LC TROUBLESHOOTING

The Case of the Mystery Gradient Peak

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Sometimes you can solve a problem without gaining the satisfaction of understanding its cause.

This month, "LC Troubleshooting" looks at a problem reported by a reader who had run out of ideas when looking for the solution. The problem of an extra peak in a gradient run can be particularly vexing because the extra peak can result from more than one cause. After several conversations and several days of work, we finally eliminated the extra peak. The cause was . . . well, we're not entirely sure.

This month's installment closes with a note of clarification. The discussion of extra-column effects in January's "LC Troubleshooting" (1) raised some questions for at least one reader. I hope the clarification resolves any confusion you might have had.

THE MYSTERY PEAK

The story begins with a reader who encountered a peak in blank gradient runs on two out of three systems, but only when one brand of C18 column was used. The gradient was 5–100% acetonitrile over 40 min. About halfway through the gradient, a peak was observed. The only thing that would make the peak go away was changing to a different brand of column, but the reader's method required the use of the original brand.

For purposes of discussion, I'll call the different LC systems system 1, system 2, and system 3. Except where otherwise noted, all the troubleshooting was done on system 1. It had two pumps in a high-pressure gradient-mixing configuration and used a diode-array detector monitored at 240 nm. The solvents were degassed by helium sparging in a batchwise mode. System 2 was nominally the same setup as system 1 but used a different brand of equipment. It used no degassing other than that accomplished by vacuum filtration of the solvents. System 3 used the same brand of pumps as system 1 but was connected to a mass spectrometer for detection. Vacuum filtration was the only form of degassing used by system 3. This was the only system that showed no extra peak in the gradient.

CONTAMINATED SOLVENTS?

By the time I talked with the reader, the organic solvent was thought to be free of problems because the peak occurred when either acetonitrile or methanol was used as the strong solvent. The problem occurred with only one brand of column; the columns' history (whether they were new or used) seemed to make no difference.

When extra peaks appear in a blank gradient, contaminated solvents are the first cause that I suspect. Because this problem occurred independently of the organic solvent type, the aqueous solvent was the likely problem. A standard way to test for contaminants in the aqueous phase is to extend the equilibration time before starting the gradient. With a longer equilibration time, a larger amount of contaminant should collect at the head of the column before the gradient is run and should produce a larger peak (in proportion to the increase in equilibration). For example, if the normal equilibration time is 10 min between runs, a 30-min equilibration time should result in peaks three times as large. When the reader performed this experiment, the results were not clear-cut — the peak size increased somewhat but not as much as was expected.

Another way to check for contaminated aqueous phase is to replace the water with water from another source. Four water sources were checked. These included two lots of HPLC-grade water (commercially purchased), laboratory-distilled water, and water from a laboratory HPLC-grade water system. All of these sources of water produced equivalent results: the extra peak remained.

SYSTEM CONTAMINANTS?

When I became satisfied that the solvents were not contributing the unwanted peak, we looked for other sources of contamination. Dirty solvent reservoirs or improperly washed glassware can contribute unwanted impurities to LC systems. We carefully ruled out these variables, and the problem peak persisted.

Although it seemed as if the autosampler could not be causing the problem (the peak showed up even when no injections were made), the reader verified its noninvolvement by changing the plumbing so that the injector was bypassed.

THE COLUMN?

With the most obvious causes of contamination eliminated, we began to consider the column as the problem source. The problem persisted when we used one brand of C18 column, but no symptoms appeared when we used another brand of C18 column. When the first brand was used, the problem occurred whether the column was new or used. This made little sense to me, because a column should contain no chemical contaminants when it is received. To my knowledge, column packing procedures use no solvents that are strongly UV absorbing at 240 nm. After columns are packed, they are flushed with reversed-phase solvents to wash out the packing solvent. Finally, the columns are tested, using reversed-phase solvents (usually methanol–water mixtures), so no previously used solvents should remain. Even if chemical residues made it through all of these cleanup steps (or if the manufacturer inadvertently skipped one or more steps), they would be washed from the column in a few gradient cycles. It seemed inconceivable to me that the peak was the result of a column contaminant.

A standard way to test for contaminants in the aqueous phase is to extend the equilibration time before starting the gradient.

But why did the problem show up with one brand of column and not another? Both brands were nominally the same (25-cm C18 with 5-μm d_p particles). The differences one expects to find between column brands might result in small changes in retention times, not in the presence or absence of a major peak. At this point, the data regarding the column were totally confusing, so we looked elsewhere in the system for a solution.

A PICTURE IS WORTH . . .

At this point, I began to suspect that the problem peak might not be a normal chromatographic peak. The reader supplied me with the chromatogram and the diode-array detec-

tor scan in Figure 1. Aha! The leading edge rises too sharply to be a chromatographic peak. Furthermore, the UV spectrum does not look like any organic compounds with which I am familiar. I suspected air bubbles.

All three LC systems used high-pressure mixing, a technique that is less likely to introduce bubble problems than low-pressure mixing. System 1 used batchwise helium sparging to degas the solvents. The other systems used vacuum degassing. In an effort to obtain more complete degassing, the reader operated system 1 with continuous helium sparging, but the problem persisted. In spite of this, Figure 1 strongly suggested a bubble problem.

In reversed-phase LC solvent systems, air bubbles are released when air-saturated solvents are mixed; the mixed solvents have a lower capacity for air than pure water or organic solvent (for example, acetonitrile or methanol). The mixture has the least capacity for air at intermediate mixtures; thus, when degassing is a problem in a gradient, the problem is most likely near the midpoint — the same place the problem peak was observed in this case. Helium sparging usually reduces the air levels in the solvents to the point at which no outgassing problems persist, but the method was unsuccessful here.

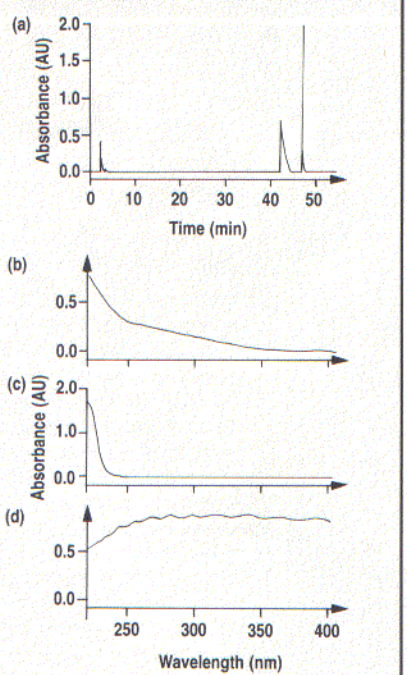

FIGURE 1: Diode-array detector data from a sample run using system 1, including (a) a chromatogram at 240 nm showing the dead volume peak at ~2 min, the problem peak at ~42 min, and the sample peak at ~47 min and spectra of (b) the peak at the solvent front, (c) the peak just after the solvent front, and (d) the problem peak.

When low-pressure mixing is used, the solvents are mixed at or below atmospheric pressure. These conditions promote outgassing of the mixed solvents, and bubbles often result. These bubbles create pump check-valve problems that produce system pressure fluctuations. When high-pressure mixing is used, the solvents are combined at much higher pressures (typically 2000–3000 psi) and air bubbles are kept in solution, which explains why solvent degassing may not be required for reliable pump operation. When the pressure drops to atmospheric pressure after the solvent leaves the system, however, the bubbles are released. If bubble release takes place in or before the detector cell, noise spikes caused by bubbles in the cell may be observed. In most systems, the release of pressure can be delayed until after the detector cell by adding a back-pressure regulator to the system. This spring-loaded device mounts on the detector waste line and keeps 50–100 psi of back pressure on the system. (Care must be taken to ensure that the back pressure does not exceed the pressure limit of the detector cell or cell damage can occur.)

When a 100-psi back-pressure regulator was added to system 1, the problem peak disappeared. When the reader checked with the operator of system 3, it was discovered that the mass spectrometer probe created about 1000 psi of back pressure on the LC system, providing the same effect as the pressure regulator.

LESSONS LEARNED

What can we learn from this problem? First of all, use the information that the chromatogram gives you. Normal chromatographic peaks are more or less Gaussian in shape. Sure, they often tail, but the peak should show a great deal of symmetry, especially on the top half. Only in two cases do real peaks have a sharp front like that observed in Figure 1. One case involves peaks at the column dead volume (solvent front); this group of "garbage" peaks shows a sharp front and a broad tail. The other case involves heavily overloaded peaks, in which the peak shape approaches that of a right triangle. Even so, the front is rarely a vertical line. Furthermore, overloaded peaks generally show very strong absorbance. The first problem, then, was that the reader failed to distinguish between a nonchromatographic peak and a sample peak (and I neglected to ask for a chromatogram early in the process).

Second, be methodical about isolating a problem when the solution is not obvious. Although the problem appeared to be with the column, the source of the problem was elsewhere. The technique of substituting a known good component for a suspect one was used here to speed problem isolation. The suspect column was replaced by a good one. Several different batches of solvents and water were used. The system was cleaned, unnecessary components (the autosampler) were removed, and the mobile phases were degassed. Eventually, a solution was found. It is likely that a hit-and-miss strategy of randomly trying solutions would have taken considerably longer to fix the problem.

Finally, don't be afraid to ask for help. Other workers, operator's manuals, troubleshooting books and brochures, and this column are all sources of information that may help you rapidly find a solution to your problem. The insight of another worker or the different perspective of a written resource may be just what you need.

OK, why did the problem occur with one brand of column and not another? I don't know. Perhaps the nature of the one column's packing promoted solvent outgassing. After working on this problem, I learned of a similar case that one of my co-workers encountered several years ago with the same brand of column. The problem went away after both of the column endfittings were tightened a little more snugly. Whether or not this really eliminated the problem source is unknown. So if anyone has any insight into the reason why one brand of column might cause such problems, please let me know.

CLARIFICATION ON EXTRACOLUMN EFFECTS

At least one reader expressed confusion with some of the information about extracolumn effects in the January 1992 installment of "LC Troubleshooting" (1). In the discussion of Case 1, I stated that substituting a smaller particle column in an otherwise well-plumbed system can result in a resolution loss for peaks eluted early in a run. Confusion arose from a misunderstanding that the particle size was important. This is *not* the case. Remember, when we talk of extracolumn effects, peak *volume* is generally the most important column contribution. With all other conditions held constant, a narrow (small-volume) peak will be more severely broadened by extracolumn effects than a broader (large-volume) peak. Think of it this way: An additional 5 s of peak width (broadening) will be much more noticeable on a 10-s peak than on a 60-s peak. Thus, any change that reduces the peak width, such as a shorter column, a narrower i.d. column, or a smaller particle column, will make the system more sensitive to extracolumn effects.

REFERENCE

(1) J.W. Dolan, *LC•GC* **10**(1), 20–25 (1992).

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