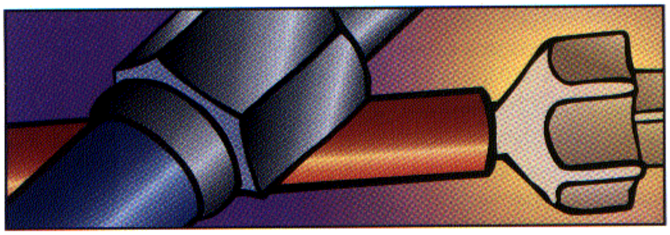
LC Troubleshooting



Problems with Gradient Methods

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Some problems have no clear solutions.

Last month, we looked at baseline problems that can occur when gradient elution is used in liquid chromatography (LC) separations (1). Many chromatographers shy away from using gradient LC methods because of their potential for problems. Although many of the instrument-related problems that gave gradient LC a bad reputation are things of the past, gradient separations do cause some unique problems. This month we look at one reader's problem. Unfortunately, we have no clear answer but only can suggest more experiments to help isolate the problem.

PEAK SPLITTING

A reader described a peak-splitting problem that occurred when one LC system was used but was absent when the same analysis was performed on another system (see Figures 1a and 1b, respectively). She uses this method for the analysis of a triazine herbicide and two of its metabolites. The peak splitting occurs only on one system and only for the metabolite peaks and not the parent herbicide.

In both cases, the reader used a 15 cm × 3.9 mm, 4-μm d_p, C18 column operated at 0.7 mL/min and 40 °C with UV-absorbance detection at 220 nm. The mobile phase comprised a gradient of water (solvent A) and acetonitrile (solvent B) with the following program steps: 0 min, 5% B; 6 min, 50% B; 10 min, 90% B; and 11 min, 5% B. She injected 5-μL aqueous samples at micrograms-per-milliliter levels. The first LC system, system A, was a popular LC model that uses low-pressure mixing for the gradient. The second LC system, system B, uses low-pressure metering pumps to deliver the solvents to the high-pressure delivery pump where the solvents are mixed. This configuration avoids some of the potential problems of low-pressure mixing noted below, and, for the present tests, it is equivalent to changing from a low- to a high-pressure mixing system.

When standards were run on system A, the reader obtained chromatograms similar to Figure 1a. The two metabolite peaks at 6.2 and 7.2 min showed a small shoulder on the tail. Her first suspicion was that the metabolite standards were impure, but mixing and testing fresh standards yielded the same results. To further test the theory that the peaks were impure, she moved the column and mobile phase to LC system B and repeated the analysis. This analysis generated the chromatogram in Figure 1b, which shows no peak doubling. Furthermore, a peak-purity test result from the diode-array detector indicated that the peaks indeed were pure.

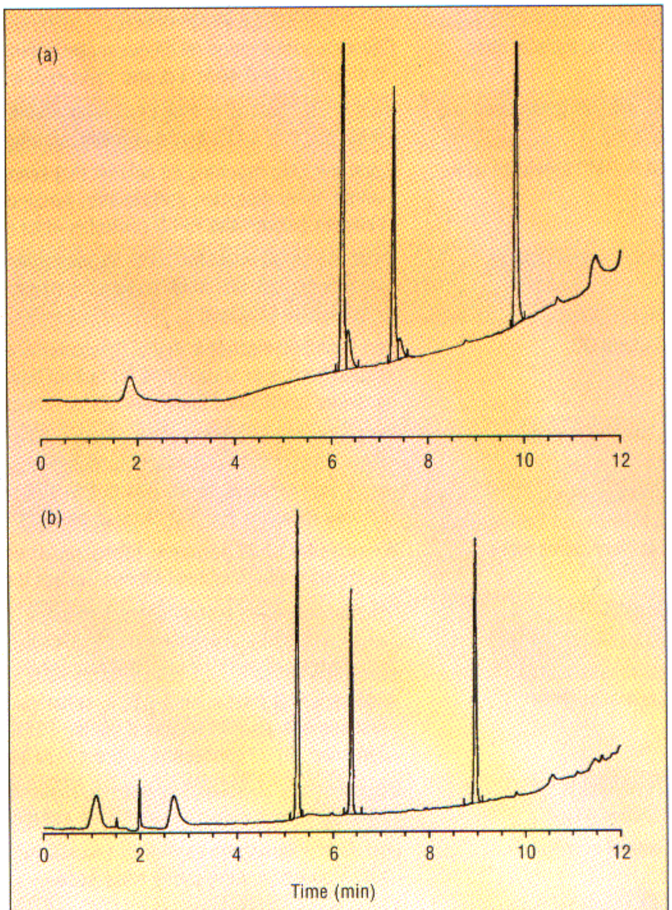

FIGURE 1: Separation of herbicide metabolites (6.2 and 7.2 min) and parent herbicide (9.8 min) using (a) low- and (b) high-pressure mixing systems. Conditions are described in the text.

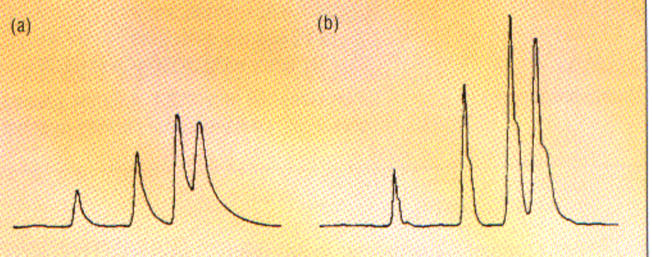

FIGURE 2: Peak (a) tailing and (b) splitting resulting from a blocked column frit or column void. (Reprinted with permission from reference 3.)

For the most part, if you are observing column-related peak-splitting problems, all peaks in a chromatogram are affected, such as in Figure 2. In this case, a void or blocked column frit caused splitting or severe tailing for all peaks. Problems related to injection solvent incompatibility tend to affect the early peaks more than later peaks, as shown in Figure 3. Here, too much of an injection solvent that was too strong severely distorted the first peak; however, the second peak is broadened only somewhat.

In an effort to isolate the problem, the reader followed the accepted practice of changing one parameter at a time. She moved the column back to system A and tried to determine the source of the problem. First, she examined the injection effects. An injection of standards in methanol made no change. She diluted the sample 1000-fold in water and injected 200 µL of diluted sample; again she observed no difference in the results. Next, she used a different brand and configuration of C18 column (10 cm × 2.1 mm, 5 µm d_p) at a reduced flow rate (0.4 mL/min) without success. She injected a different herbicide, and it too showed the doubled peak (the retention time was not reported). However, she routinely analyzed other herbicides with an isocratic method using the same column, instrument, and mobile-phase components — with no peak doubling. The reader concluded that the problem was somehow related to the instrument and not the column, injection, or mobile phase, but the problem source and solution continued to elude her.

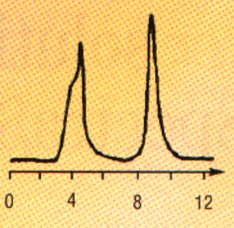

FIGURE 3: Distortion of an early peak caused by an excessive volume of a strong injection solvent. (Reprinted with permission from reference 3.)

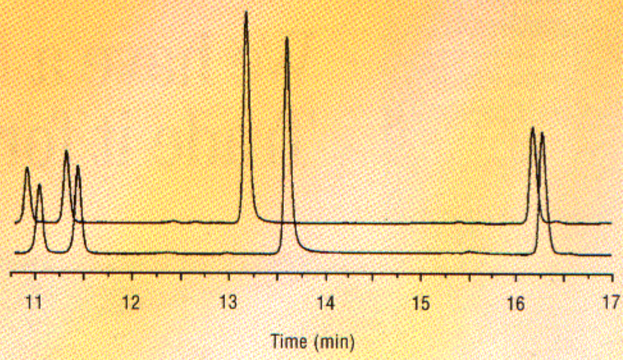

FIGURE 4: Overlay of chromatograms from two consecutive gradient runs showing larger errors for peaks near the gradient midpoint (13 min).

A RELATED PROBLEM?

I don't have a simple solution to this problem — the reader appears to have been very thorough about testing the relevant variables. However, this problem reminds me of another problem I saw recently when the gradient mixer was unreliable. The observed retention shifts were more significant for analytes eluted in the middle of the gradient than at the end.

Figure 4, which overlays the middle part of two consecutive gradient runs, illustrates this problem. It is obvious that the retention difference for the peak at about 13.5 min is much larger than for the peaks near 11 min or 16 min. The problem system used low-pressure mixing. When the same separation was performed on a high-pressure-mixing LC system, the chromatograms overlaid perfectly. I suspected a problem with the mixing system, so I ran a gradient-step test to determine the mixing accuracy (2). This test involves replacing the column with a short piece of tubing and making gradient steps between a UV-transparent A solvent (for example, water) and a UV-absorbing B solvent (for example, water spiked with 0.1% acetone). The proportioning at the midpoint of the gradient was very bad, as shown in Figure 5. The 45% B step delivered 43.2% (1.8% low), and the 50% step delivered 51.6% (1.6% high). A further problem appeared with the poor step shape for the 45–50% step, which was irreproducible from one run to the next. The remaining steps were off somewhat, but the error was consis-

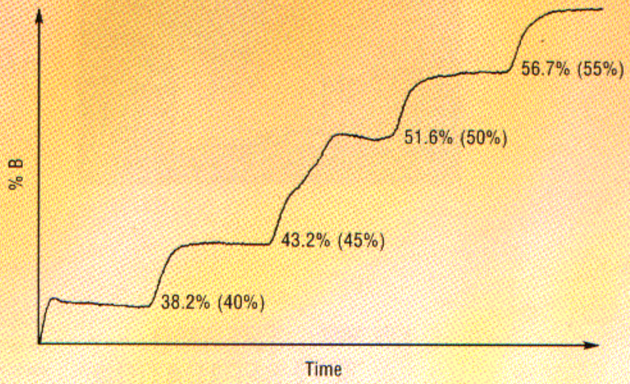

FIGURE 5: Results of proportioning step test performed near the midpoint of the gradient used for Figure 4. Theoretical values are shown in parentheses.

tent. The net result of this proportioning problem was that the composition of the gradient near 50% B was unreliable.

A similar problem with proportioning errors in the middle of the gradient could be causing the reader's problem. Because peaks at the ends of the gradient are normal, we can assume that the proportioning is good there. Depending on the nature of the midgradient error, she could observe retention time changes and split peaks. This example is not a definite cause of the current problem, but it deserves exploration.

What elements of a low-pressure-mixing LC system can fail and cause proportioning errors? The solvents generally are mixed in a proportioning manifold with computer-controlled proportioning valves. The valve seals can leak because of wear or the presence of particulate matter, and this leakage can create proportioning errors. With some systems, the open–close cycle of the valves can be fine-tuned by adjusting the controlling software. The LC system used for Figures 4 and 5 uses different algorithms to proportion solvents in the 0–50% and 50–100% B ranges. Adjusting control parameters can correct changeover problems, such as those illustrated in Figure 5.

Excessive resistance in the tubing feeding solvents into the mixer is another source of proportioning errors. If the sinker frits in the solvent reservoirs are partially blocked, the flow of solvent to the mixer can be restricted. Insufficient delivery from a restricted line followed by a surge of solvent from the less-restricted line can distort solvent proportioning. You can check for blockages by disconnecting the feed lines at the proportioning manifold — the solvent should siphon freely through these lines at several times the required flow rate. If the flow is restricted, remove the sinker frit and repeat the experiment. If solvent flows freely, the frit needs to be replaced. Replace the frit with a 10-µm or larger porosity frit. Smaller frits become blocked more quickly and are no more effective than larger porosity frits for this application.

A similar symptom of unequal solvent delivery can occur if helium-pressurized solvent reservoirs are used. If you are using this kind of degassing, be sure you have a mechanism to equalize the pressure between reservoirs, or you could get distorted proportioning.

CONCLUSION

I don't have a definitive solution to the problem of doubled peaks in the middle of the gradient. The reader seems to have been very thorough about eliminating problems that could be caused by the column, solvents, and sample. Because the problems occur with an LC system that uses low-pressure mixing and disappear when she uses a high-pressure-mixing system, I suggest that the problem could be related to mobile-phase mixing. Errors in solvent proportioning could explain the problem, but this hypothesis needs to be tested. Running the step test and linear gradient with a UV-absorbing marker will determine if the proportioning system is operating correctly. As always, I welcome suggestions from other readers who may have encountered (and solved) similar problems.

REFERENCES

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