

# LC Troubleshooting

## Obtaining Separations, Part III: Adjusting Column Conditions

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*Wait until after you have optimized the mobile phase before you change the column length, particle diameter, or flow rate.*

In the previous two installments of "LC Troubleshooting" (1,2), we examined techniques for obtaining a suitable liquid chromatographic (LC) separation by changing the mobile-phase composition, and we established a logical progression of method development steps. First, we adjusted the mobile-phase strength so the retention factors  $k$  for our sample were in the 1–20 range. This provided the best overall chromatography. Within this range, we adjusted the mobile-phase strength to obtain the best possible separation. We used the *Rule of Three* as a guideline to predictably change retention. Next, when the separation proved to be unsatisfactory, we changed the organic solvent to obtain different selectivity. We used solvent transfer rules to select the composition of this new solvent so that retention times would be approximately the same. When the second solvent also proved to be unsatisfactory, we mentally interpolated between the two solvent systems to see if there was any hope for a separation with a mixed solvent system. We deemed the mixed-solvent system unsatisfactory, so we tried a third organic solvent and obtained a satisfactory separation.

Figure 1 shows the two best separations of our sample of aromatic compounds. When we set out to develop an LC separation, we often

set goals for minimum run time ( $t_{run}$ ), minimum resolution ( $R_s$ ), and maximum pressure ( $p_{max}$ ). For routine methods,  $t_{run} < 20$  min often is an upper limit, but we would like much shorter run times if possible. Baseline resolution occurs at  $R_s \approx 1.5$  for nontailing peaks. For practical separations in which we observe some peak tailing and make allowances for separation deterioration,  $R_s > 1.7$  is a minimum acceptable value. For robust separations, most workers prefer  $R_s > 2.0$ . Pressure limitations often are less important than resolution or run time. After all, most LC systems will accommodate pressures of  $\leq 6000$  psi (400 bar). However, the days of high-pressure LC, when system pressures in excess of 4000 psi were commonplace, are over. High system pressures are hard on the LC hardware — leaks are common, mechanical parts wear more rapidly, and general system reliability is lower. For these reasons, most laboratories use methods with a  $p_{max} < 2000$ –2500 psi. So let's set the targets of our separation for  $R_s$  at 1.7–2.0,  $p_{max}$  at  $< 2500$  psi, and a minimum run time.

### WHAT TO CHANGE?

Figure 1 shows the best separations we could obtain after extensive manipulation of both the mobile-phase strength and type. On the first

line of the 30% and 25% sections, Table I summarizes the separation parameters for these two runs. The 30% run meets our pressure and time targets, but the resolution is marginal. On the other hand, the 25% run provides ample resolution and satisfactory pressure, but it yields a run time that is too long. As we might guess, a solvent strength between these two runs might satisfy all our criteria, and this is true for the 28% run at the end of Table I. However, for the present discussion, let's concentrate on the 30% and 25% runs to see how they can be further optimized.

First, let's see what other changes could improve the separation. Equation 1 shows the factors affecting separation:

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

where  $N$  is the column plate number,  $k$  is the retention factor, and  $\alpha$  is the ratio of  $k$  values for adjacent peaks ( $k_2/k_1$ ). In the previous installments, we saw that  $k$  is related to retention and is controlled by mobile-phase strength. Figure 1 illustrates the influence of  $k$  on  $\alpha$  — the separation of peaks 5 and 6 changes dramatically when mobile-phase strength changes. However, the type of mobile phase has a much stronger influence on  $\alpha$ , as we saw last month with the comparisons of acetonitrile, methanol, and tetrahydrofuran mobile-phase components (2). We have optimized the solvent type and mobile-phase strength, so there is nothing we can do to improve  $k$  or  $\alpha$ . The plate number is the remaining variable at our disposal.

### FACTORS AFFECTING PLATE NUMBER

Four easily controlled factors affect the column plate number: column length, packing particle diameter, mobile-phase flow rate, and column temperature. Because we can vary only the first three factors while holding selectivity constant, we will ignore temperature variations for this discussion. We will assume that we have the following columns at our disposal: 25 cm  $\times$  4.6 mm, 5- $\mu$ m  $d_p$ ; 15 cm  $\times$  4.6 mm, 5- or 3- $\mu$ m  $d_p$ ; 10 cm  $\times$  4.6 mm, 3- $\mu$ m  $d_p$ ; and 5 cm  $\times$  4.6 mm, 3- $\mu$ m  $d_p$ . We must also assume that the packings in these columns are chemically equivalent, a point we'll cover in more detail later.

Table I is a summary of selected combinations of these columns and changes in mobile-phase flow rate for the 30% and 25% runs. Let's work our way through Table I to see how the various factors affect the separation.

Starting out with the 30% run, we see that the initial conditions using the 25 cm  $\times$  4.6 mm, 5- $\mu$ m  $d_p$  column yield a marginal separation. In this case, our goal is to increase resolution without sacrificing pressure and run time. As we might expect, changing to a 15 cm  $\times$  4.6 mm, 5- $\mu$ m  $d_p$  column makes the run worse in terms of resolution. (Remember that plate number is proportional to column length). The 15 cm  $\times$  4.6 mm, 3- $\mu$ m  $d_p$  column improves resolution, but exceeds the maximum pressure target. We can drop the flow rate to reduce the pressure (pressure is proportional to flow rate), which also produces



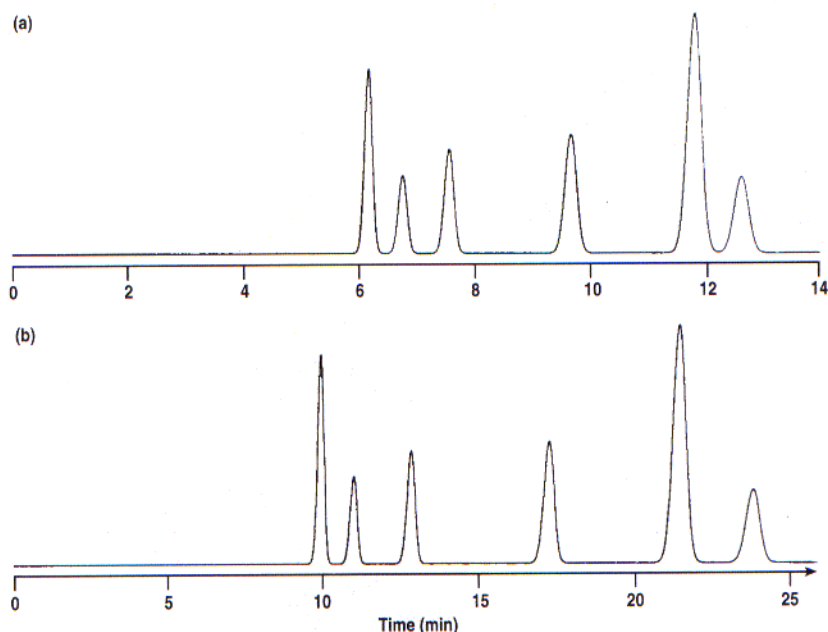
a small improvement in resolution, because for most conditions lower flow rates increase  $N$ . This separation using the 15 cm  $\times$  4.6 mm, 3- $\mu$ m  $d_p$  column at 0.8 mL/min meets all our separation criteria:  $p_{\max}$  of <2500 psi,  $t_{\text{run}}$  of <20, and  $R_s$  of  $\sim 2.0$ . We improved both resolution and run time but sacrificed the pressure of the initial run.

The separation using 25% tetrahydrofuran produces excess resolution as well as excess run time, so we need to improve these results without substantially increasing the pressure. A change from the 25 cm  $\times$  4.6 mm column to a 15 cm  $\times$  4.6 mm, 5- $\mu$ m  $d_p$  column is a step in the right direction. Because the pressure is so low, we increased the flow rate to reduce run time. The flow-rate change improves the run time as expected, but resolution drops to an unacceptable value. The 15 cm  $\times$  4.6 mm, 3- $\mu$ m  $d_p$  column seems to be a step in the wrong direction — although the run time is satisfactory, the pressure is too high, and we have more resolution than we need. Whenever we have excess resolution, we should try a shorter column because it will reduce pressure, run time, and resolution simultaneously. The 10 cm  $\times$  4.6 mm, 3- $\mu$ m  $d_p$  column illustrates this point, meeting all three of our targets. A switch to the 5 cm  $\times$  4.6 mm, 3- $\mu$ m  $d_p$  column continues this trend, but sacrifices resolution. The last line for the 25% separation in Table I shows that if we adjust the flow rate for the 10 cm  $\times$  4.6 mm, 3- $\mu$ m  $d_p$  column, all of our separation criteria are met, with  $t_{\text{run}} < 10$  min.

### WHICH IS BEST?

Some of the separations summarized in Table I are obviously inadequate, but which one is best? As with so many other aspects of LC, "best" is a judgment call that concerns several factors. Let's look at three of these factors: column availability, extracolumn effects, and method ruggedness.

**Column availability:** We have assumed two characteristics for the columns we used. First, we assumed the columns were available. This was probably a good assumption as long as we stick with the common C8 or C18 phases. Most manufacturers supply their reversed-phase columns in a variety of configurations. The second assumption is more risky — we assumed the columns were chemically equivalent. As long as the particle size remains constant and the columns are from the same manufacturer, selectivity is unlikely to change when we switch from one column length to another. It is fairly safe to assume that a manufacturer uses the same 5- $\mu$ m  $d_p$  particles to pack a 25-cm or 15-cm column. The risk increases, however, when we use columns of different particle size. By definition, the process of making 3- $\mu$ m  $d_p$  particles is different from that for 5- $\mu$ m particles because the starting silica is different, and as we might expect, these two particle batches can have slight variations in chemical characteristics. Most manufacturers take special care to keep the chemistry as consistent as possible, but we may find slight differences in selectivity even when the best control techniques are



**FIGURE 1:** Separation of six neutral aromatic compounds on a 25 cm  $\times$  4.6 mm, 5- $\mu$ m  $d_p$  C18 column at 1 mL/min using (a) 30% and (b) 25% tetrahydrofuran–water mobile phases.

**TABLE I:** Comparison of Column Conditions for the Separations of Figure 1

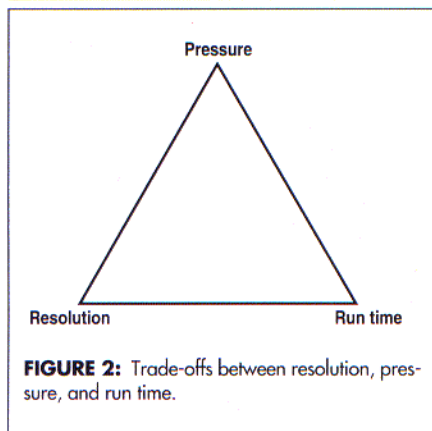
% Tetrahydrofuran	Column Length (cm)	Particle Diameter ( $\mu$ m)	Flow Rate (mL/min)	Pressure (psi)	Run Time (min)	Minimum Resolution
30	25	5	1.0	1814	14	1.71
	15	5	1.0	1088	9	1.32
	15	3	1.0	3024	9	1.87
25	15	3	0.8	2419	11	1.95
	25	5	1.0	1750	26	2.53
	15	5	1.0	1050	16	1.94
	15	5	2.0	2099	8	1.62
	15	3	1.0	2916	16	2.76
	10	3	1.0	1944	11	2.21
	5	3	1.0	972	6	1.48
28	10	3	1.2	2333	9	2.13
	25	5	1.0	1719	18	2.15

used. So what does this say about our choice of conditions? As long as  $R_s$  is  $>1.7$ , slight changes in selectivity are unlikely to have much of an impact on the separation. When we change particle size, it is wise to monitor  $k$  values for each peak — these should not change significantly.

**Extracolumn effects:** Extracolumn effects such as excessive volume in the connecting tubing and detector cell cause peak broadening. The injection solvent composition and the injection volume can also affect band broadening. These extracolumn effects are more severe for early, narrow peaks in the chromatogram (in isocratic separations) than for later, broader peaks. Narrower peaks also

result when we shorten the column length or reduce the particle size. The LC systems that are used primarily with 25 and 15 cm  $\times$  4.6 mm, 5- $\mu$ m  $d_p$  columns may yield proportionally broad peaks early in the chromatogram when more demanding columns are used. If we observe this problem, we may need to use shorter lengths of smaller inner diameter tubing, use smaller detector cell volumes, and adjust the injection volume to obtain maximum performance.

**Method ruggedness:** Whatever column length and particle size combination we use, we want a method that is tolerant to small changes in the separation parameters. We would like the column we buy next year to be



the same as the one we used to develop the method. In general, 5- $\mu\text{m}$   $d_p$  columns are more reproducible from column to column and batch to batch than 3- $\mu\text{m}$   $d_p$  columns. Manufacturers have been making and selling more 5- $\mu\text{m}$   $d_p$  columns for many years, thus they can control the characteristics of the 5- $\mu\text{m}$  material better. This statement won't always be true, and some manufacturers are better at the process than others. The same circumstances occurred when manufacturers introduced 5- $\mu\text{m}$   $d_p$  particles into a market in which 10- $\mu\text{m}$   $d_p$  particles had been the standard for years. So if all other factors are

equivalent, the 5- $\mu\text{m}$   $d_p$  column will usually be preferred over 3- $\mu\text{m}$   $d_p$  columns. In either case, we should perform the separation using at least two columns from the same packing batch and two from different batches. (Some manufacturers sell sets of three columns for this purpose.) This variation will give you an idea of what kind of column-to-column and batch-to-batch changes to expect.

### SO, WHICH IS BEST?

When we initially examined Table I, we might have chosen the 28% tetrahydrofuran run, because it met our criteria for run time, pressure, and resolution. However, we can see that for the 25% run a simple change in column length to a 15 cm  $\times$  4.6 mm, 5- $\mu\text{m}$   $d_p$  column will provide a faster separation with acceptable resolution. I would choose this column-mobile phase combination if I were concerned primarily with method ruggedness and lacked confidence in 3- $\mu\text{m}$   $d_p$  columns. The 5- $\mu\text{m}$  particles and low pressure would make this method a routine, robust separation. If we were analyzing hundreds of samples and run time was an important consideration, I would prefer the 25% run using the 10 cm  $\times$  4.6 mm, 3- $\mu\text{m}$   $d_p$  column at 1.2 mL/min. In this case, excessive resolution should improve method ruggedness, but the marginal pressure conditions suggest that we should use an in-line filter to prevent the accumulation of particles on the frit at the column head.

### CONCLUSIONS

Figure 2 illustrates the trade-offs we have observed in our efforts to optimize the column conditions. You can maximize any two of the factors, but in doing so, you will sacrifice the third factor. For example, you can obtain better resolution and lower pressure, but run time will be longer. For this reason, we must consider all of the separation application requirements and compromise when we decide which conditions will be best for a particular separation.

We have looked at manual techniques to optimize  $k$ ,  $\alpha$ , and  $N$  to obtain the best resolution for a sample. Because these are manual techniques, anyone can use them with any LC system. In the next installment of "LC Troubleshooting," we will look at the relationships between the variables we have examined and discuss how we can apply them to semiautomated or automated method optimization.

### REFERENCES

- (1) J.W. Dolan, *LC•GC* 12(5), 368-370 (1994).
- (2) J.W. Dolan, *LC•GC* 12(6), 446-450 (1994).

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