LC Troubleshooting

Resolving Minor Peaks

Is a small peak hiding under that tail?

Separating small peaks from large ones can be a major challenge when developing liquid chromatography (LC) separations. This problem occurs with many LC applications. Drug substance and drug product assays used for stability indication or impurity determination can require quantification of peaks that represent 0.05–0.1% of the area of the parent compound. Analyzing drug metabolites in plasma or tissue samples often requires the isolation of target compounds from potential interferences that are several orders of magnitude more concentrated. Pesticide residue analysis could require determination of parts-per-million or lower concentrations in the presence of high concentrations of other compounds. Trace impurities in industrial processes can compromise the quality of a product.

In each of these applications, chromatographers must separate small peaks sufficiently from larger ones for quantification to be successful. This month's "LC Troubleshooting" discusses some of the challenges of separating small peaks from large ones and gives some pointers about obtaining separations.

It's All in the Tail

One of the primary challenges in determining trace peaks occurs when they are eluted next to a major peak, especially if the major peak tails at all. Figure 1 illustrates this situation with peaks of different size and tailing factors. When peaks are symmetric, as in

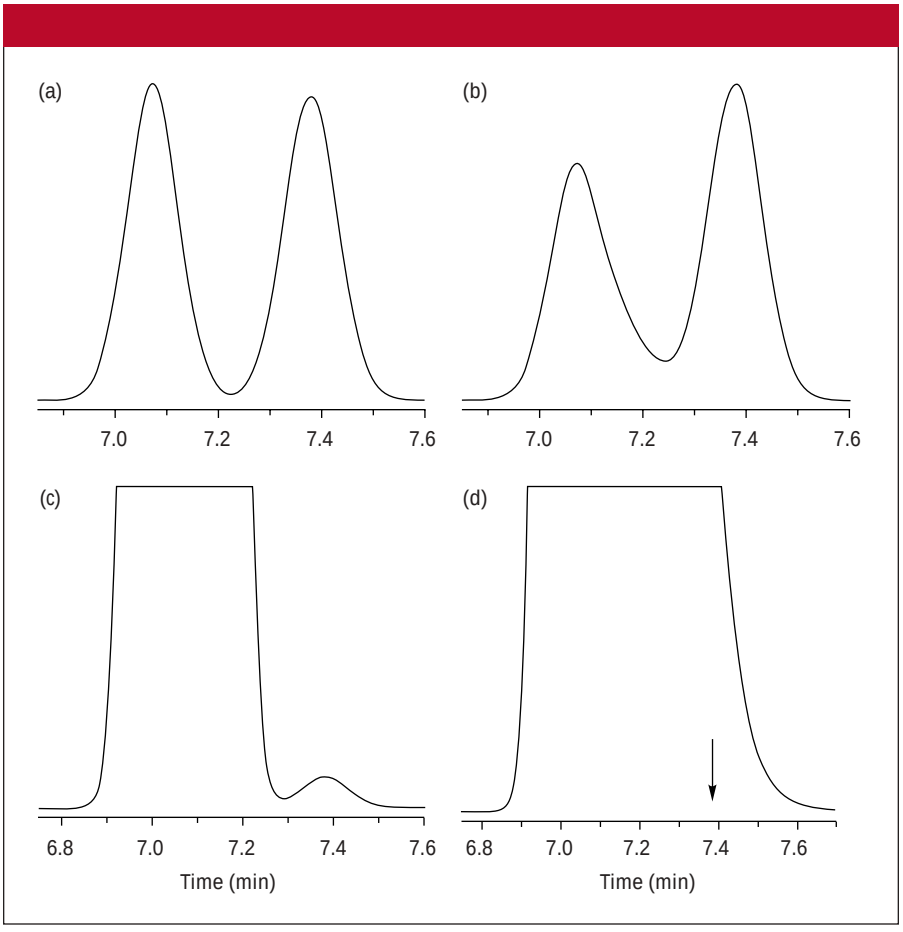

Figure 1: Simulated chromatograms showing (a) 1:1 peak areas with $A_s = 1.0$; (b) same as 1a, but $A_s = 1.5$; (c) 1000:1 peak areas with $A_s = 1.0$; and (d) same as 1c with $A_s = 1.5$.

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Figures 1a and 1c, the detection of minor peaks is easy. In each of these cases, both peaks are symmetric, and the resolution is 1.5. Whether the peak areas are the same (Figure 1a) or the minor peak is 0.1% of the major one (Figure 1c), the second peak can be quantified without problems.

Tailing peaks, however, can be much more challenging. As practicing chromatographers know, a perfectly symmetric peak is a rarity. More commonly, they find peaks with asymmetry as great as 1.5, measured as

$$A_s = \frac{\text{second half width}}{\text{first half width}} \qquad [1]$$

where A_s is the asymmetry factor and the peak half widths are measured at 10% of the peak height. When peaks are approximately the same size, as in Figure 1b, quantification of the second peak is compromised only slightly if the preceding peak has an asymmetry factor of 1.5. As the difference in peak areas increases, the problem of quantification becomes worse. Whereas the case of a 0.1% peak following a symmetric major peak (Figure 1c) was no problem, the minor peak is lost under the tail of the first peak when even a small amount of peak tailing occurs (Figure 1d). Clearly, quantifying minor peaks in the presence of large tailing peaks requires much more separation of peak centers than it does in the presence of symmetrical peaks.

Is It Pure?

Another disadvantage of tailing peaks is that they reduce chromatographers' confidence that all peaks have been separated. It is not obvious, in a case such as that shown in Figure 1d, if a small peak is or isn't present. Is the tail a result of normal peak tailing processes, or is it caused by the close elution of a smaller peak such that the merged peak pair merely looks like a tailing peak?

If a diode-array UV detector is available, analysts can use peak-purity calculations to help determine whether a second peak is present. Each manufacturer calculates peak purity using a different method with proprietary algorithms, but the basic principle is the same. UV scans are performed at various places on the peak — generally on the up slope, on the down slope, and at the peak apex. These scans are compared mathematically to determine any difference in the spectra. For example, the minor peak on the trailing edge of the large peak of Figure 1d would distort the spectra of the main peak, so a spectral difference would be detected.

Although manufacturers have some very convincing data that show determination of small peaks in the presence of large ones, several problems can confuse peak-purity calculations. The more similar the spectra of the two peaks are to each other, the more difficult it is to find a difference. Many times, the minor peak is eluted close to the major one because it is chemically similar and often has a similar spectrum. The signal-to-noise ratio for the small peak might be small, so it compromises the quality of its spectrum. With small peaks, baseline irregularities can distort the peak as well. So although diode-array peak-deconvolution techniques can be useful, they are not foolproof. Of course, if the spectra of the two peaks are quite different, it could be possible to determine the minor peak merely by changing the detection wavelength.

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In a similar manner to the diode-array scans, mass spectrometry (MS) detectors can help determine if a peak is pure or whether a minor peak is hiding under a major one. Although it is a powerful technique, MS is not an unequivocal solution to peak-purity problems. For example, LC mobile phase often is incompatible with mass spectrometers, as is the case with phosphate buffer.

Another technique that my co-workers and I occasionally use in our laboratory can help determine peak purity. We collect and reinject a fraction of the tail of a major peak. This process can change the relative concentration of the major and minor peaks and reduce the major peak sufficiently to enable detection of the minor peak. Figure 2 illustrates this procedure. The peak pair of Figure 2a is the same as Figure 1d, in which a 0.1% peak is hidden under the tail of a large peak. By collecting a fraction so the major peak is reduced 100-fold, the peak ratio became 10:1, as Figure 2b shows. The presence of the minor peak was confirmed.

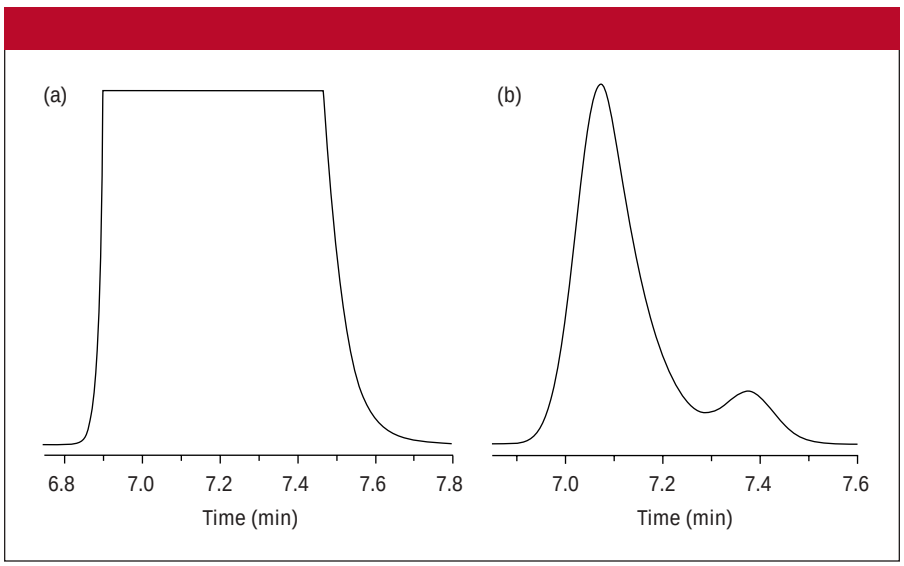

Figure 2: Simulated chromatograms showing (a) the same separation as in Figure 1d and (b) results from the reinjection of a fraction from the tail of a major peak so that the collected peak area is 10:1.

Pulling the Peaks Apart

Once you're convinced that a small peak is hiding under a larger one, the challenge is to separate the two peaks. The general technique is no different than trying to separate any two peaks; however, it might be more difficult if the two peaks have similar structures. Usually, you must explore the variables that are most likely to cause dramatic differences in selectivity. There are two schools of thought about changing variables. One suggests that analysts should make a large change in a parameter so a large change in relative retention of the two peaks will be more likely. The other approach is to make significant but small changes, so users can track the movement

of the small peak. If the chromatogram is simple and has only a few peaks, the first approach can work well. If a chromatogram has many peaks, as often is the case with stability-indicating assays or samples from environmental or biological sources, a large change can move the minor peak from under one peak to a place where it interferes with another peak. When this occurs, chromatographers easily can miss the peak movement and think that a change was ineffective when in fact a major change occurred. Unless I have reason to make dramatic changes, I prefer to start with the conservative approach and then make larger changes if the small ones are ineffective.

I recommend trying to change the solvent strength first, not because it is the most powerful tool, but because it is the easiest to change. For isocratic separations, try increasing or decreasing the organic solvent concentration a few percentage points. With gradients, change the gradient slope or gradient time by 50% and see what happens. Many times these changes will give you an idea of how difficult a separation will be.

Column temperature is a good choice for the next parameter to vary. As with solvent strength, temperature is less powerful than some other parameters, but it is easy to control and can have surprisingly effective results. A change in temperature in combination with a change in solvent strength can be especially useful. Try a change of ±10 °C to determine if the peaks move relative to each other.

Is the tail a result of normal peak tailing processes, or is it caused by the close elution of a smaller peak such that the merged peak pair ***merely looks like a tailing peak?***

After exploring the easy parameters, mobile-phase pH is a good choice to tackle next. Changes in pH can be made in small steps, so it is possible to track peak movement. Try changing the mobile-phase pH by ±0.5 pH units to see what happens to the retention of the peaks of interest. Although the buffering range isn't a major consideration when just exploring pH effects, if you make a permanent pH change in a method, be sure the buffer is operating within its normal buffering range to maintain robust conditions. Most columns also have a pH range of approximately pH 2–8; outside these limits, I recommend using specialty columns for longer column lifetimes.

A change in solvent type also can be tried, if the new solvent is compatible with the detection wavelength. Methanol can be substituted for acetonitrile and vice versa. Tetrahydrofuran is much less popular as an LC solvent today because of its undesirable physical characteristics and strong UV absorbance at low wavelengths. However, tetrahydrofuran often will provide dramatic changes in selectivity when compared with acetonitrile or methanol. The big challenge when changing solvent types is to track the movement of the peaks. One approach is to substitute 5–10% of the existing organic solvent for a new one. This method reduces the magnitude of change and makes tracking peaks easier.

A change in column type is the last parameter to explore. This variable should be the last resort because you cannot make a small change in column type, so when the column is changed, it can be difficult to track the peak movements. I usually try the conventional columns first. My first two choices are C8 (or C18) and one of the embedded polar phases. Other good candidates are cyano and phenyl columns. Generally, you can gain little by changing from one manufacturer's column to another's while keeping the stationary-phase type constant. Plenty of additional stationary phases are available for reversed-phase work, including zirconia-based columns, fluorinated phases, polymeric particles, and so forth. You might be able to improve your separation with one of these or other phases, but your choices could be limited to columns in your current inventory.

If varying the standard reversed-phase parameters fails to generate a successful separation, don't forget that normal-phase LC can yield dramatically different selectivity. Most workers will find normal-phase LC easier to use and more intuitive if they use one of the normal bonded-phase columns such as a cyano or a diol phase. Bare silica can provide larger changes in selectivity, but it also can be more challenging to work with. Optimizing a normal-phase separation is beyond the scope of this discussion. For a good set of directions for normal-phase method development, consult reference 1.

How Do You Know It's Pure?

One of the most difficult decisions to make during LC method development is when to stop. Although chromatographers often can show that a peak is not pure, it is impossible to prove that no minor peaks are hiding under a large peak. The best you can do is to explore enough variables in a stepwise fashion to convince yourself that no minor peaks are present. Using conditions that are likely to yield significant differences in selectivity — such as normal phase, tetrahydrofuran organic solvent, or pH change — will help you establish peak purity. Tools such as diode-array detectors that can perform peak-purity calculations or mass spectrometers can be invaluable in establishing peak purity. I always seem to find just one more experiment to do . . .

Once you're convinced that a ***small peak is hiding under a larger one,*** *the challenge is to separate the two peaks.*

Reference

(1) L.R. Snyder, J.L. Glajch, and J.J. Kirkland, *Practical HPLC Method Development* (John Wiley & Sons, New York, 2nd ed., 1996).

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For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at http://www.chromforum.com.