LC Troubleshooting

Broad Peaks

Do your peaks need to go on a diet?

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Peaks that are too broad can mean that analysts are not using their liquid chromatography (LC) columns very efficiently. Narrow peaks can translate into faster runs, because less time is necessary to obtain baseline separation. This month's "LC Troubleshooting" takes a look at how to determine if peaks are broader than they should be and discusses some of the most common system-related causes of peak broadening. For this discussion, I'll focus on reversed-phase isocratic separations.

What Is Fat?

As we all know from our interactions with others or examinations of ourselves in a mirror, classifying a person as being overweight involves opinion more than quantification in most cases. What constitutes an LC peak that is too broad also involves a certain amount of opinion. However, some simple quantitative measures of peak performance generally serve better than a simple visual examination of the chromatogram to determine if a peak is too broad.

The peak width is a poor measurement of a chromatographic peak, because the peak width increases proportionally with the retention time (t_R) for isocratic separations. The first step in quantifying the broadness of a peak should be to measure the plate number (N) for the peak of interest. The plate number can be measured in one of two ways:

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

or

$$N = 16\ (t_R/w)^2 \qquad [2]$$

where $w_{0.5}$ and w are the peak width measured at half the peak height and at the baseline between tangents drawn to the sides of the peak, respectively. I prefer the half-height method because it is easier, especially if two adjacent peaks are not baseline resolved. Most data systems should be able to determine N using either method.

The literature that comes with a column typically reports plate numbers of approximately 80,000/m for 5-µm d_p media and 100,000/m for 3-µm d_p media. Don't look for this kind of performance with your method, though. The manufacturers test columns under very carefully controlled conditions with test compounds such as toluene and methyl benzoate, which provide ideal chromatographic behavior. A method for analysis of a pharmaceutical compound in plasma, a pesticide in hog fat, or a synthetic intermediate will not behave in such an ideal manner. As a guide for reasonable chromatographic performance with a real compound, I use the following estimate:

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$$N \approx 3000L/d_p \qquad [3]$$

where L is the column length in centimeters and d_p is the packing particle diameter in micrometers. If I observe plate numbers within approximately 20% of this estimate, I don't worry much about column performance. A 15-cm long, 5-µm d_p C18 column probably won't generate more than approximately 9000 plates, even though its manufacturer might report N values of approximately 12,000.

Is Tailing a Problem?

Another measurement that I make before drawing any conclusions about excessive peak broadening is the amount of peak tailing. The two most common measures of peak tailing are the USP (U.S. Pharmacopeia) tailing factor (T_f) and the peak asymmetry factor (A_s). T_f is calculated as

the ratio of the peak width to twice the front half-width of the peak as measured at 5% of the peak height. A_s is the back half-width divided by the front half-width of the peak measured at 10% of its height. Generally, the tailing factor is used in the pharmaceutical industry, and the asymmetry factor for other applications — it doesn't really matter from a technical standpoint which method you use, as long as you consistently use the same method.

Once again, the column manufacturer's test compounds and real samples are different. The test compounds exhibit little, if any tailing, whereas it is rare to have a perfectly symmetric peak with a real sample. As long as the tailing factor or the asymmetry factor is no more than approximately 1.5, it generally is not worth trying to improve. Larger values of peak tailing could mean that unwanted secondary interactions are occurring. Techniques to reduce peak tailing have been discussed in previous "LC Troubleshooting" columns (for example, see reference 1).

Extracolumn Effects

Let's assume that peak tailing is acceptable. You obtained a plate number for a peak that was less than approximately 80% of the value estimated from equation 3. If all the peaks in the chromatogram are well separated, it still might not be worth addressing the problem. If resolution has suffered or if you would like to improve resolution so that you can speed the separation by obtaining the same resolution in less time, then it is time to look more closely at the chromatogram. Measure the plate number for peaks eluted at the beginning, middle, and end of the chromatogram. If the plate number improves with retention time, extracolumn effects are a likely problem source. Extracolumn effects are reflected in the peak volume:

$$V_{tot}^2 = V_{col}^2 + V_{inj}^2 + V_{tub}^2 + V_{fit}^2 + V_{det}^2 \quad [4]$$

where V^2 is the square of the peak volume (in milliliters) and the subscripts indicate the contributions from the column, the injector, the tubing, the fittings, and the detector. The total peak volume is the square root of the sum of the volumetric peak variances of the various components of equation 4. The contributions from the injector, tubing, fittings, and detector (and sometimes a term is added for the time constant or data rate) are called extracolumn effects, because they are factors outside the column.

Consider two scenarios: First, the volume contribution of the column is large compared with the extracolumn contributions. In this case, the percentage contribution to the overall peak volume by the extracolumn effects is small and usually can be ignored. Second, the extracolumn effects are much larger and are significant when compared with the contributions by the column. So, narrower peaks are more strongly affected by extracolumn effects than broader peaks. In an isocratic chromatogram, the earlier-eluted peaks are narrower than later-eluted ones (Figure 1). It follows that extracolumn effects negatively influence peaks with smaller retention times more than they do those with larger retention times. If you observed more broadening (smaller values of N) earlier in the chromatogram, extracolumn effects could be responsible.

Column Size

An additional parameter that plays a very important role in the influence of extracolumn effects is the column size. Just as the peak volume drops with smaller retention times, so do smaller volume columns generate smaller peak volumes. Refer to Table I to see this influence. I've calculated peak volumes based upon equation 3 for peaks with retention factors (k) of 1 and 5 for three column configurations. The 150 mm × 4.6 mm, 5-µm d_p column that most of us use for routine LC–UV work generates relatively large peak volumes, and, unless you are quite sloppy with the system plumbing, it is unlikely that you will notice extracolumn contributions from the tubing, fittings, or detector. If you are suspicious, make sure to minimize excess tube lengths and keep the tubing diameter no larger than 0.007-in. i.d.

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Contrast this column with the 50 mm × 2.1 mm, 3-µm d_p column that generates peaks less than one-tenth the volume of the larger column. A tube end that is poorly seated in a fitting or the accidental use of a piece of 0.010-in. i.d. tubing can dramatically increase the peak width.

If you aren't convinced, make some trial calculations using equation 4 and add 10 µL of extracolumn volume to the column-generated peak volumes shown in Table I. The small peak volumes generated by 2.1- and 1.0-mm i.d. columns are good examples of why it is difficult to obtain column plate numbers that are close to those reported by the manufacturer with test compounds. It is easy to see how chromatographers can get comfortable using 150 mm × 4.6 mm columns for routine work and get into trouble by switching to 50 mm × 2.1 mm columns, unless they take care to minimize the extracolumn contributions from the tubing, fittings, and detector.

What about the Injector?

The sample injection process can contribute to peak broadening in two different ways: First, if too large a sample volume is

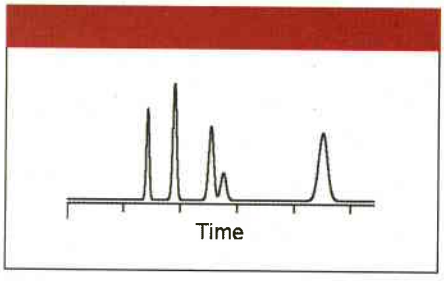

Figure 1: Isocratic chromatogram showing broadening of later peaks.

Table I: Examples of peak volumes for several columns

Column	V_m* (mL)	N†	V_1‡ (µL)	V_5§ (µL)
150 mm × 4.6 mm, 5-µm d_p	1.5	9000	125	380
50 mm × 2.1 mm, 5-µm d_p	0.1	3000	15	45
50 mm × 2.1 mm, 3-µm d_p	0.1	5000	10	35

*Column dead volume.
†Plate numbers obtained using estimate of equation 3.
‡Peak volume for peaks with $k = 1$.
§Peak volume for peaks with $k = 5$.

injected, it can cause increased peak widths. Second, if the injection solvent is stronger than the mobile phase, it can wash some of the sample molecules down the column until the solvent is diluted by the mobile phase.

For the first injection-related contribution to band spreading, think of an extreme case in which the sample volume is the same as the column volume and in which the mobile phase is used as the injection solvent. The first sample molecules injected would migrate a significant distance down the column before the last of the sample molecules were injected. This situation would generate an extremely broad band. The opposite would be an infinitely small injection size in which all sample molecules reach the column simultaneously. Realistic injection sizes are somewhere between these two extremes.

How much sample can be injected without deleterious peak broadening? If a 5% loss in resolution is acceptable, approximately 15% of the peak volume of the first peak of interest can be injected, if the mobile phase is used as the injection solvent. Table I shows that this percentage would allow approximately 20 µL for the 150 mm × 4.6 mm column, but only 2 µL for the 50 mm × 2.1 mm column. Many of the LC–tandem mass spectrometry (MS–MS) methods in my laboratory that use 50 mm × 2.1 mm columns also call for 5–10 µL injections. Extra band spreading caused by this peak volume is expected, but typical chromatograms have only two well-resolved peaks; the additional selectivity of the mass spectrometer enables users to obtain acceptable quantitative results.

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The second injection-related contribution to band spreading is the injection solvent. When a strong injection solvent washes some of the sample molecules down the column and dilutes the sample in the mobile phase, this process smears the injection along the top of the column and causes band broadening and sometimes tailing, distortion, or splitting of all the peaks in the chromatogram (see the discussion in reference 2 for an example). Again, the injection volume and injection solvent strength must be balanced. For example, a 5-µL injection of sample in a strong-solvent mobile phase is unlikely to cause much problem with a 150 mm × 4.6 mm column, but a 20-µL injection might. The best advice here is to determine the effect empirically. When the injection solvent is stronger than the mobile phase, double and halve the injection volume and see if it has any practical effect on the separation and then you can decide if modification of the method is necessary.

On the other hand, if the injection solvent is weaker than the mobile phase, you usually can inject much more than the 15% guideline. This larger injection volume is possible because the migration of peaks in the injection solvent is slower than in the mobile phase, and it has the effect of compressing the peak at the top of the column during injection. This technique of on-column concentration can be a handy tool to enable the injection of a problematic sample.

I remember a method in which the chromatographer needed to inject 50 µL of sample that was extracted into methanol, but the mobile phase was 50% methanol. By diluting the sample fourfold with water, it was possible to inject the same sample mass in 200 µL and avoid the horrible peak broadening encountered when 50 µL of 100% methanol was used as the injection solvent. Again, an empirical test should help guide you toward a suitable injection volume in a weak solvent.

And Finally, the Data System

I mentioned that sometimes equation 4 is written to include a band-spreading term caused by the data system. If the data rate is too slow, an insufficient number of data points would be gathered, and peak broadening could appear. The general rule is that 10–20 data points should be gathered across the peak. If the 150 mm × 4.6 mm column of Table I was operated at 1 mL/min, the first peak would be approximately 0.125-min or approximately 7-s wide. A data rate of 2 Hz or more should be sufficient for this case. The 50 mm × 2.1 mm, 3-µm d_p column would use a flow rate of 0.2 mL/min for the same linear velocity and would generate a peak approximately 3-s wide. To obtain 10–20 data points across this peak, the data rate would have to be 4 Hz or more.

Conclusions

I've considered only a few of the sources of band broadening in this "LC Troubleshooting" column, but these variables are ones that can influence peak broadening in any separation. Control these variables and you can feel fairly confident that you are not doing anything stupid that causes unwanted peak broadening.

Another easily controlled source of band broadening is column temperature. If the column temperature is constant, both axially and radially, you shouldn't have unwanted temperature-related peak distortion. Use a column oven and make sure the solvent at the inlet to the column is within ±5 °C of the column temperature, and you should be safe. (Reference 3 has a case study of extracolumn and temperature effects.)

If you still observe excessively broad peaks after these sources are eliminated, look to chemical interactions such as excessive interactions with surface silanol groups, slow diffusion (especially a problem with high molecular weight samples), or some other sample-related source.

References

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(3) R.M. Minikis and J.W. Dolan, *LCGC* **21**(11), 1050–1054 (2003).

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