LC Troubleshooting

Obtaining Separations, Part IV: Putting It All Together

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You can predict a separation using minimal experimental data.

In the last three installments of "LC Troubleshooting" (1–3), we examined the three stages of method development and some problems associated with them. This information is useful not only for developing new methods, but also for fixing problematic separations.

Our first goal was to obtain a separation with a reasonable retention range. Our target was to obtain retention factors of $1 < k < 20$ for all eluted peaks. We started with a strong mobile phase and worked to a weaker one until we reached the desired conditions. The *Rule of Three* helped us adjust the mobile phase to produce the desired retention.

The second step was to fine-tune selectivity, either by adjusting k or by switching to another solvent or solvent mixture. This eliminated the problem of overlapping peaks. We used a solvent nomograph to move from one solvent to another while maintaining approximately the same retention times.

These first two steps rarely provide us with the resolution, pressure, and run time we desire in a separation, so we adjusted the flow rate and column parameters to fine-tune the separation. Although all these procedures work for manual method development, the process can be tedious and time-consuming. Fortunately, software and instrument developers have designed tools to automate the process. You could write your own method development software based on a spreadsheet program, but the return on your investment would probably be lower than if you purchased a commercial system. This month we'll examine a technique that combines retention, selectivity, and plate-number information to predict what a separation will look like without performing an experiment.

KEY RELATIONSHIP

The key to simplifying method development lies in the relationship between retention (k) and mobile-phase strength. We'll look at one of these relationships for reversed-phase separations: log k versus percent organic (usually shortened to %B, for the percentage of the B solvent reservoir contents). The general principles hold for log k versus pH, gradient steepness, temperature, ionic strength, additive concentration, and ion-pair reagent. If we plot log k against percent organic solvent (for example, acetonitrile) for a given sample, we obtain a plot similar to the one shown in Figure 1. Each compound in the sample shows a linear relationship between the two variables. Plots for the individual compounds are more or less parallel.

Two features of Figure 1 are of particular interest to our discussion. First, a line is defined by two points. Because each plot is linear, retention times from two experiments of differing %B will suffice for plotting. For example, separations at 50% and 70% acetonitrile will provide all the data necessary to plot log k versus acetonitrile content. After the plot is available, we can predict the retention of each compound under any other mobile-phase concentration. Just find the log k corresponding to the desired %B and extract the retention time using:

$$t_R = t_0 (k + 1) \qquad [1]$$

where t_R is retention time and t_0 is the column dead time (see equation 1 in reference 1). With just two experiments, we can develop the relationship that allows us to accurately predict the retention of every peak in the sample for any concentration of the initial mobile phase — no more trial-and-error attempts to obtain the desired retention range. As an aside, the Rule of Three is derived from plots such as Figure 1, where k changes nearly threefold for each 10% change in organic solvent.

The second key feature of Figure 1 is that the lines for each compound in the sample are not *necessarily* parallel. Parallel lines would indicate that the separation would stay the same when we change %B. Instead, you can take advantage of the nonparallel nature of the plot to find regions of optimal separation. Consider compounds 1 and 2 of Figure 1. At 70% B, the two lines intersect — this means that retention is the same, so the two peaks overlap completely. Below 70% B, peak 2 is eluted before peak 1; above this point, peak 1 is eluted first. Thus a quick visual examination of Figure 1 allows you to determine that the peaks overlap at 39% and 70% B. The separation will be better away from these crossovers (for example, at 55% B). Unfortunately, the

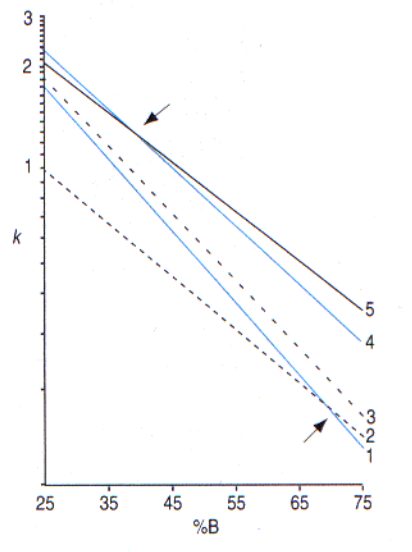

FIGURE 1: Plot of log k versus %B for five hypothetical compounds. Arrows indicate peak crossovers.

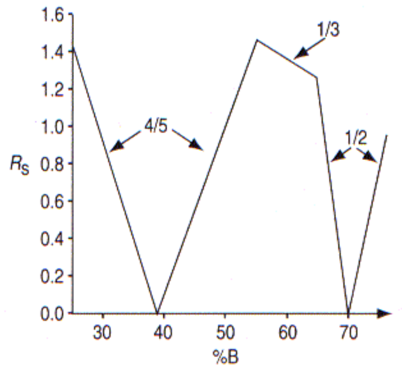

FIGURE 2: Resolution map for data of Figure 1, assuming $N = 10{,}000$. Critical peak pairs are noted on the map.

logarithmic nature of Figure 1 makes it difficult to determine the best separation conditions. This determination is further complicated because we don't know the peak width — after all, peak width is *very* important in resolution.

RESOLUTION

At this point it is useful to reintroduce the resolution equation discussed in reference 3:

$$R_s = \tfrac{1}{4}N^{0.5}(\alpha - 1)[k/(1 + k)] \qquad [2]$$

where resolution, R_s, is related to the selectivity factor, α, and retention factor, k. Recall that α is simply the ratio of k values for adjacent peaks. A plot such as Figure 1 will provide all the information, except plate number, for determining the resolution of any peak pair at any %B. All we need to know now is the plate number for any mobile-phase composition. Fortunately, we can calculate plate number without any additional experimental data. All we need to know are the column dimensions, particle diameter, mobile-phase composition, temperature, and a few other readily available parameters. A simpler, though somewhat less accurate, method is to assume that the plate number is constant for the separation as the mobile-phase content varies. For example, a 15 cm × 4.6 mm C18 column packed with 5-µm d_p particles will generate N of approximately 10,000 for acetonitrile–water or methanol–water mobile phases. Alternatively, average the plate-number measurement for several peaks in the separation.

PUTTING IT TOGETHER

If we use the data of Figure 1 and assume that N is 10,000 for the separation, we can generate a new plot of R_s versus %B to show the resolution for each peak pair of the sample for every concentration of mobile phase. We can simplify the plot if we include only the *critical* peak pairs. We define the critical peak pair as the pair of peaks that is least resolved for any mobile-phase concentration.

Figure 2 shows a resolution map plotted using these data. Now we have a powerful tool at our disposal. One glance tells us the mobile-phase composition that will yield the maximum resolution (55% for Figure 2). We can tell if we can obtain a separation from the present mobile-phase components. For example, if the resolution map shows a maximum R_s of 0.5, we can quit pursuing the current conditions because improving the resolution to 1.5 will take nearly a 10-fold increase in N (equation 2 tells us that R_s increases with $N^{0.5}$). We can also get an idea of how rugged the method is and how it will respond to changes in the variables.

For Figure 2, we can see that selecting 59% B will yield a method tolerant to a change of ±3% B with only a slight change in resolution. What if the resolution map tells us the separation is impossible? Even this information is valuable, and it only cost two experimental runs. It tells us not to waste time varying the current solvent to improve the separation. Instead, we need to make changes to the system selectivity — usually a change in solvent type, as discussed in reference 2. Two more runs will produce another resolution map for the new solvent system.

OTHER VARIABLES

We've concentrated on changes using a single organic solvent, such as acetonitrile or methanol in an aqueous mobile phase. Resolution mapping can be done for several other variables, but we must know the relationship between the variable and retention. Because most of these relationships are nonlinear, resolution maps for other variables generally require three to five experimental data points. Thus, you can determine the influence of one organic solvent, mixtures of organic solvents, pH, gradient steepness, and other variables with a relatively small number of experiments.

COMMERCIAL SYSTEMS

As mentioned above, you can generate resolution maps using spreadsheet software. Commercial software, however, improves ease of use, increases accuracy, adds flexibility, and allows direct access to chromatographic data. Some of these software packages are stand-alone (independent of any particular data system) — other software is integrated into LC system hardware. Reference 4 contains reviews of several of these products and their real-world sample applications.

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