LC TROUBLESHOOTING

Readers' Questions

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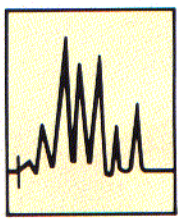

This month's "LC Troubleshooting" covers some readers' problems with the chromatographic baseline, an indirect photometry application, and a detector cell. At one time or another, we all may encounter the baseline problem discussed in the first question, especially if we assay labile compounds. Indirect photometry is used by a minority of chromatographers, but the answer to this question reminds us of the problems that can occur when we work in a marginally linear region of detector response. The final question is an odd one that illustrates the fact that the properties of the peaks eluting from the column may not be the same as those of the bulk sample that was injected.

MYSTERY HUMP

Q: I see a rather broad hump in the baseline just after the solvent peak, and this hump gets larger throughout the day as more injections are made. I'm running an isocratic method that has been working well for several years. The mobile phase is acetonitrile–water with phosphate buffer (pH ≈ 3) at 1 mL/min. I'm using a 15-cm C8 column and operating the detector at 254 nm. What could be causing this hump?

JWD: Unexpectedly broad peaks of unknown origin in liquid chromatographic (LC) separations can come from several sources. These include sample or mobile phase contamination, late-eluting components from previous injections, and injection effects. My guess is that sample contamination is the source of your problem, but let's look briefly at each of the possibilities.

Sample contamination can be introduced by a change in the sample source or in the sample preparation procedure, or can result from sample degradation. With a broad band that grows with time, as in your case, it is wise to check for sample degradation. Sample degradation products can be a poorly defined, chemically similar group of compounds that have similar, but not identical, retention times — hence the broad peak. The fact that the size of this broad band grows with time further supports the sample degradation theory. You can check sample stability by loading an autosampler tray with identical samples and injecting a normal day's sample load. If degradation is a problem, you should see regular changes in the chromatogram as the sample degrades over time. You can address the sample degradation problem in a number of ways, depending on the nature of the sample. Air-sensitive samples may require sample vials that are topped off with inert gas. You may need to use amber vials with light-sensitive samples. Temperature-sensitive samples may require that you use a cooled sample tray or locate the LC system in a cold room. If microbial degradation is the source of the problem, filtering the sample through a 0.2-μm membrane filter or adding organic or sodium azide may prevent or slow down the problem. In some cases, it will be most convenient to prevent the problem by loading just a few samples at a time into the autosampler, refilling the tray with fresh samples every hour or two.

I think that sample degradation is the most likely source of the baseline hump. The fact that you see it now, but didn't earlier, suggests a change in the nature of your sample (caused by, for example, a different sample source, coadministered drugs, or changes in sample preparation procedures).

Late-eluting components from previous injections are a common source of broad peaks. For example, if the sample has a band that elutes in 12 min, and you inject a new sample every 10 min, the peak will not show up in the first chromatogram because you injected the second sample before the 12-min peak eluted. Instead, the peak elutes 2 min into the second chromatogram. Because the peak has a true retention time of 12 min, it is much broader than expected for a 2-min peak (remember that in isocratic separations, the bands get wider the longer they are retained). If you are using an autosampler, the retention of this late peak will be very regular because the timing cycle of the autosampler is quite precise. With manual injections, on the other hand, the injection cycles tend to be somewhat unequal, so the band will move around in the chromatogram in which it appears even though its true retention time is constant. To check to see whether this is your problem, extend the injection cycle time so that the band has a chance to fully elute before the next injection is made. Sometimes these late peaks don't show up until several chromatograms later (for example, a 36-min peak in the present example would elute in the third chromatogram). You should be able to judge the approximate retention time of the late-eluting compound by using this relationship:

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

where t_R is the retention time, and w is the bandwidth for the two bands. For band 1, choose a band of known retention in the chromatogram. The problem band is band 2. To illustrate this, let's use a problem band with a bandwidth of 1.0 min and a known band with a 3-min retention time and a 0.15-min width. We can calculate that $t_{R2} \approx 3 \times 1.0/0.15 = 20$ min. Although this retention estimate is not exact (because the column plate number varies somewhat with t_R), it will let you correlate the peak with the proper injection.

Once you have determined the retention time of a late-eluting band, you should be able to adjust your injection cycle so that the band is completely eluted before the next injection or so that the band elutes in an unimportant region of a later chromatogram.

Using the wrong injection solvent can cause all sorts of unexpected peak shapes, especially at the beginning of the chromatogram. Most of us are used to the t_0 disturbance (often called the "solvent peak"), but the injection solvent can have other effects as well. If the injection solvent is different from the mobile phase and the volume is large (for example, >50 μL), changes early in the chromatogram can be expected. Large volumes of injection solvent stronger than the mobile phase cause the worst problems because they momentarily increase the mobile phase strength, often resulting in peak distortion or peak splitting. Generally, small volumes (such as <20 μL) of weak solvents (say, <50% of the mobile phase strength) are safe, but there are no hard-and-fast rules. For example, with ion-pair chromatography, injection in anything but the mobile phase is likely to cause problems. Similarly, with silica columns, if the injection solvent is not the mobile phase, the solvent can disturb the activation of the column, causing peak distortion or retention problems. Injection solvent problems are easy to check for — just dissolve the sample in the mobile phase, inject it, and see if the problem goes away.

Injection solvent problems usually are not very reproducible, so you wouldn't expect repeated injections to produce identical chromatograms. But because there is no buildup of anything in the sample or on the column, you wouldn't expect the hump to grow with time. Therefore, it is not likely that injection solvent is the source of your problem.

Contaminated mobile phase can result in a buildup of strongly retained material on the column, which may or may not be coupled with a gradual increase in the level of the baseline. Because the level of contamination is more or less steady, no peaks will be seen in the chromatogram. Eventually, the column will become saturated with the mobile phase contaminant. At that point, anything entering the column that disturbs the equilibrium may displace some of the built-up contamination, which is seen as a peak in the chromatogram. If the displacement is by a sample peak, the contaminant peak would have a normal peak shape, but if the injection solvent is displacing the contaminant, a broad or distorted peak at t_0 is possible. Buildup of mobile phase contaminants can sneak up on you, especially when you use isocratic separation and do not regularly flush the column with strong solvent. In many cases, the level of contamination is so small that the problem can be ignored if the column is flushed with strong solvent (acetonitrile in your case) at the end of each day's use. The contaminant may come from your acetonitrile (not very likely with HPLC-grade solvents), the water, or the buffer. If you suspect that this is your problem, you should systematically substitute known good mobile phase reagents for the ones that you are using and observe any differences.

VARIABLE SENSITIVITY

Q: I am measuring anions by ion-pair chromatography on a C18 column, using tetrabutylammonium hydroxide (TBA) as the ion-pairing agent. I adjust the pH of the mobile phase with potassium hydrogen phthalate, which also allows me to detect my sample components using a UV detector. The problem is that the sensitivity of my assay varies — on some days I have no problems detecting the peaks, and on other days the peaks are so small that I can hardly see them. What could be causing this?

JWD: You are using the technique called "indirect photometry" or "vacancy chromatography" to allow you to detect non–UV-absorbing compounds. This works by the use in the mobile phase of a UV absorber (phthalate) that generates a high background signal. When a non–UV-absorbing sample component elutes from the column, it lowers the concentration of UV absorber in the detector and thus gives a negative peak with a UV detector. This is a clever detection technique, but it is susceptible to sensitivity problems if you are not careful.

Most UV detectors for LC are linear only in the range from 0 to 1 absorbance unit (AU). Some models are claimed to be linear beyond this range, and some are nonlinear below 1 AU. By linear, we mean that a plot of detector response vs. amount of sample injected is a straight line, passing through the origin of the plot. Most workers do not like to use UV detectors much above 0.6 or 0.7 AU because of uncertainty about linearity above these levels. When a detector is operated in a nonlinear region, the response per unit mass of sample is less than it is in the linear region, and the reproducibility also may be worse.

Whenever the background level of the detector is raised because of UV-absorbing components in the mobile phase, the effective linear range of the detector is reduced. For example, if the mobile phase creates a 0.2 AU background, a detector with a normal 0–1 AU linearity region will have a linear range of only 0.8 AU. This is true even though you can zero out the baseline signal on your integrator. With indirect photometry, you have to be careful about the detector linearity, just as you are with direct detection. Adding so much UV absorber to the mobile phase that the detector is in the nonlinear region will cause the detector response to any sample peak, positive or negative, to be nonlinear.

How can this explain the irreproducibility that you observe? As an example, let's assume that your detector is linear only to 0.8 AU. If you add sufficient phthalate to generate a 0.75 AU background, you would expect the response to be satisfactory for your system. But a new batch of mobile phase with a little more phthalate might raise the background above 0.8 AU, resulting in nonlinear (irreproducible) response. Two possible sources of error might cause this problem. First, you may not be adjusting the pH properly. You should adjust the pH of the aqueous solution *before* you add any organic to the mobile phase. Some workers try to adjust the pH after adding organic, but pH meters are not designed to work properly under these conditions. As a result, different amounts of acid or base may be required to reach the "same" pH on repeated experiments. Second, small changes in the amount of TBA or other additives can mean that more or less phthalate is required to reach the desired pH. Different amounts of phthalate, of course, result in different background levels, which may move the detector into or out of the linear region.

I recommend a threefold cure. First, reduce the level of phthalate in the mobile phase to be sure that you are operating well within the linear range of your detector (for example, 0.5 AU or less background absorbance). Second, add a fixed amount of phthalate to the mobile phase and finish adjusting the pH with an appropriate acid or base. This will assure you of the same background absorbance from batch to batch. Finally, be sure to adjust the pH of the mobile phase before adding organic, so that you get a true pH reading. With these changes, I expect that

your method will be much more reproducible. Keep in mind, however, that many workers find that ion-pairing and indirect photometry are inherently less reliable than straight reversed-phase separations with standard UV detection. This means that it is normal to encounter more problems with your method than with standard reversed-phase applications.

DETECTOR CELL PROBLEMS

Q: I work in an industrial quality-assurance lab where we regularly assay various adhesives to be sure that they meet specifications. I was running a new lot of material and noticed that the UV detector response steadily deteriorated. Flushing the system with strong solvent did not help. After a number of other attempts at fixing the problem, I replaced the detector cell, and the system returned to normal. The windows on the old cell seemed to be cloudy, whereas the new cell had clear windows. The samples were a new formulation of adhesive that had the same stability and cure properties as previous formulations, although the chromatograms showed that it contained several extra peaks. What could have caused this problem?

JWD: This reminds me of a problem I encountered several years ago when I tried to assay a UV-curing adhesive using LC. The bulk sample contained an inhibitor that prevented the adhesive from curing in normal room light, but allowed it to cure under direct UV radiation. When the sample went through the LC column, the inhibitor and the adhesive were separated. When the adhesive reached the UV detector cell — you guessed it — instant setting occurred. You may be experiencing a similar problem, in which one or more of the sample components is polymerizing in the detector cell, clouding the windows. You might be able to get around this by using a higher wavelength or another detection technique (for example, refractive index detection). Or maybe this is one of those times when a gas chromatographic method would work better than LC.

If you have questions or problems you've encountered during routine LC work, you're invited to share them. Also, share your shortcuts and rules of thumb. Write to The Editor, "LC Troubleshooting," *LC•GC*, P.O. Box 10460, Eugene, OR 97440, USA.

"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.

Erratum: Please note that in the April 1989 installment of "LC Troubleshooting" (*LC•GC* **7**[4], 316–318), an error appears on p. 318. The second sentence of the first paragraph should read: "Thus, you should inject a methanol–water sample solvent rather than acetonitrile–water to avoid any unwanted changes in selectivity . . . ."