Troubleshooting

Starting Out Right, Part III — The Role of the Solvent in Controlling Selectivity

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LC Troubleshooting Editor

In the first installment of this series (1), the discussion centered on choosing a set of starting conditions that would be likely to obtain a successful liquid chromatography (LC) separation. Although no single set of starting conditions will guarantee success, I explained that most samples will benefit from starting with a C8 or C18 column packing based on Type B silica using a mobile phase of acetonitrile or methanol modified with water or a low-pH buffer.

There's a saying to the effect that if you don't know where you're going, you'll never know when you get there. Addressing a similar concern with LC separations, the second installment (2) emphasized the need to be able to quantitatively measure the attributes of the separation. The specific measurements of interest were retention time (t_R), retention factor (k), peak asymmetry (A_s) or U.S. Pharmacopeia tailing factor (T_f), column plate number (N), and resolution (R_s).

This month, I'll show you how these tools can be used to guide analysts to a successful separation. In particular, the fundamental resolution equation (equation 1) will help evaluate the effect of various selectivity-controlling parameters during method development.

Resolution is the Key

The goal of the chromatographic process is to separate one or more sample components from other materials in a sample. Last month, I described the measurement of resolution. At a resolution of approximately 1.5, the valley between two peaks reaches the baseline. Most workers like to have separations with resolution values of more than 1.7 to provide a little leeway for loss in separation without making the separation unusable. Similarly, when resolution is much greater than roughly 2.0, little is gained for most applications — run times increase without any additional quantitative advantage.

The chromatographic challenge is to separate two peaks.

For purposes of method development, the fundamental resolution equation below is a useful tool to guide the process of achieving a satisfactory separation.

$$R_s = \underbrace{0.25N^{0.5}}_{i}\underbrace{(\alpha - 1)}_{ii}\underbrace{[k/(k+1)]}_{iii} \qquad [1]$$

where

$$\alpha = k_2/k_1 \qquad [2]$$

and k_1 and k_2 are the retention factors of the two peaks of interest. For the balance of this installment of "LC Troubleshooting," we'll concentrate on terms *ii* and *iii* in equation 1.

Before getting into the specifics of controlling selectivity, it is useful to examine the relationship between term *iii* of equation 1 and resolution. If these terms are plotted against each other, as in Figure 1, the justification for some retention guidelines given last month becomes obvious. The data of Figure 1 confirm what most workers already know from experience. If k is quite small, the resolution is poor because peaks get crowded together, especially near the unretained material in the sample. At the other end of the separation, when retention times are quite long, little resolution is gained; however, run times increase

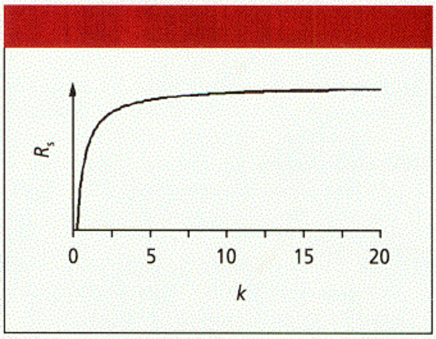

Figure 1: Plot of retention factor (part *iii* of equation 1) versus resolution.

and peaks get broader, shorter, and generally harder to detect. The remaining portion of Figure 1 represents conditions that give reasonable chromatography (acceptable retention times, peak widths, and peak heights), and a change in retention (k) in this region can make a significant change in resolution. This information is the basis of the recommendation that for good chromatography and increased likelihood of an acceptable separation, retention should be kept in the $1 < k < 20$ span, or better yet, $2 < k < 10$.

Finally, equations 1 and 2 show that as retention changes, peak spacing (α) also changes. This relationship is the source of the general improvement in resolution as retention increases (Figure 1). (Note that k in *iii* of equation 1 is the average of k_1 and k_2 of equation 2.)

Adjust Solvent Strength First

Before you worry too much about selectivity, it is important to get the retention time within a reasonable range. With isocratic separations, one simple way to adjust retention is to start with 90% or 100% solvent B (organic solvent) and reduce the concentration 10% at a time. So run 100% solvent B, then 90%, 80%, and so on until retention times fall in the $1 < k < 20$ range desired for good chromatographic behavior. Figure 2 illustrates three steps of this [illegible] centration is reduced from 80% to 70% to 60%, the retention and resolution increase as expected from equation 1 and Figure 1. The column used for the runs of Figure 2 was a 150 mm × 4.6 mm column operated at a 1.5-mL/min flow rate, so t_0 is approximately 1 min. A quick examination of the chromatogram for the 60% B mobile phase allows a retention factor estimation of $2 < k < 7$, which is very good. The peaks are baseline resolved and the run time is short, so the separation doesn't need much optimization.

Note that the peaks in Figure 2 move in a regular fashion as the organic content of the mobile phase is changed. The *Rule of Three* will guide us in the general peak movements. The general rule states that retention factors will increase roughly threefold for each 10% reduction in the organic-solvent content of the mobile phase. The k values are 1.1, 2.7, and 6.9 for the last peak in the runs of Figure 3, so the rule for this particular sample is more like the Rule of 2.5, but as a general guideline, the *Rule of Three* is quite handy. Finally, note that it is threefold for each 10%

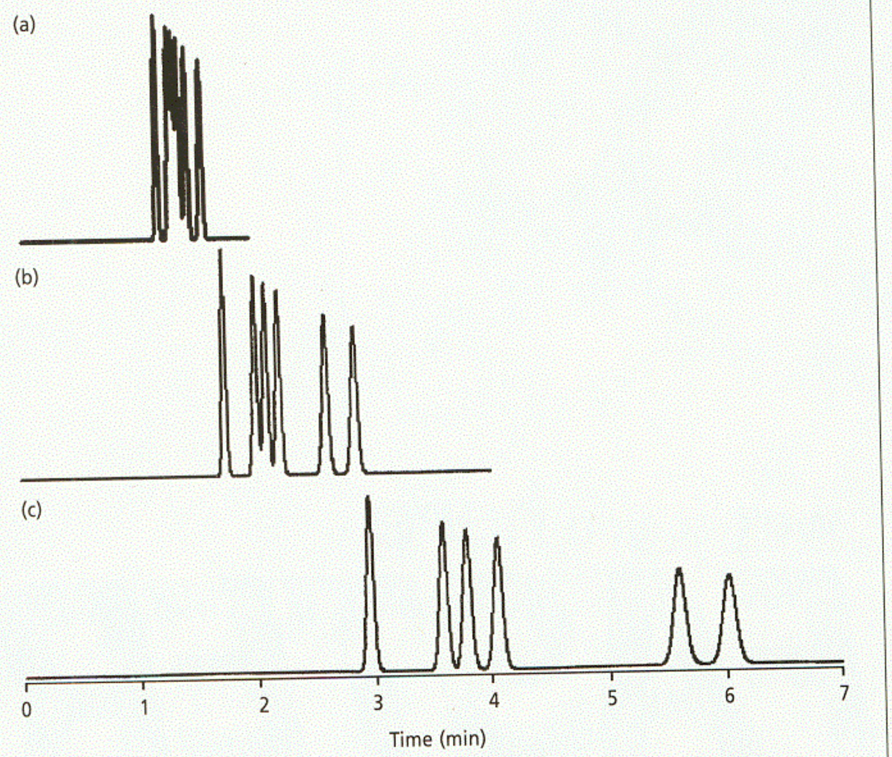

Figure 2: Simulated chromatograms generated using (a) 80% B, (b) 70% B, and (c) 60% B solvent concentrations.

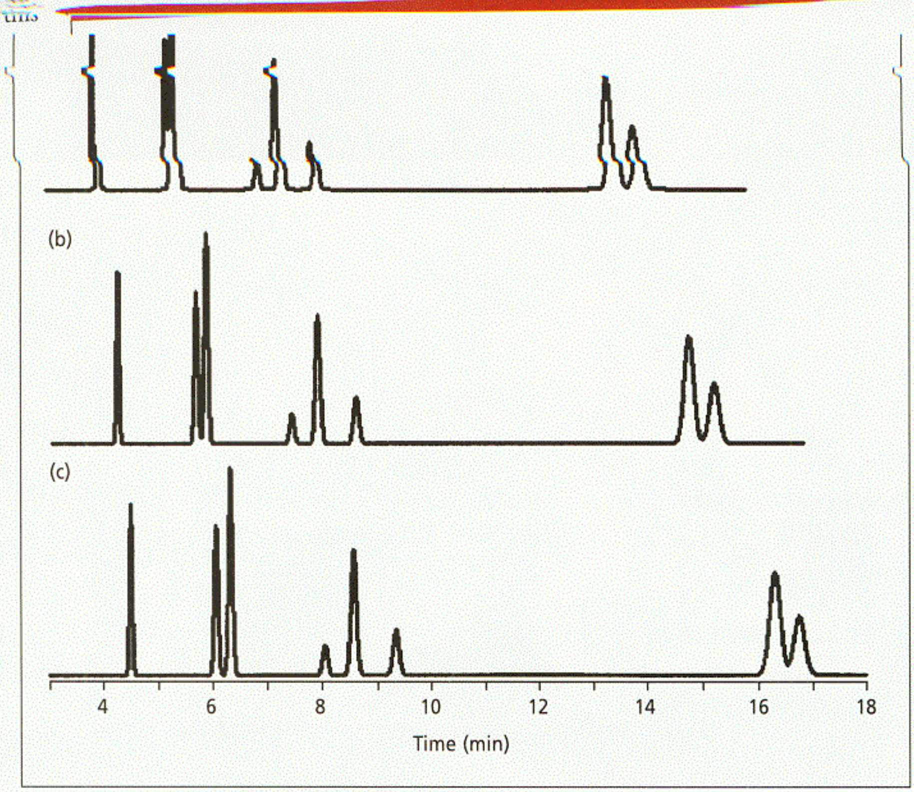

Figure 3: Simulated chromatograms generated using (a) 48% B, (b) 47% B, and (c) 46% B solvent concentrations.

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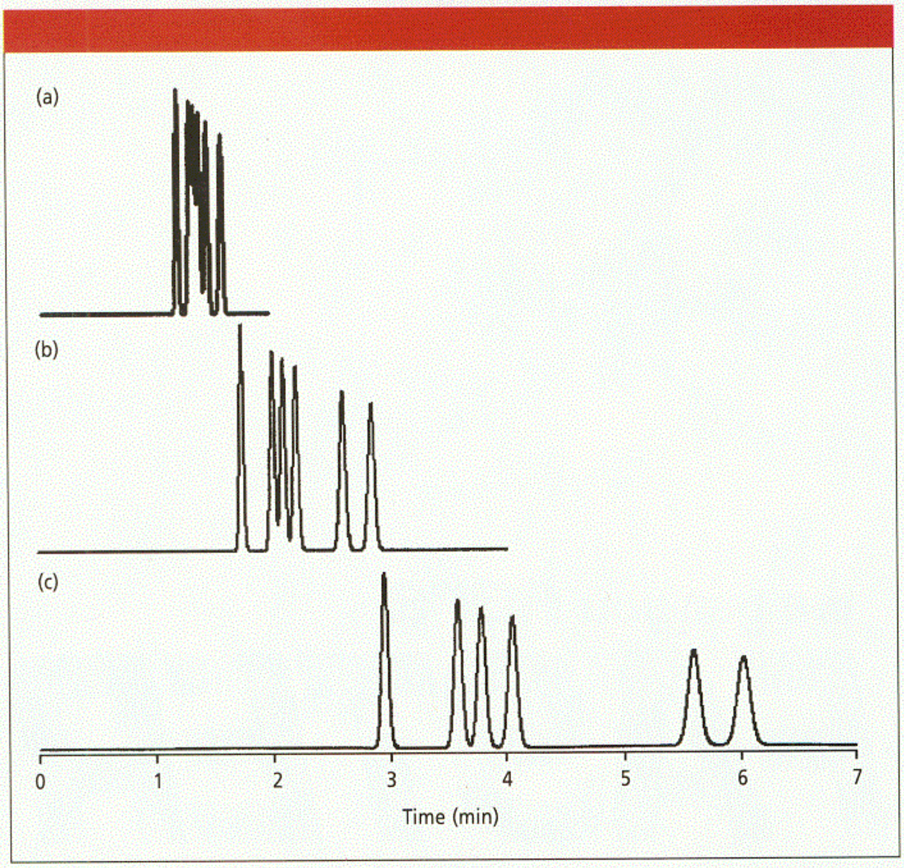

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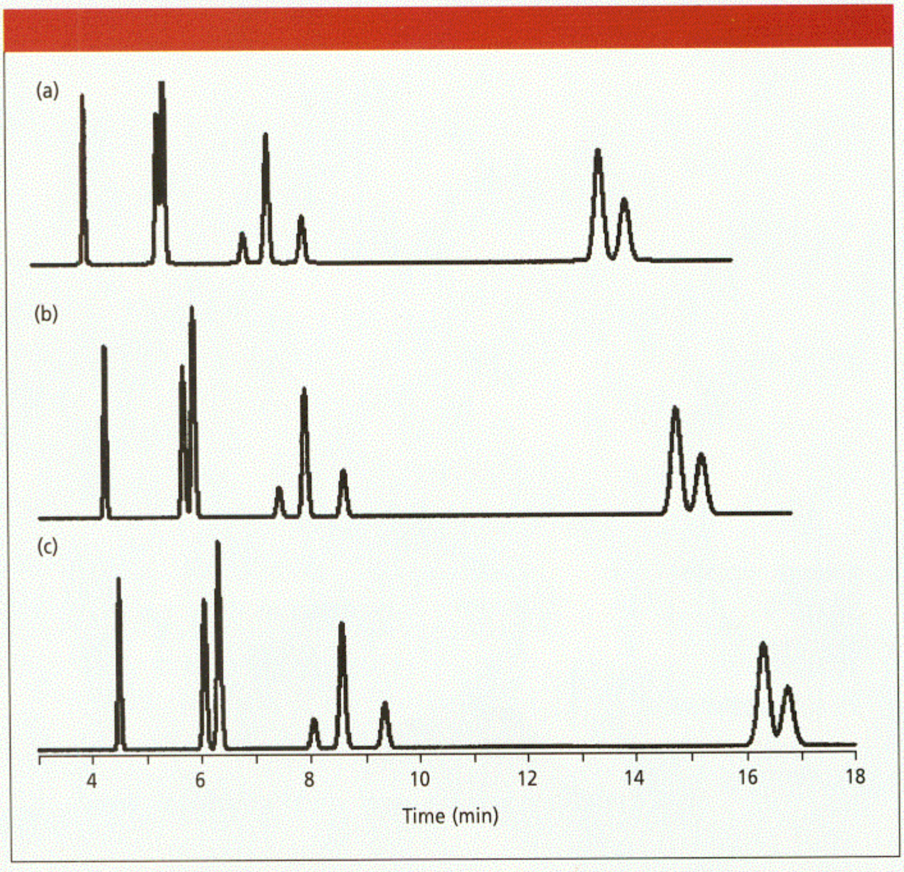


Figure 3: Simulated chromatograms generated using (a) 48% B, (b) 47% B, and (c) 46% B solvent concentrations.

change, so 20% solvent B change would cause a 3 × 3 or roughly ninefold change in retention factor.

The (Not So) Special Case

All the peaks in Figure 2 follow Figure 1's predicted pattern. That is, retention and resolution increase as the mobile-phase strength is reduced. This pattern of change is a generality, but fortunately it does not hold for every compound. The exceptions to the rule are important, because they allow chromatographers to fine-tune the mobile-phase composition and gain selectivity in a separation.

Figure 3 illustrates how you can take advantage of fine-tuning the mobile-phase composition to improve a separation. From the top to the bottom of Figure 3, the mobile phase is made weaker, much as it was in Figure 2. And in the same manner, you can see that the resolution of peaks 2 and 3 improves, and retention time increases with a lower percentage of solvent B. However, notice the movement of the last two peaks in each run. The resolution of this peak pair changes in the opposite way. These changes are common in samples submitted for LC analysis; that is, although the general pattern of longer retention and better resolution with weaker mobile phases holds for many compounds, it is quite common for resolution to decrease under the same circumstances for other compounds.

When you observe peak movements as in Figure 3, you may need to compromise to achieve acceptable separation. In the present case, the resolution of peaks 2 and 3 is better at 46% solvent B, but the last two peaks are better resolved at 48% solvent B. Midway between these two conditions, 47% solvent B, compromises the separation of both peak pairs but gives the best overall separation of this sample.

GUIDELINES FOR ADJUSTING THE CONCENTRATION OF SOLVENT B

- Adjust the concentration of solvent B for acceptable retention range
- Fine-tune the concentration of solvent B for desired resolution
- Change solvent type to change selectivity
- Blend solvents, if necessary
- Selectivity changes between runs can help you evaluate the usefulness of intermediate conditions

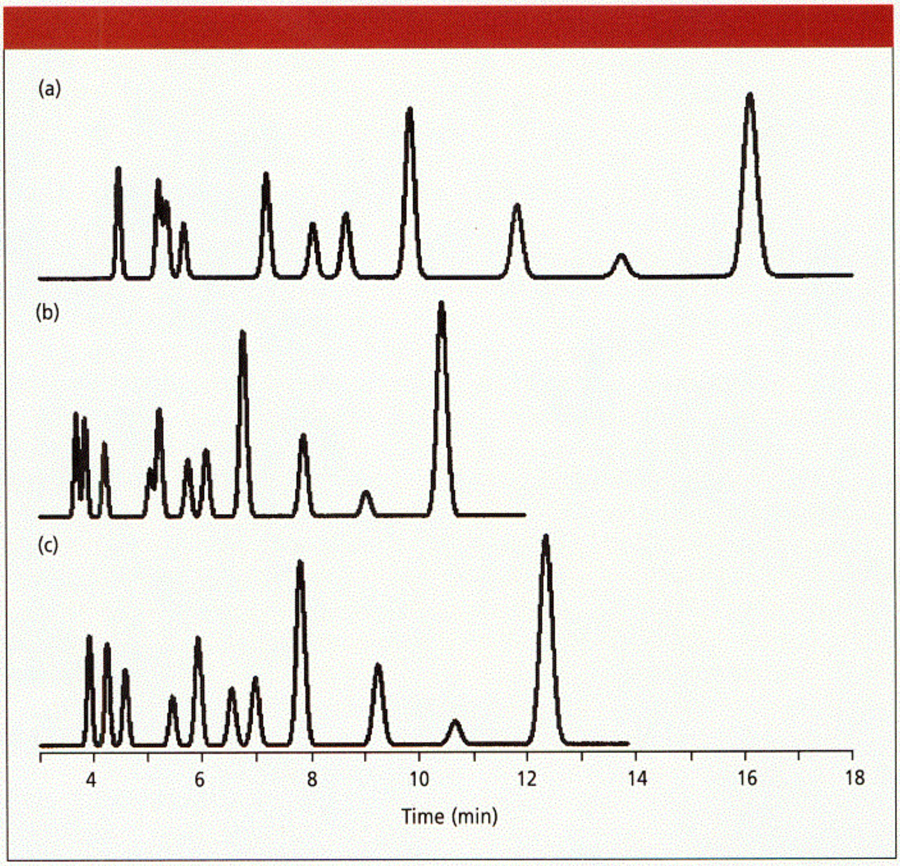


Figure 4: Simulated chromatograms obtained using (a) methanol–water, (b) tetrahydrofuran–water, and (c) blended methanol–tetrahydrofuran–water mobile phases.

Figure 3 shows one other separation feature that you should look for in method development — robustness. Robustness is the ability of a separation to withstand small changes in conditions without compromising the results. The separation at 47% solvent B in Figure 3 certainly is not robust — a ±1% change in organic solvent concentration significantly degrades the separation. In routine use, this method would require undue care in mobile-phase preparation, so these results should alert chromatographers to look for alternate conditions that would produce a more satisfactory separation.

Solvent Type

The adjustment of solvent strength alone can allow you to find satisfactory separation conditions for many samples. For other samples, however, no concentration of acetonitrile will generate a suitable separation. At this point, you should take a different approach for changing α. One powerful way to change α for many samples is to change to another organic solvent in the mobile phase.

For the sample of Figure 4, acetonitrile–water mobile phases were unsuccessful in achieving the desired separation. My next choice in mobile-phase solvents is methanol. Figure 4a shows the best separation with methanol–water (55% methanol). The resolution for most of the peaks in the chromatogram is satisfactory, but peaks 2 and 3 are poorly separated. Fine-tuning the concentration of solvent B, as was done in the previous example, was unsuccessful, so I tried still another solvent.

I substituted tetrahydrofuran for methanol for the separation of Figure 4b, and this figure shows that the resolution of the later peaks in the run is satisfactory. However, the beginning of the run still has trouble. Peaks 1 and 2 and peaks 4 and 5 are poorly resolved. Once again, fine-tuning the concentration of solvent B provided no better results.

At this point, you might want to give up — acetonitrile, methanol, and tetrahydrofuran all failed to provide a satisfactory separation. However, the separations of Figure 4 illustrate another practice in vigilance that should be the habit of successful chromatographers. Note that the critical peak pairs (those most poorly resolved) change between the runs of Figure 4a and

4b. Just as was the case for the sample of Figure 3, an intermediate set of conditions should give peak separations and retention times falling between those observed for the best methanol–water and tetrahydrofuran–water conditions. Indeed, this situation is what I observed. When the isocratic solvents of Figure 4a and 4b were blended, I obtained the separation of Figure 4c. This condition gives the desired baseline resolution of all peaks in the chromatogram.

Summary

The organic solvent plays a major role in controlling selectivity in reversed-phase LC separations. I've summarized several guidelines that you should remember from the examples above.

First, adjust the concentration of solvent B (acetonitrile as the first choice) until all the peaks fall in the $1 < k < 20$ region. In general, resolution will increase with increasing retention, but watch the movement of individual peaks to help determine when fine-tuning of the concentration of solvent B is justified.

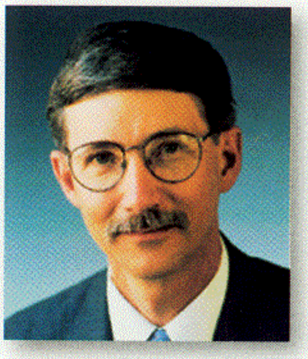
If acetonitrile is ineffective, try methanol and repeat the optimization experiments; if methanol fails, use tetrahydrofuran. Keep relative peak movements in mind as you make these changes, because changes in selectivity between conditions often indicate that an intermediate condition or solvent blend will be useful to pull apart hard-to-separate peak pairs.

Solvent strength and type are two powerful variables that affect chromatographic selectivity, but they are by no means the only variables available. Next month's "LC Troubleshooting" column will highlight some other variables that can be used to control selectivity. I will provide some guidelines about when the use of these variables is appropriate.

References

(1) J.W. Dolan, *LCGC* **17**(12), 1094–1097 (1999).

(2) J.W. Dolan, *LCGC* **18**(1), 28–32 (2000).



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