L C T R O U B L E S H O O T I N G

Capacity Factor, Plate Number, Extracolumn Effects, and More

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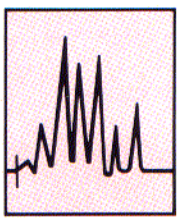

The relationships among capacity factors, plate numbers, and extracolumn effects often are confusing to workers in the liquid chromatography (LC) lab. This month's "Troubleshooting" discussion was prompted by a reader's specific problem with a lower-than-expected column plate number. Also, gradient range changes that were mentioned in earlier "Troubleshooting" columns are further explained.

LOW PLATE NUMBERS

Q: I recently purchased a polymeric reversed-phase semiprep column (30.5 cm × 7 mm, 10 μm). I checked the new column, using phenol and benzene as standards, with a 90:10 acetonitrile/water mobile phase at 1.5 mL/min. I found that for phenol the column plate number, N, was approximately half that reported by the factory (8540 vs. 18,300) (retention time, t_R = 5.7 min). Benzene, on the other hand, (t_R = 10.4) was close to the factory value (6170 vs. 7600). The shape of the phenol peak was symmetrical, and the loss in N seemed to be caused solely by peak broadening, but I would think that if it were a result of extracolumn broadening, both peaks would be equally broadened. A new column from the supplier gave similar results. Do you know what is going on?

JWD: At first glance, this looks like a classic case of extracolumn effects. After examining it closely, however, I realized that the problem is with the mobile phase and that you probably would find similar results with any LC column, not just a polymer-based one.

When bands early in the chromatogram are broadened more than later bands, you should suspect extracolumn effects. In this case, however, I think you were right to rule them out as the problem's cause — but for the wrong reasons. The idea that extracolumn effects cause equal broadening for all bands is a common misconception. I'd like to discuss extracolumn effects in detail because many workers don't understand the importance of checking the capacity factor, k', of bands in the chromatogram.

The total bandwidth of an LC peak can be expressed as

$$\sigma_F^2 = \sigma_C^2 + \sigma_{EC}^2 \qquad [1]$$

where σ_F is the final (total) bandwidth, which is made up of the band broadening within the column (σ_C) and the extracolumn band broadening (σ_{EC}). We know that for isocratic separations, the later the bands come out, the broader they get. In other words, σ_C is retention-related. On the other hand, σ_{EC} results mostly from the plumbing and the detector cell volume, which are constant for one system setup. Consider the two extremes. First, if a band elutes very early, σ_C will be small (narrow bands), and σ_{EC} will be more important; σ_F will be dominated by σ_{EC}. At the other extreme, for a sufficiently retained peak, σ_C will be large when compared to σ_{EC}, so σ_F will more closely reflect σ_C.

At what point is σ_{EC} important? Generally, the k' value should be at least 3 and preferably >5 for measurement of the plate number. The capacity factor is calculated in the normal way:

$$k' = \frac{t_R - t_0}{t_0} \qquad [2]$$

where t_R is the retention time and t_0 is the column dead time.

How do your observations fit into what we've just discussed? First, we must know the k' of each band. You've indicated t_R for your bands but not t_0; t_0 often is recognized as the first disturbance in the chromatogram, and by calculating or estimating t_0 you can verify that. With 4.6-mm i.d. columns it is easy — the column dead volume, V_D, is ~0.1 mL/cm. Thus, a 15-cm column will have about 1.5 mL of dead volume (t_0 is V_D/F, where F is the flow rate in mL/min). Your column is 7-mm i.d., so we'll have to calculate a typical t_0 value from equation 3:

$$t_0 = 0.48\, L\, d_C^2/F \qquad [3]$$

where L is the column length (in cm) and d_C is the column i.d. (in cm). (Note that this is an *estimate* of t_0; the equation must be adjusted for particle pore size to yield a true t_0 value.) In your case, t_0 = (0.48) (30.5 cm) (0.7 cm)^2/(1.5 mL/min) = 4.8 min. Now you can calculate k' for phenol: (5.7 min − 4.8 min)/4.8 min = 0.2; k' for benzene is 1.2. Phenol is practically unretained, so from equation 1, we expect σ_{EC} to dominate. This matches your observations — the measured plate number is low by a factor of two. As retention increases, σ_{EC} is less important — that is reflected in the improved N value for benzene.

So far, everything suggests that extracolumn effects are the cause of your problem. However, you are using a semiprep column (7-mm i.d.), not an analytical column (typically 4.6-mm i.d.). The peak volume increases with the square of the column diameter, so we would expect that the peak volumes for your column should be about 2.3 times those of an analytical column. These larger peak volumes should override any extracolumn effects, *if* the system plumbing is unchanged. Generally, when semiprep columns are used, no plumbing changes are made.

We can look further at your data to see how much of a change in system plumbing is required to account for your observations. These calculations can be performed manually (1,2) or with a personal computer and commercial software (3). From such calculations, we find that only a 5–7% loss in plate number is expected with a "standard" analytical LC system (20 μL extracolumn band broadening). You would have to have about 75 μL (1 σ) of extracolumn band broadening to account for the losses in N that you observed with phenol and benzene. In practical terms, this 75 μL translates into changing from an 8-μL detector cell to a 30-μL cell, which is not standard practice with semiprep columns. So, unless you've made a detector-cell change or are plumbing your system with large-bore (0.020-in. i.d.) tubing, I think extracolumn effects are not causing your problem. (For further discussion of extracolumn effects, see reference 4.)

What went wrong? I suspect that you made up the wrong mobile phase. A chromatogram for the analytical (15 cm × 4.1 mm) version of your column is shown in Figure 1. Using equations 2 and 3, we can calculate k' = 2.5 for benzene.

Notice that this is with 60:40 acetonitrile/water, not 90:10 as you used. As a general rule of thumb, reversed-phase retention increases by a factor of ~2 for a 10% decrease in organic in the mobile phase. It is not surprising that your sample compounds elute much earlier (have lower k' values) than those of Figure 1. Notice that the k' value, not the absolute retention time, is the important

factor. This is because k' is independent of the column volume and flow rate, whereas retention is not.

One final comment on k' and N: I don't have much faith in plate numbers that are reported for bands with k' values <1. This is illustrated in the chromatogram of Figure 1 and in the Knox plot of Figure 2. It is very difficult to measure the half-height bandwidth for a band that elutes with $k' < 1$ — for example, the phenol peak of Figure 1. The resulting measurement errors can have profound effects on the calculated plate number. Because of extracolumn effects, early-eluting bands should have lower plate numbers than later bands (equation 2). Whenever you see results in which early bands have significantly higher plate numbers than later bands, you should suspect measurement errors. That is the case for the factory measured plate numbers that you quoted (N = 60,000 for phenol and 25,000 for benzene) and for the bands plotted in Figure 2. Please note that this is not related to a specific column type or company; it is simply more difficult to measure the width of narrow bands than wider ones. I like to play it safe when I make plate number measurements: I use bands with k' values of at least 4, and I turn up the chart speed on the recorder so that measurement errors are minimized.

To review: First, extracolumn effects should be suspected when early bands broaden more than later ones. Second, extracolumn effects are less important with larger columns, as long as the extracolumn plumbing is not changed. Third, the k' value is more important than the retention time for determining whether a band is sufficiently retained for "good" chromatography ($1 < k' < 20$). Finally, when plate num-

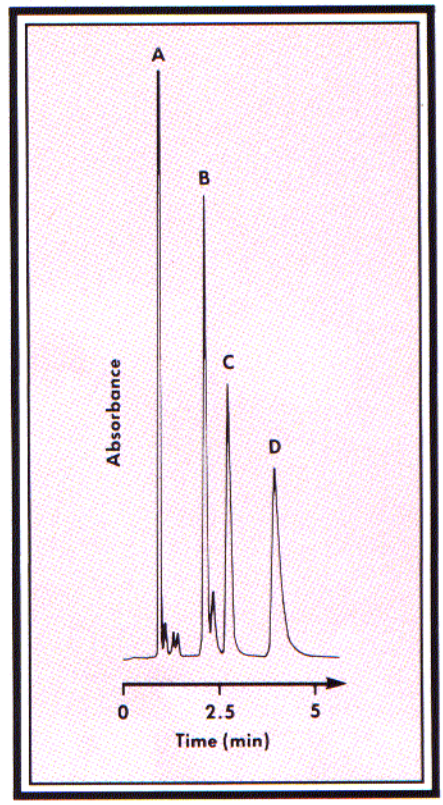

FIGURE 1: Separation of a mixture of aromatic compounds. Column: 15 cm × 4.1 mm 5-μm polymeric reversed-phase; mobile phase: 60% acetonitrile–water; flow rate: 2 mL/min; detection: UV 254 nm. Peaks: A = phenol, B = benzene, C = toluene, D = cumene. (Courtesy of Hamilton Co., Reno, Nevada.)

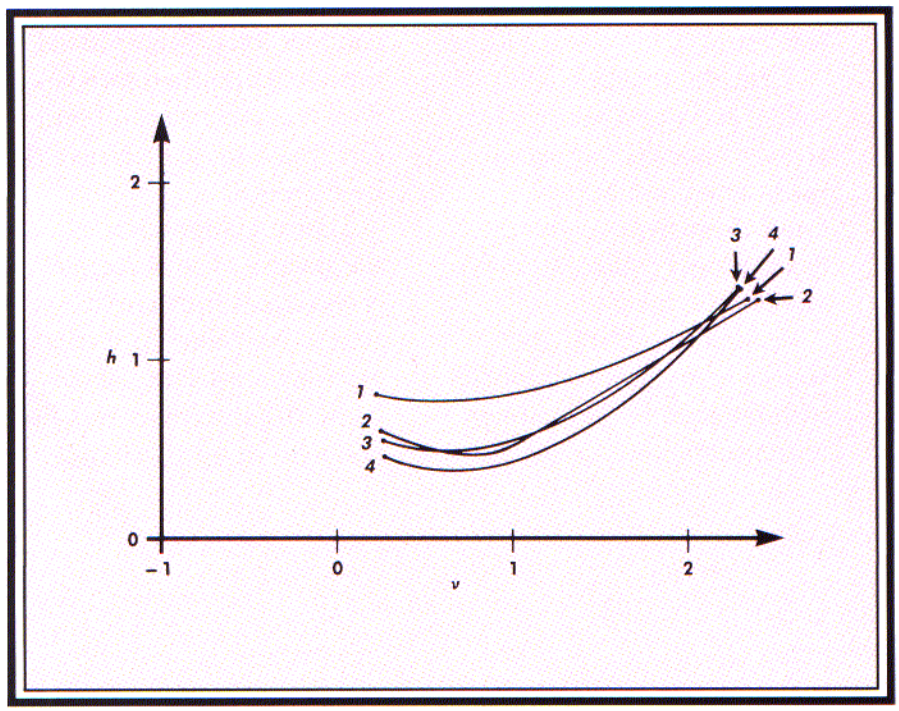

FIGURE 2: Reduced plate height (h) vs. reduced velocity (ν) for test compounds separated on columns similar to that of Figure 1. *1*: k' = 4.08; *2*: k' = 0.64; *3*: k' = 1.20; *4*: k' = 0.23. (Courtesy of Hamilton Co.)

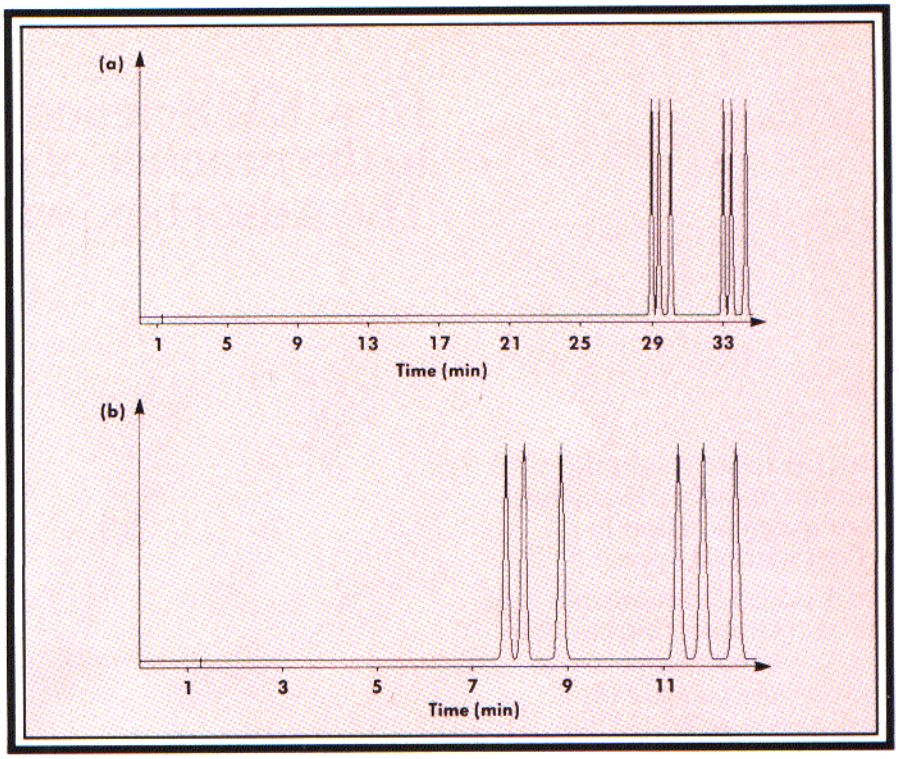

FIGURE 3: Simulated separation of six steroids on a C8 column. (a) Optimized full-range gradient, 5–100% methanol–water in 53 min; (b) optimized gradient with reduced range, 45–64% methanol–water in 11 min.

bers are measured, the mobile phase should be adjusted so that the bands of interest elute with k' values of ⩾4 in order to minimize measurement errors.

CHANGING THE GRADIENT RANGE

A reader (5) has pointed out a possible area of confusion in an earlier "Troubleshooting" column on gradient elution LC separations (6). Equation 1 of that article, repeated here as equation 4, states that the average k' in gradient elution (that is, the k' value when the sample has traveled halfway through the column) remains constant as long as the gradient range (Δ%B) is constant (all other factors unchanged):

$$k' \cong (\text{constant})\, t_G F/(\Delta\%\text{B})\, V_m \qquad [4]$$

Actually, this is not true in all cases. For example, suppose your first gradient run uses a 10–50% organic range (Δ%B = 40). You will certainly have different average k' values if you use a 50–90% organic range, even though Δ%B stays constant. There are many cases, however, when you can change the gradient range without adversely affecting the separation. That is illustrated in the two chromatograms of Figure 3, which are computer-simulated gradient elution runs (7). In Figure 3a, the separation is satisfactory, but about 25 min of run time is wasted at the beginning of the chromatogram. In Figure 3b, we reduced the gradient range from 95% to 19%. According to Equation 4, we must reduce the gradient time (t_G) from 53 min to 11 min (I've rounded to integer values). Now we have a method in which the last band elutes in about one-third the time it did originally, and we've eliminated most of the wasted time early in the chromatogram — yet the band spacing (and average k') is unchanged. This is true because in gradient elution, bands undergo no significant migration until the mobile phase reaches a certain strength. Therefore, if we can eliminate the time early in the gradient when nothing elutes plus the time after the last band elutes, we can significantly shorten chromatograms such as the one in Figure 3a. Backing up 5% in organic from the mobile phase in which the first peak elutes will get rid of the waste at the beginning of the run. Stopping the gradient as soon as the last band elutes will eliminate the waste at the end of the run; that is demonstrated in Figure 3b.

You can see that it is possible to change Δ%B without changing the resolution, but it must be done correctly. (Note: If you are looking at reference 6 for the first time, please note the correction printed in reference 8.)

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