LC Troubleshooting

Odds and Ends from the Mailbox

John W. Dolan

This month, John Dolan answers questions about column flushing, recycling and choosing mobile-phase solvents, unexpected peaks, and rust on frits.

Time to clear out my Column Ideas basket. When readers send me questions and comments, I try to answer them immediately. Of course, it takes at least a month before my answer appears in print (it is near the end of January as I prepare this column). Often, reader input sparks an idea for a column topic. Other correspondence doesn't fit in immediately so I save it, hoping to plug it into a related column in the future. The following are miscellaneous letters that I've received that didn't fit into a column topic but are worthy of sharing.

COLUMN FLUSHING

A reader presented the following problem: "At the end of a day, after running nearly 50 samples on my liquid chromatography (LC) column, it is time to wash the column and head for home. At the same time I discover that I also need to replace the guard column. Do I wash the column and then replace the guard column, or should I replace the guard column first and then flush?" The reader suggested that the second option is better.

This is a point that many chromatographers commonly overlook. If you flush a used guard column while it is connected to the analytical column, you are flushing contaminants from the guard column onto the analytical column. These contaminants may or may not pass through the analytical column, and you can unintentionally contaminate the main column. The reader is right in suggesting that the old guard column be discarded first.

But what about when the guard column is used but still in good operating condition? The safest procedure is to disconnect the outlet of the guard column and flush it directly to waste, then flush the main column independently. However, this procedure is probably too cumbersome for most workers. It involves disconnecting the guard outlet, flushing, disconnecting the guard inlet, connecting the main column inlet, flushing, then reconnecting all the fittings.

Another technique involves disconnecting the guard outlet and flushing it with 10 column volumes of solvent. For example, a 3 cm × 4.6 mm guard column has a volume of approximately 0.3 mL. Because of low back pressure, the column could be run at 3 mL/min without problem. Flushing with 10 column volumes would require only 1 min under these conditions. After the initial quick-flush is completed, rejoin the guard column and main column and flush in the normal manner.

Generally, five column volumes of solvent is sufficient to change from mobile phase to a storage solvent. To remove contaminants, use 10–20 column volumes of a strong solvent (for example, acetonitrile). For a 25 cm × 4.6 mm column coupled to a guard column, the total volume is approximately 3 mL. Flushing for about 20 min at 2 mL/min should do the job nicely.

MOBILE-PHASE RECYCLING PROBLEM

One reader reported that when he recycled the mobile phase, the baseline increased and the peak heights decreased. The separation maintained a straight baseline and constant peak heights when the mobile phase was not recycled. He recycled the mobile phase by returning the column effluent to the constantly stirred mobile-phase reservoir. Using an internal standard failed to correct the problem. The method used a fluorescence detector with excitation and emission set at 283 and 330 nm, respectively. Throughout 9 h of operation, running 30 samples at 15 min each, the baseline increased by approximately 0.5 mV per run, and the standards' peak heights decreased steadily to nearly 80% of their original value.

The solution to this problem was simple — the user quit recycling the mobile phase. I wish all problems were that simple to correct. The gradual baseline increase is not too surprising for recycled mobile phase. As fluorescing contaminants build up in a system, baselines will rise gradually, as the user observed. I suspect that the loss of signal intensity over time was the result of fluorescence quenching. That is, as the mobile phase became loaded with recycled samples, it created conditions that quenched the fluorescence of the target compound, resulting in a shorter peak.

An alternate solution to abandoning recycling might be to use one of the commercial LC solvent recyclers. These devices comprise a switching valve and an adjustable electronic switch. The switch monitors the detector signal, and when the signal drops below a set threshold, the solvent is returned to the reservoir. When the signal exceeds the threshold, the eluent is directed to waste. Thus, only clean solvent gets recycled.[1] Depending on the complexity of the chromatogram and the threshold settings on the recycler, 50% or more of the mobile phase could be reused.

WHY MORE THAN ONE SOLVENT?

Most LC column manufacturers publish numerous applications notes discussing how given columns can be used for analyzing particular samples. After studying some of these separations, one reader observed that retention order didn't vary after changing mobile-phase solvents. For example, when methanol–water was replaced with tetrahydrofuran–water, the

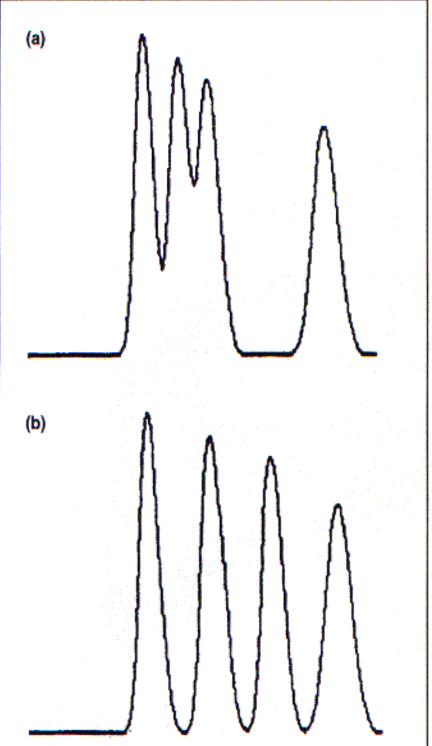

FIGURE 1: Selectivity differences in chromatograms with the same retention range. Shown are (a) marginal and (b) satisfactory separations.

peaks appeared in the same order for all the observed separations. The reader asked, "Why not use one organic solvent and adjust the water content to control retention?" Unfortunately, these conclusions are based on a sample set that was too small, and the reader's decision is similar to that of the blind man who characterized an elephant as tree-like after feeling one of its legs. Let's take a moment and examine some reasons to choose one solvent instead of another.

First, selectivity *does* change when one solvent replaces another. These subtle changes usually affect peak spacing. Occasionally, however, retention order reverses when one organic solvent is substituted for another. Figure 1 shows the importance of selectivity changes. For both chromatograms, the retention envelope for the four peaks is constant, but the resulting separation is dramatically different. In one case, the peaks are baseline resolved, whereas the other separation yields baseline resolution for only one peak. Converting a marginal separation, such as in Figure 1a, to a satisfactory one, as in Figure 1b, can be very time consuming. Chromatographers can try using additives, adjusting the pH, and changing solvent type to improve separations. Unfortunately, when one solvent is substituted for another, the result is unpredictable. We know that a change probably will occur, but we cannot predict what that change will be.

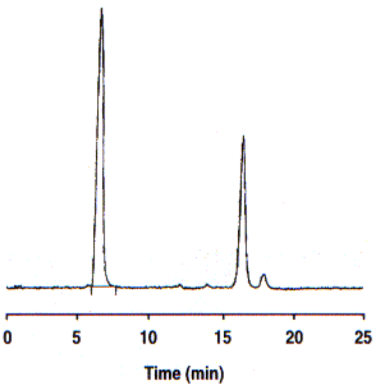

FIGURE 2: Chromatogram with unexpected contaminant.

The physical properties of a solvent are also important. Many workers prefer acetonitrile as the organic solvent for reversed-phase separations. Acetonitrile has a low UV-cutoff wavelength, low viscosity, and good stability. Tetrahydrofuran, on the other hand, must be used at higher wavelengths, creates higher back pressures under the same conditions, and is chemically unstable. These and other solvent characteristics have been covered in recent "LC Troubleshooting" columns (1,2).

INTERFERING PEAK?

Unexpected chromatogram peaks have a way of sending chills up the spines of most chromatographers. Figure 2 shows a chromatogram that was sent to me by a reader. The chromatogram normally included the large sample peak at approximately 7 min and the small unknown peak at approximately 18 min, but one day the 17-min peak appeared. The reader suspected that either the sample was contaminated or the sample matrix had changed, and she tried to isolate the problem. In the meantime, she had to continue running samples. Her boss was concerned that the new peak would compromise the quantitation of the sample peak.

When we study the influence of peak overlap, we usually assume that the peak has a Gaussian shape. This assumption does not account for peak tailing, but it is a good place to start. We calculate resolution (R_s) as

$$R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2) \qquad [1]$$

where t_{R1}, t_{R2}, w_1, and w_2 are the retention times and baseline peak widths of the first and second peaks, respectively. For example, the peaks of Figure 1b have a resolution of approximately 1.5. When the resolution is 2, the peaks are well separated with a baseline of nearly half a peak width between them. In this case, a peak overlap of less than 0.1% occurs. This overlap means that the error in peak area is also less than 0.1%, which is insignificant for most assays.

In the chromatogram of Figure 2, the peaks are spaced by more than seven peak widths, indicating that no interference is present. From a quantitative standpoint, the results of the assay should be valid even with the new problem peak present. New peaks in a chromatogram always make me nervous, so I would not ignore this problem — it is much easier to explain the results to a client if you know the source of the new peak.

RUSTY FRITS

A reader reported rust collecting on an in-line frit in the LC system and wondered how to avoid or reverse the rusting process. The solution to this problem is called *passivation.* Stainless steel, which is used widely in LC system hardware, has exposed iron inclusions at the surface of tubing or machined parts. Passivation is the process of removing the exposed iron with nitric acid. The passivated surface is much more inert and less susceptible to corrosion or chemical interaction with samples. To my knowledge, all LC systems are passivated as a part of the manufacturing process. Tubing that is purchased in precut lengths is also passivated. The passivation process is simple and was discussed in an earlier "LC Troubleshooting" column (3). Here is a summary:

Before you start, take the normal precautions for working with acids (rubber apron, face shield, gloves, and so forth). Consult the operation manual for each LC system component to see if you need to take special precautions or if you should avoid passivation altogether. Remove the column from the system and replace it with a piece of tubing. Flush the system with approximately 30 mL of HPLC-grade water to remove any traces of mobile phase (if a water-insoluble mobile phase is in the system, flush it with isopropanol first). Flush the system with approximately 30 mL of 6 N nitric acid. Follow the acid flush with approximately 100 mL of water, using at least three separate washes. Check the pH of the system effluent and flush until the pH matches that of the water at the inlet. Although it may be unnecessary, I recommend changing the pump seals after passivating the system. Reinstall the column and equilibrate with mobile phase, and you should be back in business.

REFERENCES

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"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, USA, and a member of the Editorial Advisory Board of LC•GC. *Direct correspondence about this column to "LC Troubleshooting,"* LC•GC, *859 Willamette Street, Eugene, OR 97401, USA.*