LC Troubleshooting

Gradient Problems at Low Detection Wavelengths

John W. Dolan

Gradient methods offer unique solutions to separation problems — but they can also create unique problems.

Gradient elution is used for a wide variety of liquid chromatography (LC) separations. When you encounter a sample with a large polarity range, gradient elution can be the answer to the general elution problem. Figure 1a shows an example of the general elution problem for a mixture of substituted aromatic compounds. The early peaks are crowded near the column dead time (t_0), and the later peaks are broad and flatten out into the baseline. Gradient elution provides a better separation for this sample (Figure 1b).

A quick way to determine whether you need gradient elution is to check the capacity factor (k') ratio between the first and last peaks. A shortcut is to use the ratio of the corrected retention times ($t_R - t_0$, where t_R is retention time) for these peaks. Consider the case for a chromatogram with a t_0 of 2.5 min and first and last peaks eluted at 3 min and 20 min, respectively. The ratio would be $(20 - 2.5)/(3 - 2.5) = 35$. As a rule of thumb, if the ratio is greater than ~20, gradient elution will be beneficial. Gradient elution is commonly used for biological samples, especially when polar metabolites are analyzed in the same run with less polar parent compounds.

In addition to a broad polarity range, many biological samples lack chromophores that can be used for UV detection in the >240-nm region. As a result, low-wavelength UV detection in the 200–220 nm region is the most common technique for detection. However, not only do the compounds of interest absorb at these lower wavelengths, unwanted sample components absorb as well, which results in increased interference and more-challenging separations.

Another problem that analysts often encounter with low-wavelength gradient elution separations is interference from nonsample sources. To have confidence in the results of a separation, you must have confidence that background interference is absent. System-suitability testing before sample runs can resolve any doubts. This month's installment of "LC Troubleshooting" examines some of the areas that need attention in system-suitability testing.

SOLVENT PURITY

The first area to evaluate in a gradient system is solvent purity. We usually use water–acetonitrile gradients for reversed-phase LC separations with low-wavelength detection. (Methanol and tetrahydrofuran, two solvents that are useful at higher wavelengths, absorb too strongly at wavelengths < 220 nm.) Run a blank gradient (without making an injection) under the normal analytical conditions. If any peaks appear in the gradient, verify that the results are consistent by rerunning the blank gradient. If the peaks persist, you need to identify their source and try to eliminate them. What could be the source of peaks in a blank gradient? They could be caused by a solvent impurity, residue from a previous sample, or the equipment itself.

Carryover is easy to eliminate. If you are not making injections, peaks cannot originate from the injector. If the problem is the result of sample residue sticking at the column head and gradually washing off with each successive gradient cycle (an unlikely event), the peak should get smaller with each cycle. If this happens, you may need to implement a special cleaning protocol between samples. Contamination from the system itself is rare, and when it occurs, it generally results from a recent change in the system such as installing incompatible pump seals. Most likely, the extra peaks originate with the mobile phase.

Make up new batches of the A and B mobile-phase solvents and rerun the blank gradient a couple of times. If this solves the problem, you are lucky — modify the method instructions to ensure that you make up fresh mobile phase more often, and you may have seen the last of the problem. (Don't forget to clean the reservoirs occasionally — dirty reservoirs quickly contaminate clean solvents.)

Too often, however, fresh mobile phase does not solve the problem. If the peaks aren't completely reproducible from run to run in terms of retention or peak height, solvent contamination is likely. To check the aqueous phase (solvent A) for contamination, you need to concentrate the solvent relative to the normal conditions. The easiest way to do this is to extend the equilibration phase between gradient runs. For example, if your normal procedure is to run the gradient, drop to starting conditions, equilibrate for 5 min, and then run another gradient, just extend the equilibration phase to 30 min. When the problem is with solvent A, the contaminants tend to collect at the head of the column during equilibration and wash off during the gradient. The size of the peaks should increase roughly in proportion to the equilibration time. When you concentrate the mobile phase like this, you rarely will see a smooth baseline even with the best of reagents. An example of what you might see is shown in Figure 2. The fresh mobile phase shows some disturbances, whereas the contaminated mobile phase looks terrible. Under normal gradient conditions, the good mo-

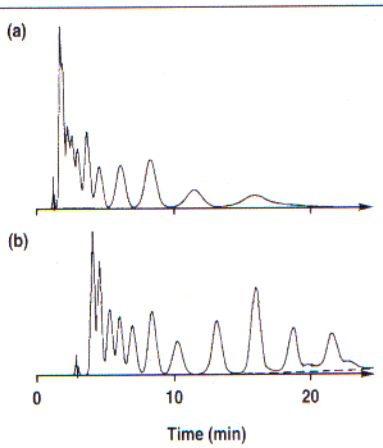

FIGURE 1: The general elution problem. Shown are (a) isocratic and (b) gradient separations of a mixture of substituted aromatic compounds. (Reprinted with permission from reference 1.)

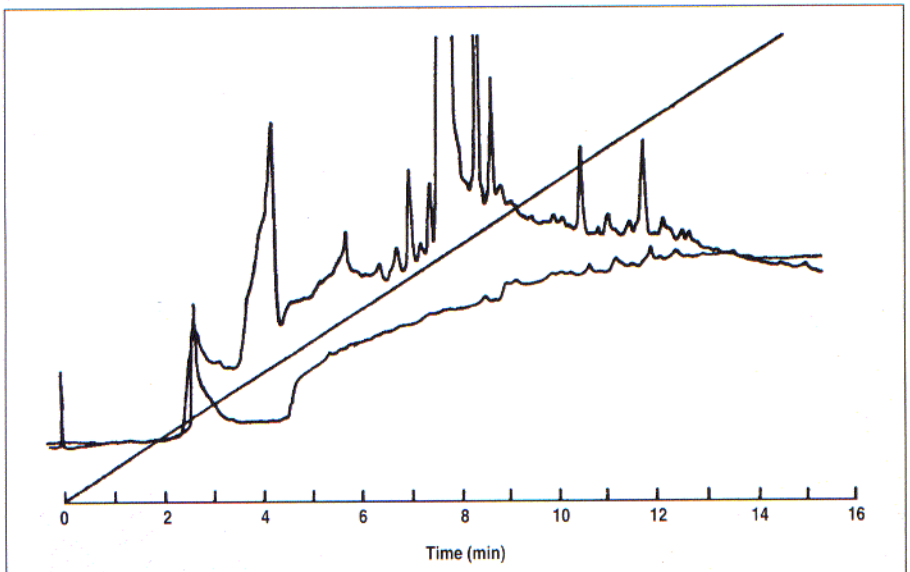

FIGURE 2: Chromatograms from a water-to-methanol gradient with contaminated solvent A (upper trace) and normal solvent A (lower trace). The diagonal line shows the gradient program.

bile phase would provide a smooth baseline, and the contaminated one would show only the larger peaks plus a little baseline noise.

Once you isolate the source of contamination to the A or B solvent, you will need to find which component is causing the problem. You should be using only HPLC-grade reagents and solvents. Use a stepwise method to isolate the problem component by making up a series of A and B solvents, leaving out one component each time. Run blank gradients with these incomplete mobile phases until you find conditions in which the unwanted peaks are missing. When you know which reagent is causing the problem, you can use a fresh bottle of reagent and the problem should be solved. Sometimes you will need to use a new lot of reagent or obtain it from another source.

Common HPLC-grade solvents such as methanol, acetonitrile, and tetrahydrofuran rarely cause problems when obtained from a fresh bottle. Our laboratory has encountered only one batch of contaminated solvents from a manufacturer. We confirmed this by checking the results with a different lot of the same supplier's solvent. Water can be more of a problem, especially if it is purified in-house. The water-purification systems used for the preparation of HPLC-grade water are quite reliable, but you need to perform maintenance regularly. In our company's previous facility, we used deionized water as the feed stock for our water-purification system, and we could go for a year or more between cartridge replacements. Our new facility has city water with rusty pipes, so we have to use a particulate filter, a carbon filter, and an ion-exchange cartridge to purify the feed stock sufficiently. Although the HPLC-grade solvents may be the source of the problem, the source of contamination is more often associated with a buffer or some other mobile-phase additive. Trifluoroacetic acid is one such additive that can be the source of many gradient problems, as discussed below.

A COMMON PROBLEM

In a previous installment of "LC Troubleshooting" (2), I asked for input regarding a problem a reader had with a gradient separation. The reader observed an extraneous peak near the end of a 40–95% B gradient between water (solvent A) and acetonitrile (solvent B) with 0.05% trifluoroacetic acid added to each solvent. I mentioned that our laboratory commonly uses a similar gradient (5–80% B) for separations of biomolecules, and we see a peak if we extend the gradient to 95% B, but we ignore it because all the peaks of interest are eluted before 80% B in our runs.

I received several letters from readers regarding this problem. The common thread was bad trifluoroacetic acid. Each of the readers had experienced a similar problem and had cured it by using fresh trifluoroacetic acid. We have had similar experience in our laboratory. As the trifluoroacetic acid oxidizes with age, some of the degradation products cause extra peaks in the chromatogram. Most users swear by a single manufacturer (not the same one), buying the purest grade of trifluoroacetic acid available. Everyone recommended buying trifluoroacetic acid in 1-mL ampules instead of in bulk. We follow this practice, although when we were using a lot of trifluoroacetic acid we bought the 25-mL bottles, and it did not deteriorate as long as we stored it in the refrigerator and carefully flushed the headspace with nitrogen before closing the bottle. Some readers reported good results as long as they replaced the mobile phase every two or three days, but when trifluoroacetic acid is used in the mobile phase it is best to replace the mobile phase every day.

You will often observe a baseline hump when using trifluoroacetic acid with detection at low wavelengths. This is caused in part by a mismatch in the absorbance of the mobile phases. The problem is minimized at ~215 nm, so the fewest problems will arise at this wavelength. You can also match the absorbance of the mobile phases by adding ~15% more trifluoroacetic acid to the aqueous phase. For example, we often mix 0.115% trifluoroacetic acid in water for solvent A and 0.1% in acetonitrile for solvent B.

Finally, gradient problems will be minimized if you avoid the extremes of the gradient and if you make the A and B solvents as similar as possible. Avoid working with organic solvent concentrations below ~5% unless necessary. When 100% water with additives is used, the environment in the column is completely different than when just a small percentage of organic solvent is present. Reversed-phase columns equilibrate poorly at 100% water. For this reason, I recommend limiting your gradients to 5–100% B unless you have a specific need to use less organic. Also, make up your A and B solvents so they contain the same concentrations of each component except the one you intentionally want to vary. For most gradients, we want to vary only the organic, so the buffer and any additives should be at the same concentration in each solvent mixture. (We violated this recommendation above when we used trifluoroacetic acid to match solvent absorbances, but it did not compromise the separation.) On-line mobile-phase mixing presents the most problems when a small portion of one solvent is mixed with another. For this reason, especially if you limit your gradients to 5–100% B, you may want to premix 5% B into your A solvent for more reliable performance.

SUMMARY

When using gradient elution for your separations, you need to be more careful than when you use isocratic methods. Minor reagent contaminants in isocratic methods may add a little baseline noise and increase the level of the baseline, but they rarely generate peaks in the chromatogram. Gradient elution, on the other hand, tends to concentrate contaminants and then release them as the gradient cycles, yielding interfering peaks in every chromatogram. When working with gradient methods that use low-wavelength UV detection, take extra care to use the best reagents available, flush the system regularly, and replace the mobile-phase mixtures regularly.

ACKNOWLEDGMENTS

Thanks to M.L. Anderson, P. Ellis, K.J. Potter, and J. Schneider for their helpful suggestions about the trifluoroacetic acid problem.

REFERENCES

(1) L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography* (John Wiley & Sons, New York, 2nd ed., 1979).
(2) J.W. Dolan, *LC•GC* **11**(4), 270–273 (1993).

"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, *P.O. Box 10460, Eugene, OR 97440-2460, USA.*