LC Troubleshooting

Extra Peaks

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Extra peaks — where do they come from? Here's a method to help you find out.

One problem that we all encounter in liquid chromatography (LC) is the presence of extra peaks in the chromatogram. The appearance of an extra peak can be the result of many different problems in the LC system or method. For example, impure samples, errors in sample preparation, carryover in the injector, strongly retained analytes, sample breakdown, and dirty glassware can introduce extra peaks in the chromatogram.

What should you do when you see an extra peak? First, practice the *Rule of Two*, which states that a problem should occur at least twice before you consider it a problem worth solving. Some sources of extra peaks, such as an occasional bubble passing through the detector, may be so rare that they cannot be eliminated reliably — save your troubleshooting energy for worthwhile problems. The simplest way to verify the problem's existence is to reinject the sample or inspect a series of chromatograms. Is the problem peak (or peaks) in every run? If it is, now you have something on which to focus your efforts.

In November's "LC Troubleshooting" column (1), I described "signature chromatograms." These chromatograms are so characteristic of a problem that they can identify it reliably. Figures 1 and 2 show examples of signature chromatograms for strongly retained peaks. In each case, a peak that is much broader than the surrounding peaks is present. For LC separations, all the peaks in a given region of the chromatogram should have approximately the same peak width, so significantly broader (or narrower) peaks are sure signs of trouble.

With isocratic separations, the peaks gradually get broader as the retention times increase, whereas gradient separations yield peaks that are of similar width throughout the chromatogram. In Figure 1, peaks 3 and 4 are noticeably broader than neighboring peaks 1 and 5. The simplest way to confirm the presence of strongly retained peaks is to extend the run. If the analyte corresponding to the extra peak is present in every sample, you will observe a peak appearing at regular intervals, corresponding to the run time of the earlier injections, even if no injection was made. Let's examine the chromatogram of Figure 1 and see what we can learn from it.

UNWANTED PEAKS

I received the chromatogram in Figure 1 from a well-known pharmaceutical company. The user was synthesizing some analogs of an existing product. The separation was satisfactory for his use, successfully isolating the parent compound (peak 2) from two minor impurities (peaks 1 and 5). The mobile phase was a ternary mixture of ~10% methanol, 10% acetonitrile, and 80% citrate buffer. The buffer strength and pH were reasonable. The separation used a 25 cm × 4.6 mm C18 column operated at a 1-mL/min flow rate.

The chromatographer started up his LC system in the morning, ran some standards to verify that it was working properly, and then started the synthetic reaction he wished to monitor. As the reaction progressed, he withdrew aliquots from the reaction vessel and injected them to follow the reaction. In the middle of the afternoon, he obtained the chromatogram in Figure 1. By the time he noticed the problem peaks, the reaction had progressed, so he was unable to obtain a duplicate aliquot, and all the original sample had been used. So he was unable to rerun the sample to satisfy the Rule of Two.

The chromatographer wasn't concerned because his experience led him to conclude correctly that the problem peaks were strongly retained peaks from an earlier run. However, this intuitive conclusion did not satisfy his manager, who was very nervous about having extra peaks in the analysis, so the worker had to construct a more quantitative argument for their source.

WHERE DO THEY ORIGINATE?

Determination of the injection point of a strongly retained peak is fairly simple if you can measure the width of the peak in question and those of some neighboring peaks. First, we determine the plate number for the normal peaks. We assume that the late peaks have approximately the same plate number. (This is not totally true, because we have ignored extra-column effects, but the assumption is good enough for this approximation.) After we know the plate number and the peak width, we can calculate its retention time. Let's see how this works using the chromatogram of Figure 1.

The first step is to determine the plate number, N, for neighboring peaks. Peaks 1 and 5 are good candidates for this measurement. Because the baseline is difficult to determine for peak 1 and slants for peak 5, using the half-height calculation minimizes errors. The equation is

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

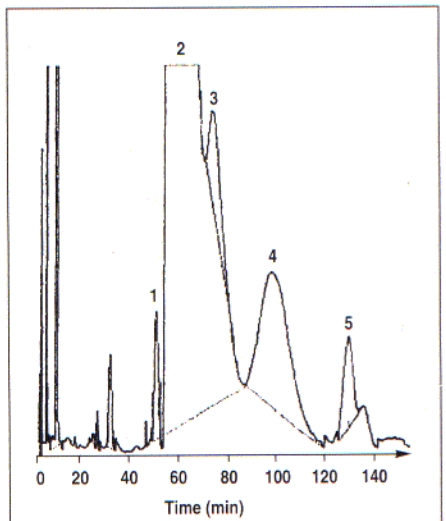

FIGURE 1: Chromatogram of a reaction mixture. Peaks 1 and 5 are minor impurities, peak 2 is the product compound, and peaks 3 and 4 are strongly retained peaks from an earlier injection.

TABLE I: Retention and Peak Width Data for Figure 1

Peak	t_R (mm)	$w_{1/2}$ (mm)
1	40	1.5
3	59	?
4	78	10
5	105	4

where t_R is the retention time and $w_{1/2}$ is the peak width at one half of its height. Because the units cancel out, we can measure the retention time and peak width in any units we like. I usually do this calculation manually, so I use a ruler and make the measurements in millimeters. My measurements for the various peaks are summarized in Table I. I have not included the width of peak 3, because I could not measure it due to the severely slanting baseline. Peak 3 clearly should be classified with peak 4 in terms of width rather than with peaks 1 or 5.

Now the plate number calculations are done for each peak:

$$N_1 = 5.54(40/1.5)^2 = 3939$$

$$N_4 = 5.54(78/10)^2 = 337$$

$$N_5 = 5.54(105/4)^2 = 3817$$

These results support our visual conclusions: Peaks 1 and 5 belong in the same chromatogram, and peak 4 is much broader (lower plate number). If we now combine peaks 1 and 5, we obtain an average plate number of (3939 + 3817)/2 = 3878.

The next step is to extract the retention time for peak 4 from the plate number and width information. Because we are interested in the retention time, not its square, we take the square root of both sides of equation 1 before rearranging it to a more useful form:

$$t_R = N^{1/2} w_{1/2}/2.35 \qquad [2]$$

For peak 4 we have

$$t_R = (62.2)(10)/2.35 = 264 \text{ mm}$$

To convert from millimeters to minutes, we use peak 5, with a retention time of 125 min or 105 mm. This gives (125 min/105 mm) = 1.2 min/mm. So (264 mm)(1.2) = 316 min for peak 4. Because of the degree of extrapolation and the nature of manual measurements, I would guess that this prediction is accurate within roughly ±10%.

The data are insufficient to perform similar calculations for peak 3, but based on the general appearance of peaks 3 and 4, I would expect similar results. Next, we should compare these predictions with the system-use history to see if they are in agreement. The user examined his injection times for the day and confirmed this hypothesis. He had indeed made an injection of his reaction mixture ~5 h earlier.

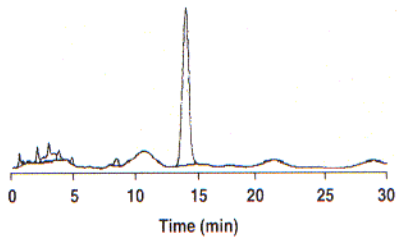

FIGURE 2: Chromatogram of a routine sample showing a parent peak at 13.5 min, numerous small impurities at 0–8 min, and strongly retained interferences at 3, 10, 21, and 29 min.

WHAT TO DO ABOUT IT?

The goals of the analysis influence the correction of problems caused by strongly retained peaks. In the case at hand, the user was only analyzing a few samples each day, but the sample composition varied from sample to sample. The easiest way to correct his problem would be to fully elute the unwanted peaks before the next injection.

Elution can be accomplished by two methods. The simplest but most time-consuming method is to wait until the peaks are fully eluted before injecting another sample. But clearly, waiting 5 h or more between injections is not a satisfactory solution for most users.

An alternative method is to wait until the last peak of interest is eluted and then change the mobile phase to quickly elute the unwanted materials. The chromatogram of Figure 1 appears to end at ~150 min, so a steep gradient or step change to strong solvent should quickly flush the unwanted material from the column. I would try flushing with ~10 column volumes (~25 mL of mobile phase for the present column) of a 40% methanol, 40% acetonitrile, 20% citrate buffer mobile phase. Then return to the starting conditions and reequilibrate before the next injection.

Routine analysis of the same sample type tens or hundreds of times a day requires a different strategy to combat strongly retained peaks. Strongly retained materials are by definition much less polar than the sample components, so enhanced sample cleanup based on peak polarity should be straightforward. Two common polarity-based cleanup techniques are solvent–solvent extraction and solid-phase extraction (SPE). Either technique should remove strongly retained materials with a minimum of method development time. Use of a gradient flush after each sample usually is too time-consuming to be practical for routine samples, but sometimes it is necessary. Another alternative is adjusting the run times slightly so the extra peaks come out in an unimportant portion of the chromatogram.

ANOTHER CASE

Figure 2 shows another example of strongly retained peaks. Three obviously broad peaks are eluted at ~10, 21, and 29 min. Another peak, buried under the group of small peaks, appears at ~3 min. Faulty integration marks in the 2–4 min region illustrate the potential for problems. If no interference were present, the peaks would likely be integrated with a baseline–baseline technique. The present integration parameters will grossly overestimate at least two of the peaks that are riding on top of the interference. This situation is another case of strongly retained peaks that should easily be removed with a solvent extraction or SPE.

If you would like to try your hand at calculating the retention time of a strongly retained peak, start with the peak at 10 min in Figure 2. The measurements from my original copy of the chromatogram are 0.8 mm for the half-height width of the 10-min peak and 2.8 mm and 63 mm for the peak width and retention of the 13.5-min peak. My answer appears at the end of this column.

CONCLUSION

So what have we learned about strongly retained peaks? First, make sure that you really have a problem by reinjecting the sample and examining other chromatograms for interfering peaks. If interfering peaks are found, compare their widths to those of neighboring peaks. If the peak widths of the unexpected peaks are noticeably broader than those of their neighbors, you have fairly good evidence of strongly retained peaks.

The easiest way to confirm strongly retained peaks is to extend the run. If the peaks continue to appear for one or more run cycles, although no injections have been made, you have confirmed the presence of strongly retained peaks. If you need to know which injection was the source of a strongly retained peak, use the technique described above to estimate the undesired peak's retention time.

Unwanted strongly retained materials can be removed from the chromatogram by improved sample cleanup that prevents them from entering the system. Alternatively, use a strong solvent wash to remove the interference before the next injection.

ANSWER FOR FIGURE 2

I calculated N = 2804 for the 13.5-min peak. This result allowed me to calculate the true retention of the 10-min peak as 180 mm or 84 min. This retention suggests that the peak comes from an injection made (84 − 10) = 74 min earlier. The injection cycle is 30 min, so the peak is probably from an injection two (60 min) or three (90 min) cycles before the current injection.

REFERENCE

(1) J.W. Dolan, *LC•GC* **11**(11), 790–792 (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, *859 Willamette Street, Eugene, OR 97401, USA.*