LC TROUBLESHOOTING

Extracolumn Effects

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Chromatographic-peak broadening that occurs outside of the column can ruin an otherwise satisfactory separation.

When developing a liquid chromatography (LC) method, chromatographers usually concentrate on adjusting the mobile-phase selectivity so that sample compounds are well separated. This adjustment is perhaps the most important aspect of developing the separation. If extracolumn effects are ignored, however, the job can become unnecessarily difficult. Extracolumn effects can cause unexpected problems when traditional 25-cm columns with 5-µm d_p particles are exchanged for shorter columns containing 3-µm d_p particles. This month's "LC Troubleshooting" covers the basics of extracolumn effects and discusses how to determine them and avoid problems.

EXTRACOLUMN EFFECTS — WHAT ARE THEY?

When a sample band passes through a chromatographic column, it gradually broadens so that a plug injection at the beginning of the column emerges with a Gaussian profile. This band broadening results from diffusion in the solvent, interactions with column particles, and other physical characteristics of the system. We usually don't measure the individual peak widths; instead we measure the plate number of the column, N. For a given column, larger theoretical plate numbers indicate less band broadening (narrower peaks). Generally, we like columns that provide large N values because narrow peaks are easier to separate than wider peaks.

Although we tend to think that all band broadening takes place within the column, it actually occurs throughout the entire LC system through which the sample travels. The sample injector, connecting tubing, and the detector flow cell are the three major sources of band broadening outside of the column.

In a microscopic sense, band broadening is a result of the broadening in each of the system components through which the sample passes. This is usually expressed as

$$W_T^2 = W_i^2 + W_t^2 + W_c^2 + W_d^2 \quad [1]$$

where W_T is the observed (total) bandwidth and W_i, W_t, W_c, and W_d are the contributions to band broadening by the injector, tubing, column, and detector, respectively. Technically, the contribution to band broadening by the fittings should be included, but it is minimal with well-assembled zero-dead-volume fittings and can be ignored.

It is important to use the same measurement units throughout the system when assessing extracolumn effects. Volume units usually are the most convenient; just multiply the bandwidth in minutes by the flow rate:

$$W_{(mL)} = W_{(min)}F_{(mL/min)} \quad [2]$$

Because the volume contributions by various system components are small, bandwidths in this context generally are discussed in microliters instead of milliliters.

SOURCES OF BAND BROADENING — A CLOSER LOOK

Let's look more closely at each of the major contributors to the total bandwidth as noted in equation 1.

Sample injector: If the sample is dissolved in the mobile phase or another solvent of similar strength, the contribution of the injector to the overall bandwidth is about two times the injection volume. (Ideally, this contribution should be ~1.15 times the volume, but the flushing characteristics of sample valves usually result in a larger value.) Thus, a 10-µL loop would be expected to yield a W_i^2 of 20^2, or 400 µL^2. Some tricks can reduce the contribution of the injection volume, including using a weaker solvent for injection or introducing a bubble into the loop to minimize sample dispersion (1).

Connecting tubing: Dispersion in open tubes has been studied very thoroughly and is well defined. For practical purposes in LC, the important factors are the tube dimensions and the flow rate. The connecting tubing's contribution to band broadening can be estimated by

$$W_t^2 = 2000 d_t^4 L_t F \quad [3]$$

where d_t and L_t are the tubing internal diameter and length in millimeters, F is the flow rate in milliliters per minute, and W_t^2 is expressed in microliters squared (2). Thus, 10 cm of 0.01-in. (0.25-mm) tubing used at a 1-mL/min flow rate will give a W_t^2 of $(2000)(0.25^4)(100)(1)$, or 781 µL^2. The equation shows that the contribution to band broadening by the tubing is proportional to the flow rate, so changes in the flow rate can have dramatic effects on this factor. An even stronger factor is the tubing diameter, which has fourth-power dependency.

Column: A well-packed 25-cm column containing 5-µm d_p particles should provide 15,000 theoretical plates, exclusive of extracolumn effects. Similarly, you should expect 10,000 and 5000 theoretical plates from 10-cm and 5-cm columns packed with 3-µm d_p particles. The bandwidth varies with the retention time in isocratic LC, so an equation is needed to convert retention into bandwidth:

$$W_c = 4V_m(1 + k')/\sqrt{N} \quad [4]$$

Here, V_m is the column dead volume (estimated as 100 µL/cm of length for 4.6-mm i.d. columns), and k' is the capacity factor, which can be calculated using the following equation:

$$k' = [(t_R F) - V_m]/V_m \quad [5]$$

So the column contribution to the bandwidth of a peak with a capacity factor of 1 (t_R = 5 min at 1 mL/min) on a 25-cm column (V_m = 2500) is $(4)(2500)(1 + 1)/\sqrt{15{,}000}$, which equals 163 µL; $W_c^2 \approx 26{,}500$ µL^2. The three major contributors to band broadening in the column, therefore, are the column dimensions (or column dead volume, V_m), the retention time (or capacity factor, k'), and the column plate number (N). Each of these factors can play an important role in the overall bandwidth.

Detector: With flow rates in the normal 1–3 mL/min range and with well-designed detectors, band broadening in the detector (W_d) is about eight times the detector cell volume (3). Many cell flow paths are 10 mm long and have diameters of 1 mm, which corresponds to an 8-µL cell volume. This would yield a W_d^2 of $[(8)(8\ \mu L)]^2$, or ~4100 µL^2. The discussion so far shows that the detector cell generally is the most important factor in extracolumn band broadening. Poorly designed detectors and old detectors with large heat exchangers can contribute much more to band broadening than can be calculated using the relationship described above. Smaller flow cells can improve the situation dramatically but usually also reduce detection sensitivity.

PUTTING IT ALL TOGETHER

Now that we've looked at the individual components of band broadening, let's see what it means in practical terms. Table I shows examples of system configurations along with some resolution values. (Note the tubing i.d. conversions: 0.020 in. = 0.5 mm, 0.010 in. = 0.25 mm, 0.007 in. = 0.18 mm, and 0.005 in. = 0.13 mm.) Resolution values are listed for three column configurations. First is the most common column configuration in use today, a 25 cm × 4.6 mm column packed with 5-μm d_p particles; it generates ~15,000 plates and has a void volume of ~2.5 mL. Next is a 10-cm, 3-μm d_p column; it yields ~10,000 plates and has a void volume of 1.0 mL. Finally, values for a 5-cm, 3-μm d_p column are shown; this column provides ~5000 plates and has a void volume of 0.5 mL. The values in the table were calculated using a 1.0-mL/min flow rate.

To obtain the best chromatographic results, the mobile phase should be adjusted to produce k' values of 2–10; the data for the k' value of 5 represent this target. When k' is <1.0, the bands are usually difficult to separate from each other and from the unwanted substances eluted at the column void volume. Early peaks like these are highly susceptible to problems from extracolumn band broadening.

The table also shows data for a peak that has a k' of 15. This value is approximately the maximum acceptable retention for routine analysis because the analysis takes too long and later bands are wider, which sometimes makes detection difficult. (Table I shows, however, that the larger the capacity factor, the smaller the impact of extracolumn effects. As W_c^2 gets larger, the remaining factors in equation 1 become less significant.) To get an idea of how these k' values translate to retention times, peaks having capacity factors of 1, 5, and 15 would be eluted at approximately 5, 15, and 40 min, respectively, on the 25-cm column operated at 1 mL/min.

For each column, we selected reference conditions that yield resolution (R_s) values of 1.25 for a typical system configuration. The examples in Figure 1 show that a resolution value of 1.25 is approximately the minimum acceptable resolution for a routine quantitative method. Many labs prefer resolution values of 1.5–2.0. From a practical standpoint, a loss of 5–10% in resolution can occur without causing a noticeable change in the separation (compare resolution values of 1.25 and 1.15 in Figure 1).

Each of the following examples refers to the cases listed in Table I.

Case 1: This is our reference situation for a standard 25-cm column. A 10-μL injection loop and a detector with an 8-μL flow cell are used. The column is connected to the injector and the detector by 10 cm of 0.010-in. i.d. tubing (5 cm at each end). Note what can happen if a smaller-particle column is substituted in this system without making any other plumbing changes: A significant loss of resolution occurs, especially for early peaks (for

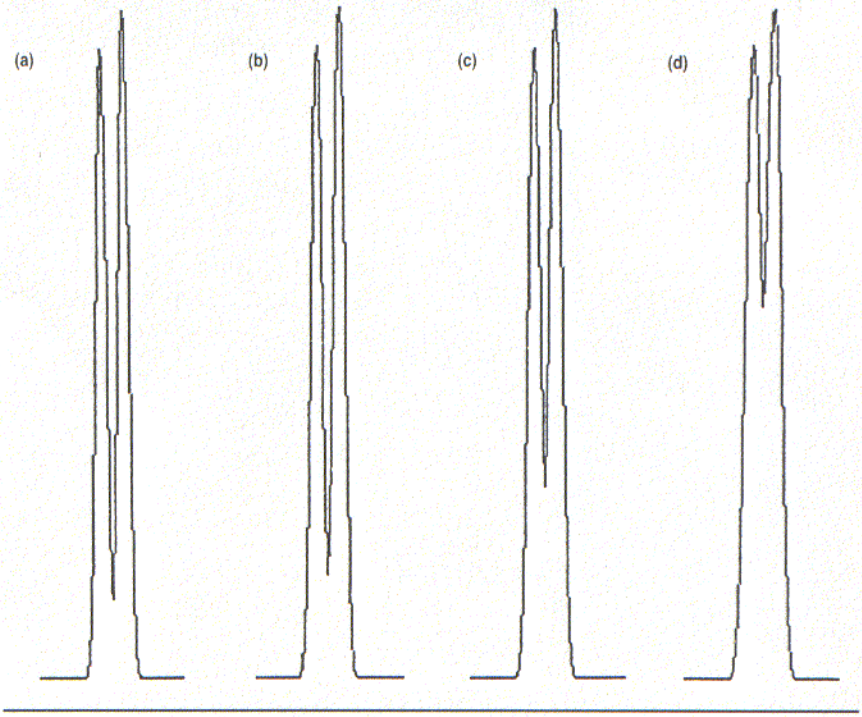

FIGURE 1: Simulated separations with resolution values of (a) 1.25, (b) 1.15, (c) 1.0, and (d) 0.8.

TABLE I: Effect of Extracolumn Contributions on Resolution

Case	Injector Volume (μL)	Tubing Size: Length (cm)	Tubing Size: Inner Diameter (mm)	Detector Flow-Cell Volume (μL)	Resolution Values: 25-cm, 5-μm Column* k' = 1	k' = 5	k' = 15	10-cm, 3-μm Column† k' = 1	k' = 5	k' = 15	5-cm, 3-μm Column‡ k' = 1	k' = 5	k' = 15
1	10	10	0.25	8	1.25	1.25	1.25	0.99	1.21	1.24	0.81	1.16	1.23
2	10	10	0.18	2	1.35	1.26	1.25	1.25	1.25	1.25	1.18	1.24	1.25
3	5	10	0.13	2	1.36	1.26	1.25	1.29	1.26	1.25	1.25	1.25	1.25
4	10	20	0.25	8	1.24	1.25	1.25	0.95	1.20	1.24	0.78	1.15	1.23
5	10	100	0.25	8	1.13	1.23	1.25	0.78	1.15	1.23	0.60	1.06	1.21
6	10	100	0.18	8	1.23	1.25	1.25	0.93	1.20	1.24	0.76	1.14	1.23
7	100	100	0.18	8	0.83	1.16	1.24	0.46	0.94	1.18	0.34	0.78	1.13
8	10	100	0.5	8	0.57	1.02	1.21	0.29	0.70	1.09	0.21	0.54	0.98
9	10	10	0.5	8	1.07	1.22	1.25	0.70	1.11	1.22	0.53	1.00	1.20
10	10	10	0.5	2	1.12	1.23	1.25	0.76	1.14	1.23	0.59	1.04	1.21

*N = 15,000; V_m = 2.5 mL.
†N = 10,000; V_m = 1.0 mL.
‡N = 5,000; V_m = 0.5 mL.

example, a drop in resolution value from 1.25 to 0.99 for a capacity factor of 1 with the 10-cm column). Just because the system works well with a standard 25-cm column doesn't mean it will work well with smaller columns.

Case 2: Reference conditions for the 10-cm, 3-μm d_p column are similar to those for the 25-cm column, but 0.007-in. i.d. tubing is used, and a 2-μL flow cell is used instead of the 8-μL flow cell. When this configuration is used with the 25-cm column, early peak resolution improves, but not dramatically. Similarly, the 5-cm, 3-μm d_p column is only slightly compromised by these conditions.

Case 3: In general, analysts who use 5-cm, 3-μm d_p and other small-volume columns must be sure to minimize extracolumn effects. In these reference conditions, we've selected a 5-μL injection loop and 0.005-in. i.d. tubing to help minimize problems. As expected, we see minor improvements in the performance of the 10-cm and 25-cm columns under these conditions.

At this point, you may ask, "Why not use the minimum plumbing configuration with all of the columns?" Here are several good reasons for not universally minimizing plumbing configurations: Sample-component concentrations often dictate the injection volume, so few options may be available for limiting the injection volume. Sections of tubing <5 cm long at each end of the column are not very convenient for making column connections, and in many cases longer lengths are required. Decreasing the diameter of the connecting tubing increases dramatically its susceptibility to blockage from particulates. (My advice is to avoid 0.005-in. i.d. tubing unless it is clearly required — I find it more trouble than it is worth in most cases.) And as was mentioned earlier, smaller detector cells may reduce band broadening, but they also reduce the signal, which may preclude their use in many analyses.

Next let's look at some cases in which the reference configurations are modified for convenience, because of method requirements, or by mistake.

Case 4: In this situation, 10 cm of connecting tubing would have been sufficient to plumb in the 25-cm column, but 20 cm was used instead. The extra tubing had no practical impact on the separation.

Case 5: An autosampler was used in this case, and 100 cm of connecting tubing was required for connections. We see a noticeable drop in resolution for the early peaks but little or no change for the later peaks. If a long run of tubing is needed, we need to make sure that the method is adjusted for better early peak resolution or that the retention of the bands of interest is sufficient (for example, $k' = 5$) and extracolumn effects are minimal.

Case 6: An alternative to adjusting the method mentioned in Case 5 is to use smaller diameter tubing. Tubing with an inner diameter of 0.007 in. was substituted for Case 5's 0.010-in. i.d. tubing, and resolution was maintained, even for early peaks.

Case 7: When large injection volumes are used, as with the 100-μL injection used here, loss in resolution of the early peaks is inevitable unless some other adjustments are made. The best way to get around this problem in isocratic analysis is to use an injection solvent that is ≤50% as strong as the mobile phase (for example, using 20% methanol as the injection solvent and 50% methanol as the mobile phase). When weak injection solvents are used, bands compress at the head of the column, canceling the band broadening observed up to that point.

Case 8: Sometimes we make mistakes, and pieces of large internal diameter tubing are used by accident. In this example the autosampler was connected with 100 cm of 0.020-in. i.d. tubing. The results are devastating for all but very strongly retained peaks. Even when short lengths of large-bore tubing are used, as in Case 9, the effect can be a significant loss in system performance. If a similar mistake is made with the smaller volume system that uses the 10-cm and 5-cm column, as shown in Case 10, the separation can be ruined. The potential for problems created when 0.020-in. tubing is used in the sample-contacting parts of the system dictates that all of the 0.020-in. i.d. tubing in the laboratory be labeled. An inadvertent substitution such

as this can spoil a day's work wasting time trying to locate the problem source.

SOME FINAL THOUGHTS

In addition to the examples discussed above, it is useful to study other combinations of system configurations and columns shown in Table I to know when plumbing changes are most likely to cause problems and when they are of little importance. Some conclusions that can be drawn from Table I and the above discussion are obvious. First, 0.020-in. i.d. tubing should be used only in the parts of the system that do not contact the sample, such as those that connect the pump to the injector or autosampler. Very small internal diameter tubing (for example, 0.005-in. i.d. tubing) has little practical application, except when very small peak volumes are encountered, as with bands eluted early from small-bore or short columns. On the other hand, 0.007-in. i.d. tubing can be used to improve the band-broadening characteristics of the system without the blockage problems encountered with smaller tubing.

Chromatographers often ask whether most of the tubing should be placed before or after the column when long lengths of tubing must be used. When using the mobile phase as the injection solvent for isocratic analyses, the location of the excess tubing makes no difference. With gradient elution or when weak solvents are used with isocratic work, however, on-column concentration occurs. In these cases, the effects of band broadening in the injector and tubing before the column are for the most part canceled out, so there is an advantage to having the excess length upstream from the column.

With some detectors, use of an electronic filter or time constant can also contribute to band broadening, so excessive time-constant values should be avoided. As a rule of thumb, the time constant should be set to no more than 10% of the baseline bandwidth of the earliest peak of interest (4).

As a practical matter, extracolumn band broadening can be minimized by using small injection volumes (or concentration techniques), short runs of small internal diameter tubing that connect the column to the rest of the system, and a method-appropriate detector cell volume. Furthermore, the data in Table I indicate that extracolumn problems are most severe with peaks eluted early in the run. If at all possible, mobile-phase conditions should be selected so that the first band of interest is eluted at a capacity factor > 2. Fortunately, extracolumn band broadening does not change unless some physical change in the system is made, so when a sudden loss in resolution occurs, look elsewhere for the problem source. This fact suggests that care must be taken to select the appropriate components when reconfiguring a system from applications with conventional 15-cm or 25-cm columns to short, small-particle columns.

For more information about band broadening, consult the general discussion of Dolan and Snyder (5) or the detailed discussion of Bakalyar (2). Band broadening in the detector is covered in detail by Martin et al. (3) and Scott (4).

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