# LC TROUBLESHOOTING

## Readers' Questions

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This month's installment of "LC Troubleshooting" addresses a variety of readers' questions and responses to earlier articles. These include injection problems, extra peaks in the chromatogram, mobile phase preparation, and equipment repair.

### INCREASING INJECTION PRECISION

In a previous installment of "LC Troubleshooting" (1), some practices that help to maximize injection precision were discussed. The column stated that, because of laminar flow, the loop is poorly filled when sample volumes between ~50% and 300% of the loop volume are used. In other words, for a partially filled loop, it is best to use <50% of the nominal loop volume (for example, use <10 µL in a 20-µL loop). For filled-loop injection, use at least 300% of the loop volume (for example, 60 µL in a 20-µL loop) in order to ensure that the loop is completely free of its previous contents.

A reader has reminded me of a technique that allows accurate partial loop filling up to the full loop volume (2). The trick is to place a small bubble (for example, 0.2 µL) in the syringe needle before the sample is injected into the sample loop. When the bubble enters the loop, it isolates the sample from the previous loop contents so that laminar flow does not cause washout problems. When the injection valve is placed in the *inject* position, the bubble will dissolve and will not cause any problems with the analysis. Because the bubble creates a condition of plug flow in the loop, you can place any amount of sample (up to the loop volume) in a sample loop; injection precision now depends primarily on the operator's syringe-filling technique. A detailed discussion of this and other injection loop filling techniques can be found in reference 3.

### NEGATIVE PEAKS

Another reader (4) responded to an "LC Troubleshooting" column on negative peaks (5) with still another cause of negative peaks. A pharmaceutical formulation was assayed on a C18 column using an isopropanol/buffer mobile phase and detection at 215 nm. A negative peak was observed that did not appear in the original method. The retention time was consistent, and the peak was independent of the type of sample being analyzed. However, the negative peak intensified when methanol was injected, which suggested that methanol was the source of the peak. When manual injections were used instead of the standard autosampler, the problem disappeared.

The source of the problem was traced to the methanol/water wash solvent and the partial loop injection technique used by the autosampler. The 200-µL injection loop of the autosampler was rinsed with methanol/water, but the method called for only 20-µL injections, so the autosampler injected 20 µL of sample plus 180 µL of methanol/water. When a 20-µL loop was used in the autosampler, the problem disappeared. Changing the autosampler wash solvent to mobile phase also could have solved the problem.

This experience calls our attention to the fact that external factors, seemingly unrelated to the actual assay, can often create problems observed in the chromatogram.

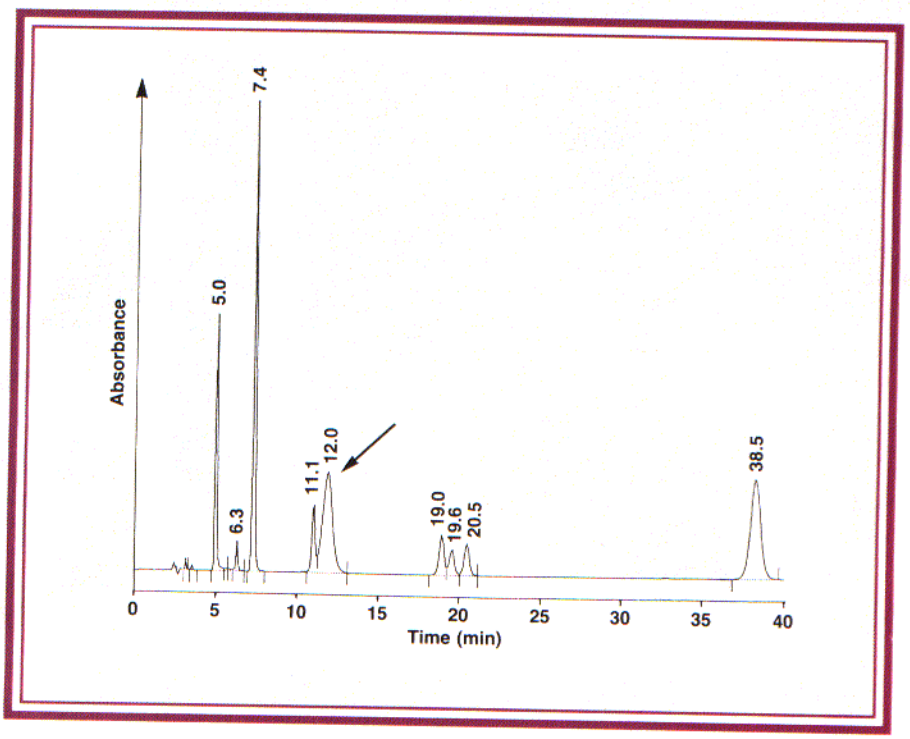


**FIGURE 1: Chromatogram showing interfering "mystery" peak at about 12 min (arrow). Corresponding peak at its true retention time is seen at 38.5 min. See text for discussion.**

### LATE PEAKS

In another column (6), we discussed a simple method to estimate the retention of late-eluting peaks that appear in subsequent chromatograms. A reader (7) submitted an excellent example. The separation shown in the chromatogram of Figure 1 usually was terminated at 25 min, just after the small triplet of peaks. In one set of samples, however, a "mystery" peak at about 12 min was observed (marked with an arrow in Figure 1). This peak was much broader than the neighboring peaks, suggesting that it was a late-eluting band from a previous chromatogram. In reference 6 we suggested that the elution time of such bands can be estimated from the relationship:

$$t_{R2} \approx t_{R1} \times w_2/w_1$$

where $t_R$ refers to the retention time and $w$ refers to the bandwidth of the problem band (band 2) and a reference band (band 1). Let's try this with the chromatogram of Figure 1,

using the band at 19 min as the reference peak. The half-height bandwidth of the problem band is $w_2 = 3.25$ mm, and that of the reference band is $w_1 = 1.5$ mm. (For convenience, I measured the width at half-height, and because the units cancel out, I left them in mm rather than converting them to min.) Thus, $t_{R2} \approx 19 \times (3.25 / 1.5) \approx 41$ min, which suggests that we should let the chromatogram run longer to see if there is a late peak at about 41 min. As we can see in Figure 1, a peak comes out at about 38.5 min. This peak closely matches the appearance of the peak at 12 min, so the problem band has been located.

We can solve the problem of the interfering 38-min peak in one of two ways. First, we can let each chromatogram run for 40 min. This is a simple solution, but if we are analyzing more than just a few samples, adding an extra 15 min to each assay has little appeal. An alternative solution is to adjust the injection time so that the band elutes in an unimportant region of the chromatogram. In this example, shortening the injection cycle by three minutes will solve the problem. Stopping the chromatogram at 22 min rather than 25 min would allow the last triplet of interest to elute completely, and the problem band to elute three minutes later in the next chromatogram, or at about 15 min. Because this region has no peaks, no problems should occur. Thus, by shortening the injection cycle time, we've reduced the overall run time and simultaneously solved the problem of the late-eluting interference.

## LEAKY COLUMN

**Q:** Recently, I replaced a column-inlet frit because I suspected that the old one was causing high system back pressure. Now, however, the endfitting leaks. Tightening the nut even more does not solve the problem. What could be causing this leak?

**JWD:** The primary cause of column leaks of this nature is a contaminated sealing surface. When you replace a frit, it is easy to get a few particles of packing material on the ferrule or inside the endfitting on the tapered cone where the ferrule seals. It takes only a few particles to cause a leak, so before you reassemble the column, be sure to rinse the fitting thoroughly with solvent and wipe the ferrule with a damp paper towel. In your case, take the fitting apart, clean it, and reinstall it. I expect that the problem will be solved.

An alternative cause of the problem could be using a frit that is too thick. An overly thick frit acts as a spacer and prevents the ferrule from seating properly in the cone. If this is the case, you can tighten the nut all you want and the ferrule will never seat properly. Although this problem is not as common as the contaminated seat, the wise chromatographer should verify, every time a frit is replaced, that the replacement frit has the same thickness as the original.

Another good practice to be aware of when you change column frits is holding the column properly: it is best to use a vise or other mechanical grip, rather than your hand. This minimizes the chances of dropping the column while it is open and, more importantly, keeps the column at a constant temperature. When you hold the column, the heat conducted from your hand can warm the column enough to cause problems. As the column warms up, the packing expands, and with the fitting removed, packing will extrude like toothpaste. This irreversible process can ruin the column, so be careful handling the column during this critical operation.

Finally, the risk and hassle of changing the column frit can practically be eliminated by the use of an in-line filter. A 0.5-μm porosity frit will trap any particulate matter before it can reach the column frit and cause problems. In-line filter frits are easy to change and create no risk to your column packing. Guard columns also help to protect the analytical column from chemical contamination and blockage due to particulate matter.

## MIXER PROBLEMS?

**Q:** I recently changed the motor in our LC mixer from 1/600 hp to 1/500 hp. All other parts of the protocol remained unchanged, yet the retention time of the standard increased by about 4 min. Does the speed of the mobile phase mixer affect retention time?

**JWD:** The mixer's speed should not affect the retention time to the degree that you observe. In some mixer designs, I suppose that mixing speed affects the uniformity of the mobile phase, and thus would change the baseline noise, but it would have little or no effect on retention. Furthermore, you have changed the strength, but not necessarily the speed, of the motor. The new motor that you used is stronger than the old one, so there is no risk of stalling due to insufficient power. You should check to be sure that the speed of the new motor (this should be written on the motor casing — for example, 60 rpm) is the same as that of the old motor.

Large changes in retention, such as you observed, typically are caused by changes in the flow rate or in the mobile phase composition. You can check the flow rate volumetrically by doing a timed collection of the column effluent in a graduated cylinder. If the flow rate is too low, check the pump settings; also, check carefully for leaks. Air in the pump also can cause both a low flow rate and low or fluctuating system pressure.

You can check for changes in the mobile phase composition by mixing up a new batch of mobile phase or by checking the capacity factors of the bands in the chromatogram. Capacity factor, $k'$, is calculated as

$$k' = (t_R - t_0) / t_0$$

where $t_R$ and $t_0$ are the retention times of the peak of interest and of an unretained peak (column dead time), respectively. The $k'$ should remain constant if the mobile phase is unchanged, even if the flow rate changes. If changes in $k'$ are observed, a change in mobile phase composition is the most likely cause. Other possible candidates are a change in the column chemistry (by contamination or aging) or a change in the temperature of the column.

## pH ADJUSTMENT

**Q:** We use several reversed-phase liquid chromatographic methods that were developed in other laboratories. Several of these require adjustment of the mobile phase pH *after* addition of the organic component. We usually specify adjustment *before* addition of the organic component. Is one method preferred over the other?

**JWD:** You should always adjust the pH of a solution before the organic component is added. Even if the pH of an organic/water solution had any useful meaning, it is unlikely that you could get a good reading from a pH meter that uses electrodes designed for aqueous solutions.

Another often-overlooked aspect of pH adjustment is the ionic strength. In many separations, the ionic strength of the mobile phase is important in determining retention. It is possible to unknowingly change the mobile phase ionic strength by using the wrong pH-adjustment technique. For example, to make a 20 mM phosphate buffer at pH 4.0, one lab may make a 20 mM solution of $KH_2PO_4$ and then adjust the pH to 4.0 with phosphoric acid. A second lab may make the "same" buffer by adding a solution of 20 mM phosphoric acid to a solution of 20 mM $KH_2PO_4$ until the desired pH is reached. It is easy to see that the second lab obtained a true 20 mM phosphate buffer, whereas the first lab's buffer was greater than 20 mM because of the addition of concentrated acid — even though both buffers might be labeled "20 mM phosphate, pH 4." In other words, you should make both the acidic and basic components of your buffer at equimolar concentrations and then mix them to obtain the proper pH. One additional pointer for good method development practice: always report exactly how your buffer was prepared so that the next user can prepare it properly.

## REFERENCES

(1) J.W. Dolan, *LC•GC* **7,** 102–106 (1989).
(2) M.C. Harvey and S.D. Stearns, *J. Chromatogr. Sci.* **20,** 487 (1982).
(3) J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems* (Humana, Clifton, New Jersey, 1989), Chapter 10.
(4) D.A. Williams, personal communication, 1989.
(5) J.W. Dolan, *LC•GC* **7,** 316–318 (1989).
(6) J.W. Dolan, *LC•GC* **7,** 384–387 (1989).
(7) D.C. Lommen, personal communication, 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.