instrument-dependent, the original user may have had no problems, but the new worker can have all sorts of trouble. Again, my intention is not to criticize the original authors but to remind us all that we should take extra care when working with methods that may be sensitive to small changes in operating conditions. If we develop methods that may be used on other instruments or in other laboratories, it is wise to explore the impact of small changes in the operating conditions and to include a brief summary of the findings in any published report about the work.

## FITTING LEAKS

**Q:** I recently inspected the plumbing on my LC system and discovered white deposits on the nuts on some of the pump fittings. I have noticed no problems with pump leaks before, but the deposits indicate that the fittings are leaking. What is going on?

**JWD:** As I'm sure you have concluded, the deposits are residues from buffer in the mobile phase. Your observation indicates that the fittings have indeed been leaking. However, the leakage rate is probably so slow that the mobile phase evaporates before a visible drop forms. Thus, over several days or weeks a mobile phase residue will form. A leak of this magnitude probably has no practical effect on the performance of your system or the quality of your results.

To solve this problem, tighten the fitting nuts slightly. However, because you observed buffer residues, disassemble each fitting and rinse it with water to remove the deposit before reassembling. Allowing the residues to remain on the fitting threads may eventually result in seizing or corrosion. Remember to watch the fitting for the next few weeks to be sure the problem no longer exists. Sometimes leaks will continue even after the fitting parts have been cleaned and retightened. Such a leak may be caused by a

defective or damaged fitting or the presence of a small contaminant (such as a silica particle) that prevents the ferrule from sealing properly in the fitting body. If this problem is observed, cut the tube end to remove the old ferrule and install a new one. Alternatively, try using one of the finger- or wrench-tightened fittings that use a polymeric ferrule. The polymeric ferrule will deform enough to seal even when the mating surface is imperfect.

***If the leak doesn't stop after one tightening, something is wrong.***

A final note on tightening fittings: Although all fittings manufacturers give specifications for proper assembly (for example, three-quarters of a turn past finger-tight), I don't know of anyone who pays much attention to these guidelines. Generally, we give them a "good" snugging up after we have assembled the fittings by hand. When they leak, we tighten them a little more, and a little more, and snap! My experience has taught me that if the leak does not stop after one such additional tightening, something is wrong; the fitting should be disassembled and cleaned or replaced as described above.

## PRESSURE PROBLEMS

**Q:** I'm using a dual-piston pump with a premixed mobile phase that is fully degassed by helium sparging. When I run samples, the pressure sometimes drops momentarily from the normal 800 psi (5.5 MPa) to ~400 psi (2.75 MPa). Then the pressure quickly returns to normal. I have not observed this problem on a regular basis — it's one of those gremlins that never seem to appear when I want to show someone else — but the pressure dip occurs every few samples. What could be the source of this problem?

**JWD:** This sounds like a classic check-valve problem. If possible, try to determine which pump head is working when the pressure drop occurs. You should be able to identify which check valves are causing the problem. Replace the inlet and outlet check valves on the problem head with new or factory-rebuilt check valves. If your pump uses cartridge-style check valves, just replace the old cartridge with a new one. If you want, you could replace one valve at a time to find out which one is causing the problem, but this may not be worth the trouble. Discard the old check valves or return them to the manufacturer for reconditioning. I have had poor success with check-valve rebuilding kits because our laboratory does not have a clean-room environment comparable to that in which manufacturers assemble check valves.

The key to minimizing future problems is to prevent particulate matter and air bubbles from reaching the check valves. You should continue to degas the mobile phase. Also, before using the mobile phase, be sure to filter it through a ≤0.5-μm-porosity membrane filter. Use a 10-μm-porosity inlet-line frit in the reservoir to prevent particulate matter from contaminating the pump. Mount the reservoir above the pump to maintain a slight hydrostatic pressure; this arrangement can improve check-valve function. If you suspect that the solvent-inlet tubing is restricted, loosen the tubing at the inlet check valve. In a properly configured system, solvent will freely siphon from the tubing. If solvent does not siphon freely, check for a blocked inlet-line frit or a kink in the line. On systems that use low-pressure mixing, the siphon test should be performed upstream from the mixing manifold or proportioning-valve block.

Another practice that can extend check-valve life is flushing buffered mobile phase from the system at the end of each day and discarding the remaining buffer. Flushing the system with nonbuffered mobile phase will prevent buffer crystals from forming in the system if the mobile phase evaporates. Buffer precipitation can shorten the lifetime of pump and injector seals and can block filters and frits. Also, many buffers support microorganism growth, which is a problem that can be largely prevented by using fresh buffers each day.

## REFERENCES


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(3) J.W. Dolan, *LC•GC* **8,** 212–215 (1990).
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*"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. *Correspondence concerning this column can be sent to "LC Troubleshooting,"* LC•GC, *P.O. Box 10460, Eugene, OR 97440, USA.*


## Prize Winner!

Earl D. Fluharty, Senior Chemist at Central Pharmaceuticals Inc., Seymour, Indiana, won the 1991 *LC•GC* Buyer Intention Survey drawing. One of the first 500 respondents, Fluharty won a Panasonic portable compact disc player.