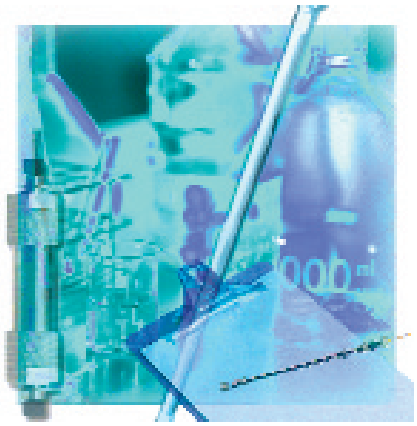

Unexpected Peaks

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Where did that extra peak come from?

One of the most vexing problems with liquid chromatography (LC) separations is the presence of unexpected peaks in a run. The sample components eluted from an injection before the current run are one source of these peaks. Some workers refer to these peaks as carryover peaks because they come from a previous run. However, the more widely accepted definition of carryover refers to the injection of residual sample remaining in an autosampler; for example, when an analyst sees a peak that is 1% the size of the peak in the previous run. I will discuss carryover in a future "LC Troubleshooting" column. This current instalment will focus on late-eluted peaks.

Late Elution

Late elution is characterized by the presence of unexpected peaks in a blank or non-injection run. Often, late-eluted peaks are observed first as the presence of unexpected peaks in a chromatogram. This problem tends to be more frequent with isocratic methods than with gradient ones, because gradient elution programmes typically end with strong mobile-phase conditions that tend to flush strongly retained materials from the column. The peak at approximately 2 min in Figure 1(a) is an example of a late-eluted peak. In this instance, the solvent peak appears at 1.3 min and eight additional peaks appear before the run stops at 5 min.

As a general rule, all peaks in a given portion of a chromatogram should be approximately the same width. With isocratic separation, the longer a peak is retained, the broader it is. The last seven peaks in Figure 1(a) show those two characteristics: similar widths and a gradual increase in width. When a broad peak appears among narrower ones, as in Figure 1(a), the peak width pattern is broken, which provides a hint that its true retention time is much longer than that of the narrow peaks.

The easiest way to determine whether a peak is a late eluter is to extend the chromatogram beyond its normal stopping time. If the peak is truly a late eluter, it will also appear later in the chromatogram. That is, the peak at 2 min in Figure 1(a) is probably a late-eluted peak from a previous run. For this reason, one would expect the peak to appear at approximately 2 min plus the normal run cycle time. In Figure 1(a), the run time is 5 min, so in an extended run, I would expect to observe the peak at 2 min and at 7 min (2 min + 5 min). This hypothesis is confirmed by allowing the run to extend to 10 min, as Figure 1(b) shows. The peak appears where I would expect it, at 7 min. Therefore, the peak at 2 min is from the previous injection.

Fix Number 1: Extend the Run Time

The simplest fix to the current problem is to extend the run time so that the extra peak is eluted at its normal position in the chromatogram. Figure 2 shows the first three chromatograms under new conditions with the run cycle time set at 10 min. Oops! Something isn't right. The unknown peak doesn't appear at its expected retention time in Figure 2(a). But it shows up at the 2 min position instead of at 7 min in subsequent chromatograms. I must have made a mistake.

If I had allowed the run of Figure 1(b) to run longer, I would have observed a peak at 12 min, as well, which is the true retention time of the unknown peak. (I'll leave it to the readers to figure out why the peak also appears at 7 min in Figure 1(b).) Armed with knowledge of the true retention time, I can extend the run still further — to 15 min — to accommodate the elution of the problem band. The chromatograms of Figure 3 show the first three runs with the 15 min cycle time. Now, the chromatograms appear as expected, with all the peaks in the same position in each run. The peak widths increase in a regular fashion as retention time increases and this observation provides additional confirmation.

Although the extended run time of Figure 3 solves the current problem, the solution may be impractical, especially in a production environment. For example, a 100 sample run would require just over 8 h with a 5 min cycle time, but 25 h if 15 min runs were used. This approach would preclude the use of a single LC system to analyse a 100 sample sequence every day. I'll look at some alternative solutions later.

Calculating Retention Time

As the situation above showed, it is possible that the late peak originated

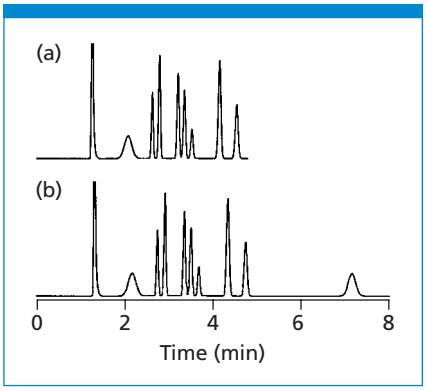

Figure 1: *Simulated isocratic chromatograms for an eight-component mixture. Shown are (a) a normal 5 min run with an unexpected peak appearing at approximately 2 min and (b) the next injection with a 10 min run time.*

several runs earlier. Extending the run until the peak appears at its normal retention time is not the only way to determine the retention time of a late-eluted peak. Chromatographers can make an educated estimation of retention time by using the column plate number calculation. This calculation assumes that, as a first approximation, all retained peaks in an isocratic run have approximately the same plate numbers. First, calculate the plate number (N) for a normal peak:

$$N = 5.54(t_R/w_{0.5})^2 \quad [1]$$

where t_R is the retention time and $w_{0.5}$ is the peak width at half height. Assuming that the plate numbers are the same, Equation 1 calculations for two peaks can be combined and rearranged as

$$t_{R2} = (t_{R1}w_2)/w_1 \quad [2]$$

where t_{R1}, t_{R2}, w_1 and w_2 are the retention times and half-height widths of peak 1 (the narrow peak) and peak 2 (the broad one).

My measurements of Figure 1 yield t_{R1} = 2.37 min, w_1 = 0.04 min and w_2 = 0.20 min. Thus $t_{R2} \approx$ 11.8 min, or, at 5 min/run, the broad peak originates two runs earlier (5 + 5 + 2 ≈ 11.8). After it first appears, I would expect the peak to appear with a retention time of approximately 1.8 min in each subsequent run. Don't be too exacting with this calculation, but it can be useful as a guide. In the present instance, it estimated the true retention time (12.16 min) fairly closely, but a small error in peak width measurement can have a significant impact. For example, if w_1 were measured as 0.05 min instead of 0.04 min, the estimated retention time would change by almost 2 min. For best results, expand the peaks on the data system display for more accurate width measurements.

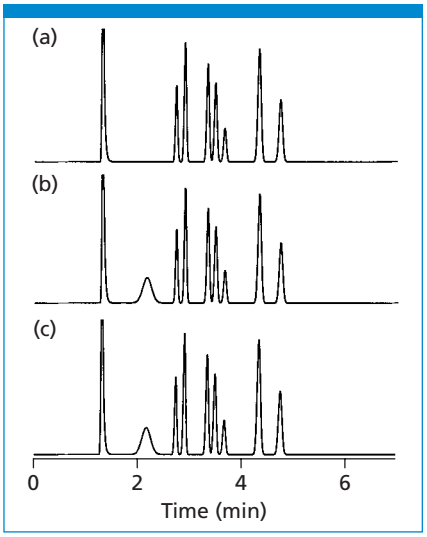

Figure 2: *Chromatograms for (a) the first, (b) second and (c) third runs of the sample of Figure 1 with the run cycle time increased to 10 min.*

Alternative Fixes

Now that I've identified the source of the late-eluted peak, how can I eliminate the problem? This problem has many possible solutions. The simplest, but most expensive, solution from a time standpoint would be to extend the run time to 15 min, as I did in Figure 3. This tripled the run time, which is an unsatisfactory fix in most instances.

At the other extreme, I could remove the unwanted peak through sample pretreatment. The retention time of the problem band is more than twice that of the other peaks in the run. This longer retention time suggests that the peak is significantly less polar (assuming a reversed-phase separation). A large polarity difference might enable removal of the peak by solid-phase or liquid–liquid extraction techniques. If the problem band had a pK_a that differed from those of the other peaks, then pH adjustment could enhance the performance of either extraction technique.

One common technique for dealing with late peaks is to add a strong-solvent flush at the end of each run. For example, the current 50:50 acetonitrile–water mobile phase could be stepped to 90:10 acetonitrile–water for a couple of minutes to flush the column. Then, the column would be re-equilibrated before the next injection. These steps might double the present cycle time, but they would probably save time compared with the 15 min run option. An added advantage of this flush is that it could improve baselines because other strongly retained materials might be removed as well.

Another simple fix that would work in the present example, but might not work for other samples, would be to ignore the peak. The broad peak is eluted in a section of the chromatogram that contains no peaks of interest. It appears to me that the problem peak and the peaks of interest have plenty of resolution between them, so integration should not be a problem. As long as the peak's position remains constant, I should be able to obtain good analytical results for the other peaks. Sometimes it is possible to change the cycle time slightly to move the peak's apparent retention time to an unimportant region. For example, if the present run

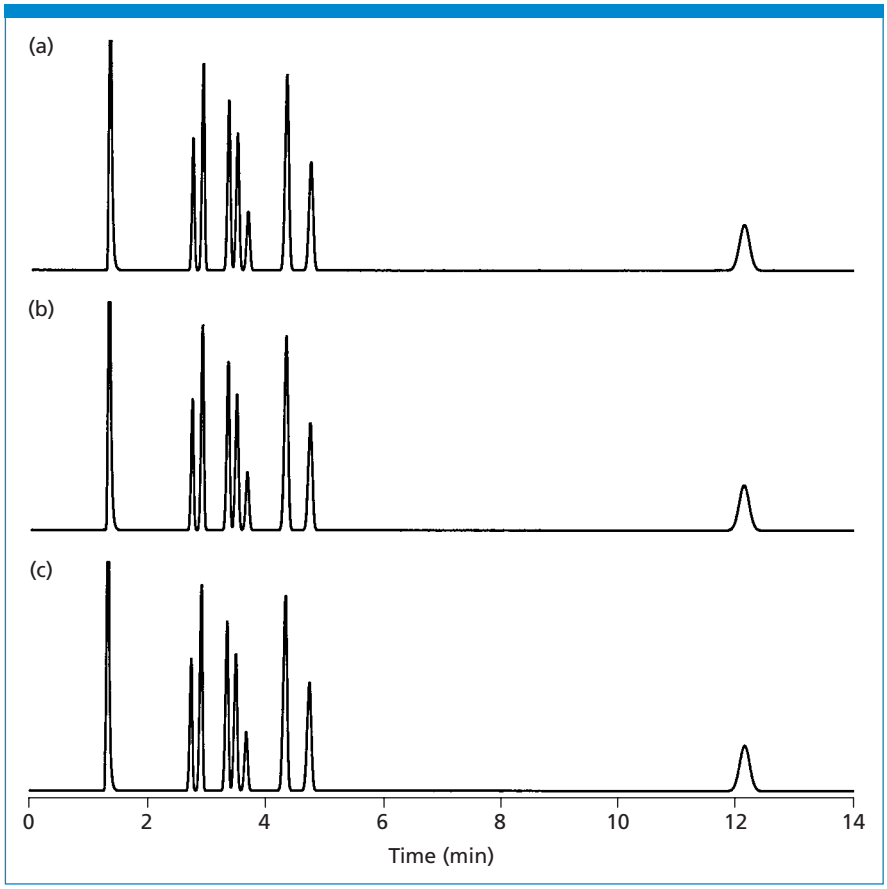

Figure 3: *Same as Figure 2 but with a 15 min run time.*

time were increased from 5 min to 6 min, the late peak would come off the column at the end of the run or before the solvent peak in the run where no peaks of interest are eluted.

A final alternative is to modify the method conditions to move the true peak's retention time closer to those of the peaks of interest. This alternative might involve a change in mobile-phase composition or the use of a different column type. Redeveloping method conditions in a situation such as this one can be a major undertaking — I would consider the other alternatives first.

Column Flushing

In the present example, the late-eluted peak was reasonably large compared with other sample components and its retention was not excessive (the retention factor [k] was approximately 8), so it appears as an obvious peak in the chromatogram. Because peak width increases in proportion to retention time and peak height decreases in proportion to peak width (assuming constant peak area), many strongly retained peaks may not be obvious. For example, if the problem peak were retained with a k of 20 ($t_R \approx 27$ min), the increase in peak width would result in a peak approximately 40% as high as those shown in Figures 1–3. It is easy to see that very strongly retained peaks can appear as indistinct broad humps in the baseline. Rolling, bumpy baselines will reduce signal-to-noise ratios and degrade precision near the limit of quantification for a method.

Because it is common for samples to contain very strongly retained materials of no analytical interest, a regular flush with a strong solvent is wise with nearly every LC method. A simple expedient is to perform this flush once a day when the LC system is shut down or changed to another method. First, flush the system with non-buffered mobile phase. For example, if the mobile phase is 50:50 acetonitrile–buffer, flush with 10–20 mL of 50:50 acetonitrile–water. This flush will remove buffers from the system and will help to avoid precipitation of buffer salts when a strong solvent flush is used. Next, flush with 10–20 column volumes (typically 15–30 mL) of strong solvent (such as acetonitrile in the present example). This second flush should remove strongly retained materials from the column. The system can be stored containing the strong solvent.

As mentioned above, gradient elution inherently includes a strong-solvent flush. Although many gradient methods do not extend to 90–100% organic solvent, the programme can be modified for flushing with a strong enough solvent to remove strongly retained materials after each run.

The Keys to Success

I've shown that peaks remaining on the column from previous injections often appear as broad peaks in a subsequent chromatogram. Late-eluted broad peaks often stand out like a sore thumb because the widths of all peaks in a section of the chromatogram tend to be of similar width, and, for isocratic separations, they gradually increase in width as retention increases.

Although you may be fortunate and be able to fix the problem by extending or adjusting the run time so that you can ignore the peak, this situation is the exception rather than the rule in most instances. Perhaps one of the alternative solutions proposed above may apply to your separation. Whatever changes you make to correct a problem created by a strongly retained peak, don't forget to consider the impact of that change on the method's certification status. Some changes may require revalidation of a method, whereas others may be less important.

"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 Europe. *Direct correspondence about this column to "LC Troubleshooting,"* LC•GC Europe*, Advanstar House, Park West, Sealand Road, Chester CH1 4RN, UK, e-mail: dhills@advanstar.com*

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