# LC Troubleshooting

## Obtaining Separations, Part II: Adjusting Selectivity

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*After we obtain reasonable retention times for a separation, we may need to adjust selectivity.*

Last month's "LC Troubleshooting" column (1) covered the basic steps for obtaining an initial separation. The simplest manual technique starts with a strong isocratic mobile phase and proceeds stepwise to weaker mobile phases until the retention range for the sample fits within $1 < k < 20$ (where $k$ is the retention factor). As we saw in last month's column, this procedure requires no special equipment or software and is likely to obtain a satisfactory separation (in terms of retention) in ≤10 runs — a day's work. We also saw that each peak in the chromatogram moved in a regular manner as we changed the mobile-phase conditions. This consistent peak movement provides confidence for interpolating the chromatogram's appearance between two runs.

### WHEN RETENTION ISN'T ENOUGH

We achieved the best separation of the six aromatic compounds in our sample using a 35% acetonitrile–water mobile phase (Figure 1, which is the same as Figure 2f of reference 1). For most purposes, this separation is inadequate because of the poor resolution ($R_s$) for peaks 1 and 2 ($R_s \approx 0.8$). Clearly, in this case retention adjustment alone is insufficient to obtain a satisfactory separation. The next step is adjusting the selectivity or relative peak separation of our sample.

In reversed-phase liquid chromatography (LC), we control retention primarily by adjusting the mobile-phase strength, or organic solvent–water ratio. On the other hand, selectivity is a function of chemical interactions between the sample molecules, the mobile phase, and the stationary phase. The user controls factors such as mobile-phase chemistry (organic solvent, pH, and additives) and the stationary-phase type (for example, C18 versus cyano). For neutral samples such as ours, adjusting the mobile-phase organic solvent is usually the best way to change selectivity. The most popular organic solvents are acetonitrile, methanol, and tetrahydrofuran. Tetrahydrofuran is often the last choice because it is unpleasant to work with, tends to form explosive peroxides, and is much more difficult to flush from the LC system than the other solvents.

### CHANGE TO METHANOL

Our separation was unsatisfactory with acetonitrile–water mobile phases, so we should try methanol–water next. We could start over with 90% methanol and follow the same stepwise procedure to determine the best conditions for methanol. However, we should use the information we gained from the acetonitrile experiments for a more efficient approach. Fortunately, we can generalize solvent strength conversions, although specific retention predictions are usually inaccurate. Figure 2 shows one example of these relationships, a nomograph based on averages for a large number of compounds with differing properties.

Just like with the *Rule of Three* from last month, we can save time by using the solvent nomograph to make useful, but not necessarily precise, conversions from one solvent to another. To use the nomograph for determining the new mobile-phase composition, draw a vertical line from the solvent strength of the current mobile phase (35% acetonitrile in the present case) to the desired solvent system. The nomograph indicates that 45% methanol–water should yield approximately the same retention times as 35% acetonitrile–water mobile phases. We have no information about sample selectivity changes, but experience tells us that a change from acetonitrile to methanol generally affects selectivity.

Figure 3a shows the chromatogram for our 45% methanol–water run. Oops! The chromatogram took approximately three times longer than we expected. Does this mean that the nomograph is no good? Although incorrect for this sample, it is only an approximation and in fact works much better for many other samples. We do, however, see some encouraging results in the chromatogram. First, all peaks are baseline separated ($R_s > 1.5$). Second, the critical (least separated) pair of peaks has changed from peaks 1 and 2 (Figure 1) to

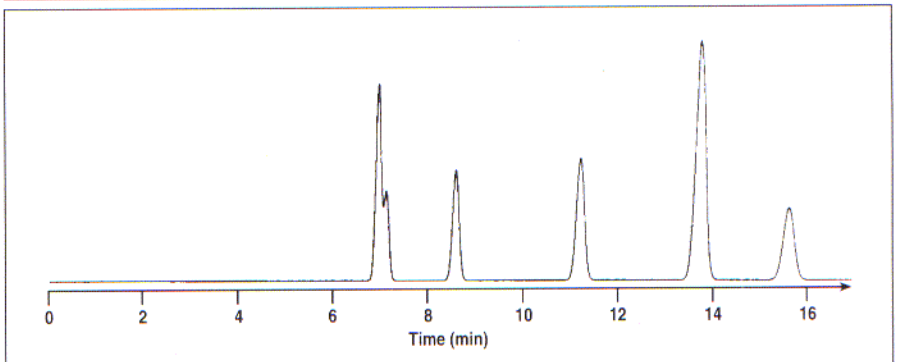


**FIGURE 1:** Separation of six neutral aromatic compounds on a 25 cm × 4.6 mm C18 column using a 35% acetonitrile–water mobile phase at 1 mL/min.

peaks 4 and 5. The retention factors $k$ are acceptable ($6 < k < 20$), but the retention times are longer than we'd like for a six-component sample.

To reduce the retention times, apply the Rule of Three. We need to decrease retention by a factor of approximately three, so an increase of ~10% methanol should work. Figure 3b shows the run for 55% methanol. Now the retention times are better, as are the retention factors ($2 < k < 6$), but the resolution has decreased to ~1.3. By examining the chromatograms of Figures 3a and 3b, we face a tradeoff. As we increase the percentage of methanol, retention decreases (good), but the separation of peaks 1 and 2 also decreases (bad). A robust separation with resolution of 1.7–2.0 will require run times of >50 min with methanol as the organic solvent; we saw last month that $R_s$ values of this magnitude are impossible to achieve using acetonitrile. Therefore, if we want to maintain a reasonable run time, as in Figures 1 and 3b, methanol–water or acetonitrile–water mobile phases will be unsatisfactory.

## SOLVENT MIXTURES?

At this point, we can take two routes to improve the separation. We can try mixing acetonitrile, methanol, and water to see if a ternary mixture will yield a better separation. Alternatively, we can try tetrahydrofuran. We can avoid running the solvent mixtures by carefully examining the results shown in Figures 1 and 3. Just as we found by adjusting the organic strength, changes between organic solvents will yield chromatograms that show regular peak movements, although the changes aren't quite as linear with ternary systems as for binary solvent mixtures. For example, if we blend the mobile phases from our best acetonitrile (35%) and methanol (55%) runs in a 50:50 ratio, we will produce a mobile phase of 17.5% acetonitrile, 27.5% methanol, and 55% water. The run time will be ~17 min, the average of the acetonitrile and methanol runs. However, the resolution will be worse than the methanol run because any acetonitrile added to the methanol mobile phase will yield acetonitrile-like separation characteristics, including poorer resolution. So a quick visual examination of the chromatograms shows us that blending acetonitrile and methanol will not produce the desired results. Our remaining choice to obtain the separation is changing to tetrahydrofuran.

## TETRAHYDROFURAN

We need to determine the starting composition of the tetrahydrofuran–water mobile phase. After the poor predictions for methanol using the nomograph of Figure 2, you may be hesitant about using this technique, but once again we can use our experience to obtain better results. The nomograph converts 35% acetonitrile to ~25% tetrahydrofuran. Similarly, 55% methanol converts to ~35% tetrahydrofuran. Earlier, we saw that the acetonitrile–methanol conversion for our sample was low, so an average of the two tetrahydrofuran predictions would seem reasonable — 30% tetrahydrofuran–water.

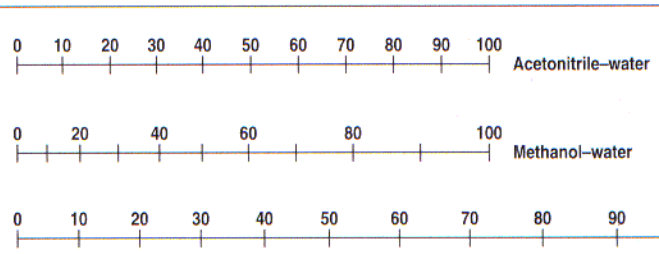


**FIGURE 2:** Solvent-strength nomograph for reversed-phase LC. See text for details. Reprinted with permission from reference 2.

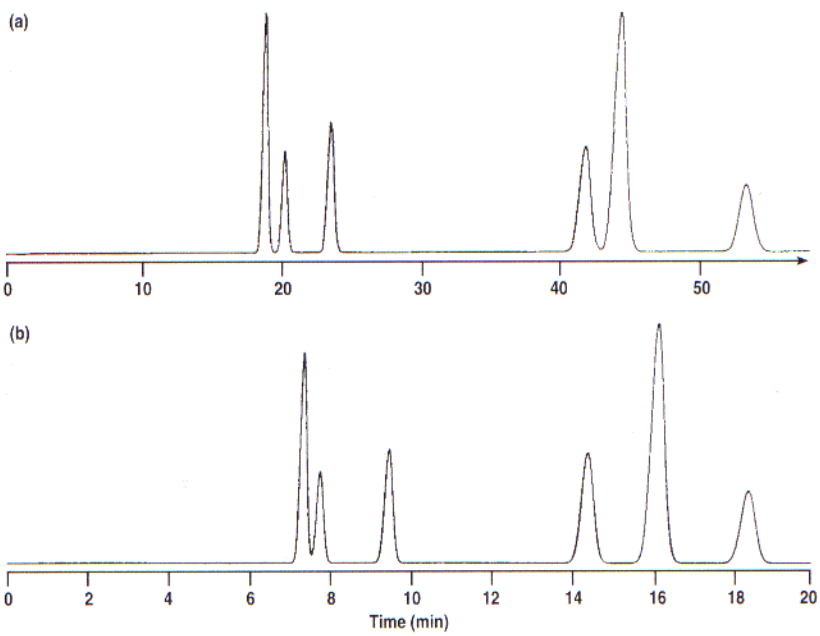


**FIGURE 3:** Separation of the sample of Figure 1 using (a) 45% and (b) 55% methanol–water mobile phases.

Figure 4a shows the 30% tetrahydrofuran separation. This separation is much better than any of the chromatograms we've seen so far. The run time is short and the worst resolution is ~1.7 for peaks 5 and 6. This separation would be acceptable according to our criteria ($R_s > 1.7$, run time < 20 min). Even so, running another separation with a different mobile-phase strength will help us understand how the peaks behave with tetrahydrofuran. We can apply the Rule of Three to choose the appropriate solvent. We probably don't want to decrease the retention any more because the peaks will become crowded, so we should decrease the organic solvent. A 5% decrease in tetrahydrofuran should increase retention by $3^{0.5}$ (~1.7), which should yield a run time of ~20 min. Figure 4b shows the chromatogram for 25% tetrahydrofuran. Resolution has increased to >2 for all peaks at a run time of ~25 min.

By careful examination of the results in Figure 4, we can see that the critical peak pair has changed from peaks 5 and 6 for the 30% run to peaks 1 and 2 for the 25% run. These results indicate that the maximum resolution (when peaks 1 and 2, and peaks 5 and 6 are equally separated) occurs for conditions between these two runs.

We could examine mixtures of tetrahydrofuran and methanol or acetonitrile for further improvements. The tetrahydrofuran–acetonitrile mixtures would suffer the same problems of the methanol–acetonitrile mixtures mentioned earlier. It may be possible to improve the separation with tetrahydrofuran–methanol mixtures because the critical peak pairs change when the solvents are changed. I tend to abide by the chromatography principle "better is the enemy of good enough." The separation using tetrahydrofuran is satisfactory, and a binary tetrahydrofuran–water mobile phase is

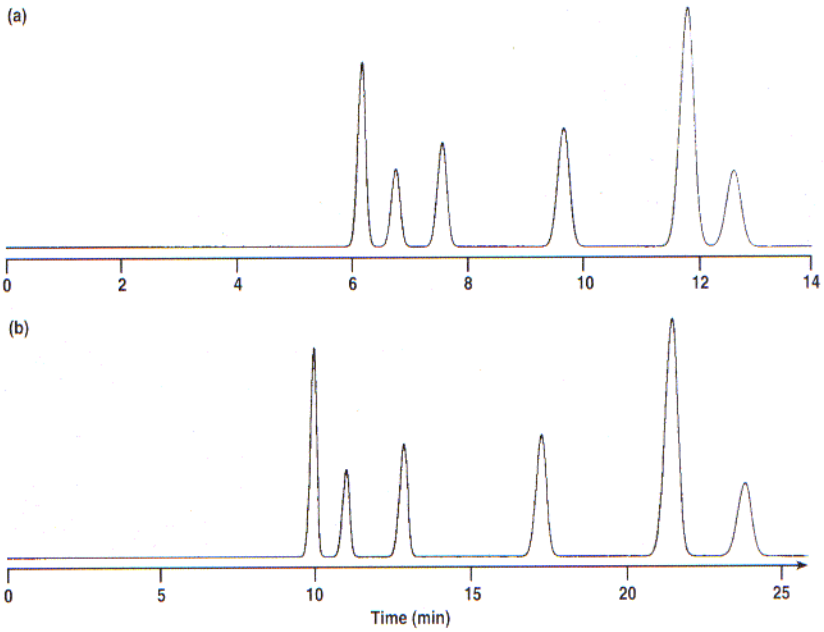


**FIGURE 4:** Separation of the sample of Figure 1 using (a) 30% and (b) 25% tetrahydrofuran–water mobile phases.

simpler to use and less error-prone than a ternary tetrahydrofuran–methanol–water mobile phase. For these reasons, I would disregard the ternary mobile phase.

## SUMMARY

In last month's column, we worked step-by-step to dilute a strong acetonitrile–water mobile phase until we obtained the best possible separation in acetonitrile. This separation was unsatisfactory, so we used a solvent-strength conversion tool (Figure 2) to estimate the methanol–water mobile phase that would yield the same approximate retention with different selectivity. The retention estimates were inaccurate, so we adjusted the mobile phase using the Rule of Three as a guideline to produce the best methanol–water separation. This separation was much better than the acetonitrile one, but it was still inadequate. Comparison of the best acetonitrile and methanol chromatograms (Figures 1 and 3b) led us to conclude that mixtures of these solvents would not improve the separation, so we switched to a tetrahydrofuran–water mobile phase. Once again, we used the transfer rules but adjusted them based on our experience with this sample. The tetrahydrofuran–water separations were satisfactory, so we stopped exploring changes in solvent strength and type.

We made heavy use of three tools in developing this method. First, the Rule of Three enabled us to adjust the solvent strength in an intelligent, time-saving manner. Second, the solvent-strength nomograph helped us transfer from one solvent system to another. Both of these tools offered only estimates, and both were inaccurate, but in spite of the inaccuracies, we saved time. Finally, with the knowledge that peaks move in a regular and predictable manner between two sets of mobile-phase conditions, we predicted what would happen if we performed intermediate runs.

The steps we took to obtain the separation of our sample form the basis of manual method development procedures. Although we only examined solvent-strength and -type changes, the same principles apply for adjusting mobile-phase additives, pH, and temperature. A thorough understanding of this process will allow you to obtain better separations in less time.

Next month's "LC Troubleshooting" column will examine further adjustments you can make to tailor the chromatogram to your requirements and will offer some techniques to further speed up the method development process using software and hardware that you may already have at your disposal.

## REFERENCES

(1) J.W. Dolan, *LC•GC* **12**(5), 368–370 (1994).
(2) L.R. Snyder, J.L. Glajch, and J.J. Kirkland, *Practical HPLC Method Development* (John Wiley & Sons, New York, 1988).

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