LC Troubleshooting

Worth Repeating

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Like a dental checkup, a regular review of common LC problem sources helps remind us to strive for better separations.

I am often asked where I get my source material for this column — it's from you. Another popular question is how often I rerun my "LC Troubleshooting" columns — I've never run the same column twice, although certain problem types are common enough that they are covered in one way or another on a fairly regular basis. One such problem relates to peaks appearing too early in the chromatogram. I discussed the diagnostic tools for retention problems a couple of years ago (1), and you may find that reviewing that column will complement this month's installment.

A month rarely goes by in which I am not asked a question that points to retention time problems. The complaints are poor resolution, tailing peaks, or interferences. When I inspect chromatograms for problems, retention factor is one of the first diagnostic tools I use. Most of us are familiar with *capacity factor,* but as we learned in July's *LC•GC* (2), the preferred International Union of Pure and Applied Chemistry (IUPAC) nomenclature is now *retention factor,* with the abbreviation k instead of k', so we'll have to deal with unlearning as well as learning.

RETENTION FACTOR

The retention factor is a measure of the distribution of a sample component between the mobile and stationary phases. The larger the retention factor, the more time the component spends in the stationary phase relative to the mobile phase, thus it is eluted later. Major factors affecting retention times are changes in the column dimensions, flow rate, and mobile-phase composition. I like to use the retention factor as a tool to look at retention times independent of these changes. The result is a diagnostic tool that helps determine the likelihood of problems with a given separation.

The retention factor is defined as:

$$k = (t_R - t_M)/t_M \qquad [1]$$

where t_R is the retention time of the peak of interest (generally measured in minutes or seconds) and t_M is the mobile-phase holdup time. (IUPAC suggests the use of t_M rather than the more traditional t_0, which we referred to as column dead time.) The retention time is readily available from the data system. We can identify t_M as the first disturbance in the chromatogram, but an estimate of t_M is just as useful for diagnostic purposes. Just use the relationships

$$V_M \approx 0.1L \qquad [2]$$

and

$$t_M = V_M/F \qquad [3]$$

where V_M is the volume of mobile phase in the column in milliliters, L is the column length in centimeters, and F is the flow rate in milliliters per minute. Equation 2 is true only for 0.46-cm i.d. columns, which is the most common size. For other column diameters, it will probably be easiest to measure t_M directly. Thus, for a standard 25 cm × 0.46 cm column, $t_M \approx 2.5$ min at 1 mL/min.

Now we can calculate (or estimate) the retention factor for any peak in the chromatogram. For example, a peak with a retention time of 10 min on a 25 cm × 0.46 cm column operated at 1 mL/min would have $k \approx (10 - 2.5)/2.5 = 3$. For our purposes, estimates of k within ~0.5 units are fine.

RESOLUTION

To make practical use of the retention factor in problem diagnosis, we need to see how it fits into the overall separation process. The resolution equation is most useful for this:

$$R_S = 0.25N^{0.5}(\alpha - 1)[k/(1 + k)] \qquad [4]$$

Here R_S is the overall resolution or separation, N is the column plate number, and α is the selectivity. For the current discussion, we are only concerned with the relationship between the retention factor and resolution. This is illustrated in Figure 1.

RETENTION FACTOR VERSUS RESOLUTION

The plot of Figure 1 shows that there can be dramatic changes in resolution with small changes in retention if the retention factor is small. For example, if $k < 2$, a small change in retention may double or halve the resolution. For a routine method, this is clearly something to avoid. A small error in mobile-phase preparation, or even a temperature change of a few degrees, can be enough to make a previously good method unusable.

When $k < 1$, other factors also have a negative influence on the separation. One group of these factors is extracolumn effects. For most separations performed with 15- or 25-cm columns, we ignore the potential for problems resulting from extracolumn effects because the peak volumes of reasonably retained samples are sufficiently large that extracolumn effects

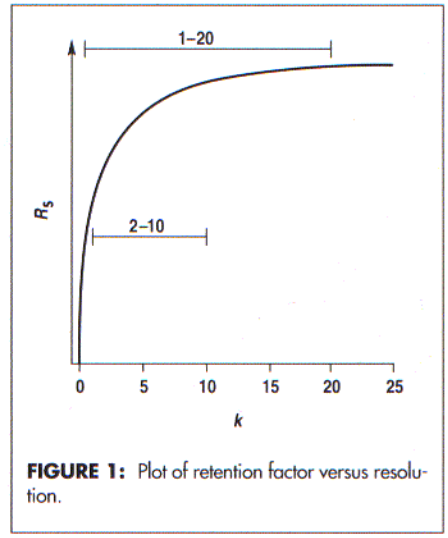

FIGURE 1: Plot of retention factor versus resolution.

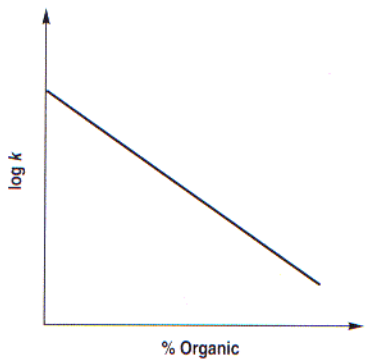

FIGURE 2: Plot of log (retention factor) versus mobile-phase %-organic.

have no practical importance. (The peak volume can be measured by multiplying the baseline peak width in minutes by the flow rate in milliliters per minute.) When we make changes that result in smaller peak volumes, the potential for extracolumn effects is more pronounced. Physical changes to the system, such as using columns with inner diameters < 0.2 cm or columns shorter than ~10 cm, raise the risk of extracolumn effects. A more subtle way of reducing the peak volume is to reduce retention time. Earlier eluted peaks are narrower, so they have smaller peak volumes. Because the broadening effect of extracolumn volume is more pronounced for narrow peaks, one clear sign of extracolumn problems is the broadening of early peaks relative to later ones.

Another problem plaguing early peaks is interference caused by extraneous sample components. Often this garbage consists of highly polar materials that are eluted in a large tailing peak. This result makes quantitation difficult for peaks that are eluted during the first few minutes of a chromatogram. Chromatographers using a method that normally elutes analytes with $k < 1$ can be in trouble if the sample matrix changes unexpectedly.

Even if the system is free of extracolumn effects and sample-matrix problems, equation 4 and Figure 1 show us that resolution will be poor for early peaks. For the retention factor, the more-is-better philosophy holds true — up to a point. Figure 1 also shows us that if $k > 20$, there is little increase in resolution for a given increase in retention.

The Law of Diminishing Returns rapidly catches up as retention time increases. Three problems are apparent. First, and perhaps least important, is the observation that little gain in resolution is made when retention changes occur at $k > 20$. Second is the negative effect of increased retention times, and thus longer run times. This is especially important with high-throughput routine methods. After all, time is money. Finally, quantitation is compromised as retention increases. The longer a compound is retained, the broader the peak becomes. Because area is conserved, this means that the peak height is decreased. The short, broadened peaks are more difficult for a data system to quantitate than narrow, sharp peaks, so detection limits are generally poorer for late-eluted peaks.

GETTING THE MOST

Now that we've reviewed the problems, the middle region of the plot in Figure 1 is clearly the best place to work. If you are trying to develop or improve a separation, the most dramatic changes in resolution will occur when changes in retention are made for $2 < k < 10$. The $1 < k < 20$ region is acceptable for many applications, but it is more prone to the early and late-elution problems discussed earlier. If your goal is a rugged method that is less susceptible to resolution changes if small retention changes occur, you may want to work in the flatter portion of the curve ($5 < k < 10$). Sometimes you can speed up a separation by increasing the retention factor to increase resolution, and then increasing the flow rate to reduce the retention time. As is noted below, increasing the retention factor is no guarantee of improved resolution, but the relationship in Figure 1 yields a good first assumption.

CONTROLLING RETENTION

So we know what retention factors are desirable for the best resolution — how do we control the retention? The plot of Figure 2 shows the general relationship between retention (expressed as log k) versus the organic content of the mobile phase for reversed-phase isocratic separations. The slope of the plot varies by a factor of approximately four for the small compounds (<1000 Da) that most of us analyze. This slope variance can lead to peak reversals and other changes in relative retention when the mobile-phase strength is changed (which can be an advantage during method development). However, the general pattern of decreased retention with increased mobile-phase organic is true. This generalization leads to what I call *the rule of three*. It states that k changes by a factor of approximately three for each 10% change in mobile-phase organic. Thus, if your first peak of interest is eluted at $k = 0.5$ at 50% methanol:water, a change to 40% methanol:water should increase the k-value to ~1.5. Note that because Figure 2 is a log plot, a 20% change in mobile phase would cause a ninefold change in the retention factor (3×3, not $3 + 3$).

THE COMMON PROBLEM

The retention-related problem that I see most often is caused by small retention times. If you check the methods you now use, I think you'll be surprised at how many of them routinely specify conditions that result in retention times with $k < 1$. In most of these methods, you will see improved resolution, better peak shape, fewer interferences, and better overall method performance if the mobile phase is adjusted so that the first peak is eluted at $k > 1$. As mentioned, larger k-values coupled with higher flow rates can often yield improved resolution without increased run time. Excessive retention times are a less common problem, probably because the peak-height problems are obvious, and most of us are impatient, so we don't like long runs anyway.

WHAT ABOUT GRADIENT ELUTION?

The above discussion focuses on isocratic LC separations, but the same factors hold true for gradient elution as well. You can't measure the chromatographic parameters in the same way, because the mobile-phase strength changes during the run. The result is that the retention factors and peak widths are fairly constant throughout the chromatogram. In fact, gradient elution is often used to overcome the problem of broad, late-eluted peaks. It is still important, however, to make sure that the first peak is sufficiently retained: The retention time of the first peak should be greater than $(t_D + 2t_M)$, where t_D is the dwell time, or time it takes the mobile phase to travel from the point where it is mixed to the head of the column. (Typical values of dwell volume are 0.5–2.0 mL for high-pressure mixing systems and 3–7 mL for low-pressure mixing systems. These volumes provide values of t_D at 1 mL/min of 0.5–2.0 min and 3–7 min, respectively.) Allowing the gradient to reach the column and an additional two column volumes of mobile phase to pass through before the first peak appears is roughly equivalent to making sure that $k > 1$.

REFERENCES

(1) J.W. Dolan, *LC•GC* **9**(7), 466–469 (1991).
(2) L.S. Ettre, *LC•GC* **11**(7), 502–506 (1993).

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