LC Troubleshooting

Reader Questions

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The daily operation of an LC system can create perplexing problems. Trivial problems for one operator can be roadblocks for another.

Liquid chromatography (LC) can be applied to a variety of problems. Some workers use LC as a tool for quantitative and qualitative analysis. For these analysts, LC is the centerpiece of their job function. For others involved in organic synthesis, LC is merely a tool for monitoring a reaction. Some mass spectroscopists, for example, consider LC a sophisticated cleanup technique. With such diverse applications, it is not surprising that problems in one application may never occur in another. This month we examine a smattering of puzzling problems that readers brought to my attention.

MEASURING COLUMN DEAD TIME

Question: You often mention the importance of calculating retention factors, k, for a separation. The retention time is easy to measure, but I'm unsure of how to measure the dead time, t_0. It seems that there is much room for error. What do you recommend?

John W. Dolan: The column dead time, as you are aware, is the time that it takes something to pass through a column if it does not interact with the column's stationary phase. I like to use the garden hose analogy to illustrate t_0. If you hook up an empty garden hose to a faucet and turn on the water, a certain amount of time passes before any water comes out the end. The same thing happens in the column. Unretained materials require a minimum amount of time to pass through the column — this is the dead time.

Many techniques will measure dead volume — just take a look at the literature and you will see that analysts don't agree on any one technique. However, a few practical techniques will work well for determining t_0. The easiest way to obtain a value for dead time is to estimate it. For the standard 4.6-mm i.d. column, you can use the estimate

$$V_m \approx 0.1L \quad [1]$$

$$t_0 = V_m/F \quad [2]$$

where V_m is the column dead volume, L is the column length (in centimeters), and F is the flow rate (in milliliters per minute). Thus a 25-cm column would have a dead volume of approximately 2.5 mL and a dead time of 1.25 min at a flow rate of 2 mL/min. This estimate is good within approximately 10%, which is fine for most method-development and troubleshooting applications.

An alternate calculation for columns of other diameters is

$$V_m \approx 0.5Ld_c^2 \quad [3]$$

where d_c is the column internal diameter (in centimeters). For example, a narrow-bore 15 cm × 2 mm column operated at 1 mL/min will have a dead time of 0.3 min.

An alternative to calculating dead times is to determine them using the chromatogram. Figure 1 shows two possible t_0 disturbances. Figure 1a shows a refractive index disturbance, which is characterized by a negative and positive deflection. Measure t_0 at the point where the signal crosses the baseline in this deflection. More often you will see a large peak, as in Figure 1b. In this case, the best measure of dead time is the point at which the signal first rises above the baseline. Once you obtain this measurement, you can roughly verify it using one of the two calculations mentioned above.

Be aware, however, that t_0 is not necessarily the first disturbance in the baseline. The dead volume represents the total volume inside the column accessible to the mobile phase, including the volume inside the pores but not the space occupied by the packing particle structure. If you inject a compound that cannot enter the pores, it will be eluted before t_0. When would this happen? If you have conditions in which ion exclusion takes place, such as when

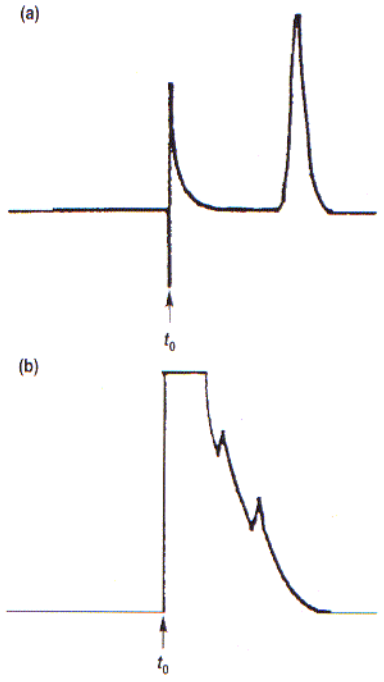

FIGURE 1: Measurement of column dead time, t_0, from chromatograms containing (a) a refractive index disturbance and (b) a large initial peak. (Reprinted with permission from reference 1.)

negatively charged sample molecules cannot enter the pores of a negatively charged stationary phase, analytes can be eluted before t_0. In a size-exclusion separation, t_0 is the last peak in the chromatogram. Only the smallest molecules have full access to the pores in size exclusion, so they will come out last at t_0.

WASHING WITH WATER

Q: The mobile phase for my separation comprises 37% acetonitrile and 63% 40 mM phosphate buffer, pH 3.5. I'm using a C18 column at 1 mL/min. At the end of the day, I wash the system with water to remove the buffer, then switch to acetonitrile and leave the system for the night. One of my coworkers told me that I should never wash a column with water. Is this correct?

JWD: The answer is no — and yes. Although you are unlikely to harm the column by washing it with water, I would use straight water in a reversed-phase column only if I had a specific reason. Two points are important to keep in mind.

First, in 100% water the C18 phase collapses, trapping some of the acetonitrile–buffer mobile phase. (You can find more information about this phenomenon in reference 2.) A water wash intended to remove the buffer residue could actually trap buffer so it would not wash off the column.

A second complication is that reequilibration takes longer after the column has been subjected to 100% water. This reequilibration time is the reason most workers do not use less than approximately 5% organic in their mobile phases.

So how do you wash out the buffer? My recommendation is to replace the buffer portion of the mobile phase with water for the initial washout. The buffer is soluble at this organic level or you wouldn't be using the mobile phase in the first place. In your case, you should use 37% acetonitrile plus 63% water (instead of buffer). Wash with approximately five column volumes of this mobile phase to remove the buffer. According to equation 1, this volume would be $(0.1 \times 25 \times 5) \approx 12.5$ mL for your system. Remember that whenever you are flushing or equilibrating, it's the volume, not the time, that is important, so you could cut the wash time in half by doubling the flow rate if the pressure is reasonable.

After you've removed the buffer, you can safely change to conditions that will strip strongly retained materials from the column. For reversed-phase conditions, this means increasing the organic in the mobile phase. Your acetonitrile wash will work fine for this purpose.

The only precaution is to be careful when switching from 100% acetonitrile to your acetonitrile–buffer mobile phase. You should never create conditions that will precipitate the buffer. Acetonitrile is notorious for causing buffer-solubility problems.

HOW LONG DOES HELIUM SPARGING LAST?

Q: I degas my mobile phase off-line using helium sparging, then I move the degassed mobile phase to the LC system for use. How long must I operate the helium sparger to adequately degas the solvent? How long will the degassing be effective?

JWD: Degassing effectiveness depends largely on the LC system you are using. Some systems operate reliably without degassing, whereas others are unreliable unless you continuously degas the mobile phase. However, every LC system that I know of will operate more reliably if you degas the mobile phase.

First, let's examine adequate degassing. For helium sparging of reversed-phase mobile phases, such as methanol or acetonitrile mixed with water or buffer, only one volume of helium is required to fully degas (remove 99% of the air) an equal volume of mobile phase (3). This means that 1 L of helium bubbled through 1 L of mobile phase is satisfactory, assuming the helium is well distributed. For practical purposes, a vigorous 3–5 min helium sparge is probably sufficient to fully degas the mobile phase.

How long will the mobile phase remain in a degassed state? Obviously, air will begin to diffuse back into the mobile phase as soon as sparging is stopped. As I mentioned earlier, the degassing requirements vary with each LC system, but many workers degas batchwise once a day and operate their LC system satisfactorily.

INJECTION PROBLEMS

Q: I am using a size-exclusion method to quantify several polymers present in a production stream. I use methylene chloride as a mobile phase and as a sample solvent. I program the autosampler to inject 500 µL of the 1-mL sample contained in each 1.5-mL sample vial, but I can't seem to get the injector to work properly. It injects approximately 350 µL even when it is programmed to inject 500 µL. The sampler works well with other samples prepared in aqueous injection solvents, so I know it is working properly. What's the problem?

JWD: I think the problem is the result of cavitation, the vaporization of the methylene chloride during injection. When the syringe attempts to remove 500 µL of sample, it is removing approximately half the sample from a vial with very little headspace. If the vial is well sealed, this could create a partial vacuum in the vial and the syringe. Under these conditions, methylene chloride, which is quite volatile, may vaporize, filling part of the syringe with vapor instead of liquid. Even though the syringe drew 500 µL, less than 500 µL of sample would enter the syringe because of the bubble of methylene chloride vapor.

You could test this theory by injecting a sample from a vial with a loose cap. This approach, however, would not offer a permanent solution to the problem because a loose cap

would allow methylene chloride to evaporate while the sample sat on the sample tray.

Several approaches could correct this problem. Using a larger sample vial (for example, a 3-mL vial) would create a larger headspace and lessen the vacuum problem because a smaller portion of the total vial volume would be removed. You could also use a slower syringe speed during sampling if the autosampler has a syringe-speed adjustment. This might reduce the cavitation problem.

In any event, it would be wise to add an internal standard to your sample. With a sample solvent as volatile as methylene chloride, some evaporation is inevitable, so sample concentration will change. An internal standard allows you to correct for solvent evaporation and injection-volume errors, which are proportional for both the sample compounds and the internal standard.

ABNORMAL FIRST INJECTION

Q: I am running a method for the analysis of aflatoxins in corn. The method requires a fluorescence detector for adequate lower detection limits. Each day when I start making runs, the fluorescence response of my sample compounds is low for the first injection of sample or standard, then it increases and is consistent for the rest of the day. The method seems to work, but I cannot use the first injection. What is happening here? Can I do something to correct this problem?

JWD: Although I have not seen this problem for fluorescence, similar problems are fairly common in LC analyses. Commonly, retention time changes a little for the first few injections and then stabilizes. Both of these problems are caused by the heterogeneity of the packing surface. Retention shifts occur because more than one mechanism enables sample retention. For example, the primary retention mechanism might be reversed-phase interactions with a C8 bonded phase, but there are secondary interactions with exposed silanol groups on the silica surface. We generally like to have a single retention mechanism so that response curves are linear and peak shapes are symmetrical. One way to correct the problem is to add something to the mobile phase to overload the unwanted reactive sites. When exposed silanols are a problem, we often add triethylamine to the mobile phase to swamp out these secondary interactions.

Response or retention changes within the first few injections are the result of deactivation of some secondary interaction site. In other words, something in the sample is sticking very strongly to those secondary interaction sites. After those sites are totally blocked, the primary interaction site is predominant, and the behavior of the system stabilizes. In your case, something on the column is quenching the fluorescence of your compound. When those fluorescence quenchers are overloaded, the signal stabilizes.

It is fairly common to intentionally load up the column at the beginning of a series of runs to overload unwanted reactive sites. For example, I have used methods for serum analysis requiring several injections before retention stabilizes. To overcome this problem, I inject a large blank serum sample. If the normal injection volume is 10 µL, I might inject 50 or 100 µL of serum. The components of this first injection effectively block the column's problem sites so that it behaves normally. I have also used a protein analysis method that requires the initial injection of a large sample of bovine serum albumin to overcome unwanted interactions.

Your observation of changing fluorescence is consistent with the overloading of a secondary interaction site. If it were my method, I would inject a big slug of sample before I started running samples or standards of interest. This sample could be a matrix blank or leftover sample from previous runs.

I am interested in hearing about ways readers have successfully combatted fluorescence quenching. I can be contacted in care of *LC•GC*.

REFERENCES

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