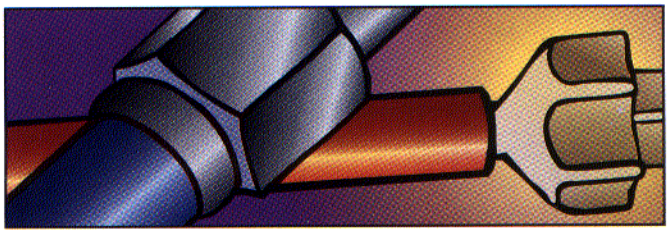
LC Troubleshooting



Using a Gradient Scouting Run to Get Started

John W. Dolan

Sometimes knowing where to start is half the battle.

Where do you start when it is time to develop a new liquid chromatography (LC) separation? For simplicity and reliability, the preferred method usually will be based on an isocratic separation. Analysts generally perform one of two common steps to find approximate starting conditions: They either check the literature or make stepwise mobile-phase adjustments.

LITERATURE METHODS

In the first procedure, a literature search may locate an existing separation for a similar sample. Although this technique often will reveal a set of conditions that someone else has found useful, the method can be loaded with problems. (A previous "LC Troubleshooting" column covered some of the pitfalls associated with using published methods [1].) In my experience, many of these methods are incomplete because they omit critical information such as the system dwell volume. Further problems can arise from what I refer to as *genetic drift*, which can happen something like this: A successful separation for Sample A uses an acetonitrile–phosphate buffer (pH 6) mobile phase. Someone uses this method for Sample B but has to add a little tetrahydrofuran to get the necessary selectivity. Someone else adjusts the pH to 3 and adds some triethylamine to reduce tailing for Sample C. Finally, you use this method to start with an unknown sample of similar structure to Sample C. Which of the mobile-phase ingredients is important? You really don't know, and as a result, you may have a much more complicated, and thus more error-prone, mobile phase than is required. We should all apply the KISS principle (Keep It Simple, Stupid) when designing mobile phases — the fewer the ingredients, the fewer the variables that can cause problems. Usually, we are better off starting a new method from scratch rather than relying on a literature method. I find that a literature method may be helpful in selecting the organic solvent or the approximate organic solvent concentration, but it is not much more useful than that.

STEPWISE CHANGES

A second way to find the approximate starting conditions for a separation is to adjust the solvent composition systematically to obtain reasonable retention times. Choose a 15 or 25 cm × 4.6 mm C8 or C18 reversed-phase column and make runs with successively weaker solvents until the desired retention is obtained. For example, start at 100% organic, then run 90%, 80%, and so forth. A couple of guidelines are helpful here. First, remember that the retention factor, k, should be in the range of 1–20 for all compounds of interest. Better yet, use retention factors between 2 and 10 for the best chromatographic behavior. The retention factor is calculated as

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

where t_R and t_0 are the retention time and the column dead time, respectively. A second useful tool is the *Rule of Three*, which states that k changes by approximately three times for a 10% change in the organic solvent concentration. So an analyte that is eluted at a retention factor of approximately 1 by 80% acetonitrile–water will be eluted at a retention factor of approximately 3 by a 70% acetonitrile–water mobile phase. By using the Rule of Three, you quickly can find conditions that give retention factors within the target region. With thoughtful selection of conditions, you can find near-optimum conditions in a few hours of laboratory work — *if* you are using an isocratic method. What happens if an isocratic method is not possible? Most of the work you've done to this point won't help you select conditions for a gradient method. However, there is an alternative.

GRADIENT VS. ISOCRATIC

A somewhat arbitrary dividing line exists between separations that require gradient elution and those that can be accommodated with an isocratic LC method. You can use an isocratic method when the ratio of k values for the first and last peaks is less than 25–30. If the range is larger than 30, the separation generally will be unsatisfactory under isocratic conditions. A simple example illustrates this point. Consider a separation for which the first peak is eluted with a k value of 1 and the last peak has a k value of 20. This gives a ratio of 20 for this separation. If the column is a 25 cm × 4.6 mm column operated at 1 mL/min, t_0 will be 2.5 min, so the retention time will be 5.0 min for the first analyte; determine the retention time by rearranging equation 1:

$$t_R = kt_0 + t_0 \qquad [2]$$

The last analyte will be eluted at 52.5 min. This is about the maximum run time we would like for an isocratic separation, in terms of both convenience and band-broadening.

A GRADIENT SCOUTING RUN

Rather than start with a series of isocratic runs, I prefer to use a gradient scouting run as the first run for an unknown because it offers three distinct advantages. First, we know that all analytes will be eluted in a reasonable period. By starting our isocratic search pattern at 100% organic, we assure ourselves that everything will come off the column fairly quickly, but this solvent likely will be too strong for most separations. With a gradient, on the other hand, we have the assurance of some level of separation coupled with the knowledge that nothing is likely to remain on the column, because the run ends with a strong solvent.

A second advantage of a gradient scouting run is that gradient theory allows us to calculate a set of gradient conditions that will give us reasonable chromatography for any sample. With an isocratic separation, we get the best chromatography in the $2 < k < 10$ region, with an ideal value of approximately 5. With gradient separations, we are concerned with the average k value, k^*, defined as the instantaneous k value for a compound when it has traveled halfway through the column. For a scouting run, a k^* value of approximately 2 will be adequate to give a reasonable separation. These conditions can be obtained with a full-range gradient run on a 25 cm × 4.6 mm column run at 1 mL/min over 20 min. It is best to avoid 100% water (or buffer) with reversed-phase columns, so for most purposes consider a 5–100% gradient a full-range gradient.

Based on information obtained from this standard scouting gradient, we can determine if an isocratic or gradient method is needed. We also can estimate the best isocratic mobile-phase composition or gradient range. Reference 2 distills the use of the scouting run into a simple procedure. We can use the results of the scouting gradient run shown in Figure 1 to illustrate an application of this technique.

GRADIENT OR ISOCRATIC?

The first decision we need to make from the scouting run is whether to proceed with an isocratic or a gradient method. We do this by determining what portion of the chromatogram is occupied by eluted peaks (refer to Figure 1). First, find the retention range, Δt_g, in this case 18.0 min − 9.5 min = 8.5 min. Then divide this result by the gradient time, t_G, to get 0.425. Finally, apply these guidelines:

- if $\Delta t_g/t_G$ is less than 0.25, use an isocratic run, and
- if $\Delta t_g/t_G$ is greater than 0.25, use a gradient run.

This means that if the peak envelope occupies more than 25% of the scouting gradient, a gradient method is needed; otherwise, an isocratic method will work. For the example in Figure 1, the peak envelope occupies more than 40% of the gradient, so a gradient method will be required.

DETERMINE THE GRADIENT CONDITIONS

Once we have decided that a gradient is necessary, we can use the information from the scouting run to determine the gradient range for the next gradient experiment. The chromatogram in Figure 1 shows that roughly 8 min is wasted at the beginning of the gradient. Another 1–2 min is wasted after the last analyte is eluted. We could make more-efficient use of the separation time by starting the separation at a higher percentage of organic solvent and ending the run a little faster. The chart in Figure 2 can help us determine the starting and ending points of the gradient. To use the chart, first determine the type of LC system being used. In the present case, we used a 25-cm column, and let's assume the system used a single pump in a low-pressure mixing mode. The fifth column in the chart corresponds to this system. Use this column to find the retention time of the first peak (9.5 min). Trace this location to the left-hand column to determine the starting solvent strength — approximately 15%, in this case. Similarly, find the final retention time (18 min) in the 25-cm, one-pump column and trace right to find the final mobile-phase strength of approximately 67%. This finding means that subsequent optimization of gradient steepness should be based on a 15–67% organic gradient.

DETERMINE THE ISOCRATIC CONDITIONS

If the scouting run indicates that an isocratic method is possible, we perform a similar exercise to find the isocratic conditions for the next separation. For this purpose, let's assume that the separation in Figure 1 contains only the first five peaks. The retention range is 13.5 min − 9.5 min = 4 min, which is less than 25% of the gradient range. This means that an isocratic method is possible. Next, refer to Figure 3 and find the appropriate column in the chart to determine the time corresponding to the midpoint of the gradient elution profile (11.5 min for the present example).

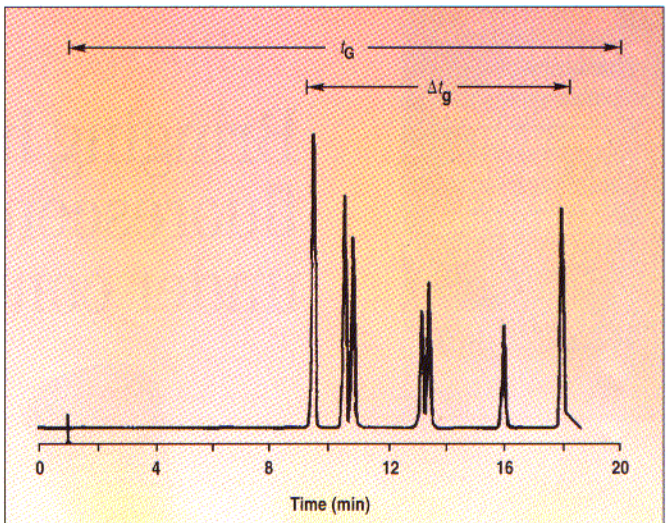


FIGURE 1: Chromatogram for a gradient scouting run. See text for details. (Reprinted from reference 2 with permission.)

Then trace to the left column to find the suggested isocratic mobile phase, which is approximately 10% organic solvent in the current example.

FURTHER ADJUSTMENTS

Figures 2 and 3 assume acetonitrile as the mobile phase. If methanol or tetrahydrofuran is used, you will need to make some minor adjustments with the help of the solvent nomograph shown in Figure 4. For example, if the scouting run used methanol instead of acetonitrile, you would need to adjust each of the values according to the nomograph. In the situation described above, this means a gradient of 20–75% methanol or an isocratic mobile phase of 17% methanol.

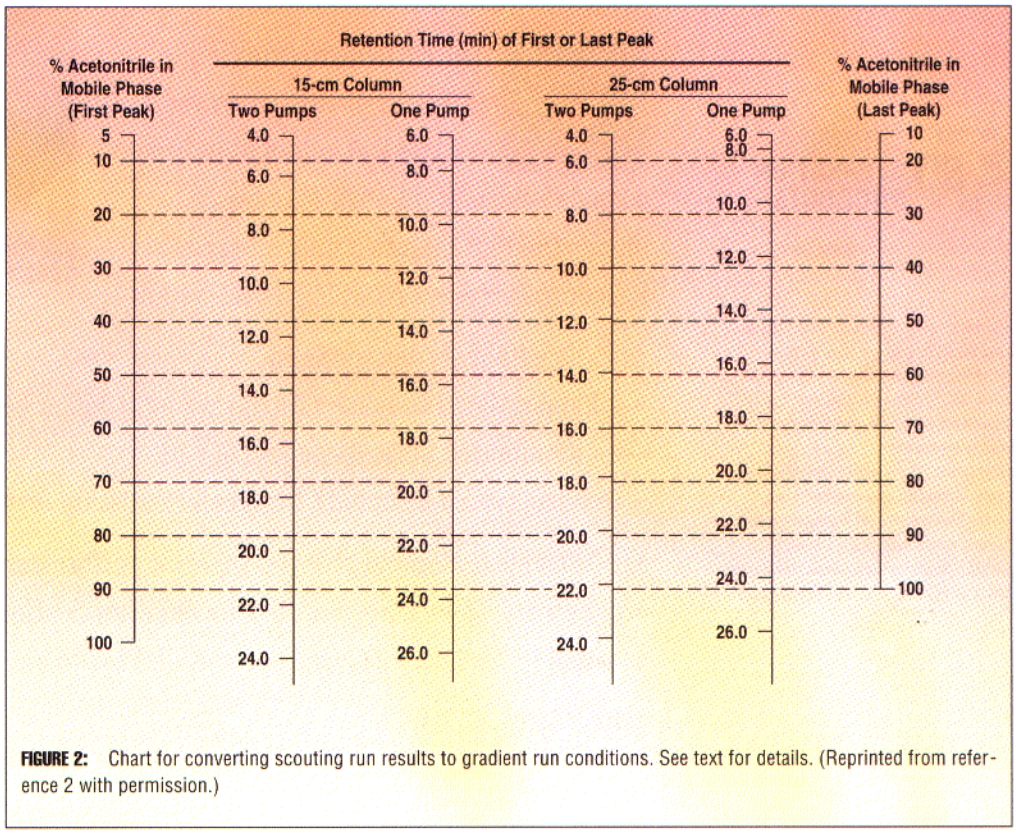


FIGURE 2: Chart for converting scouting run results to gradient run conditions. See text for details. (Reprinted from reference 2 with permission.)

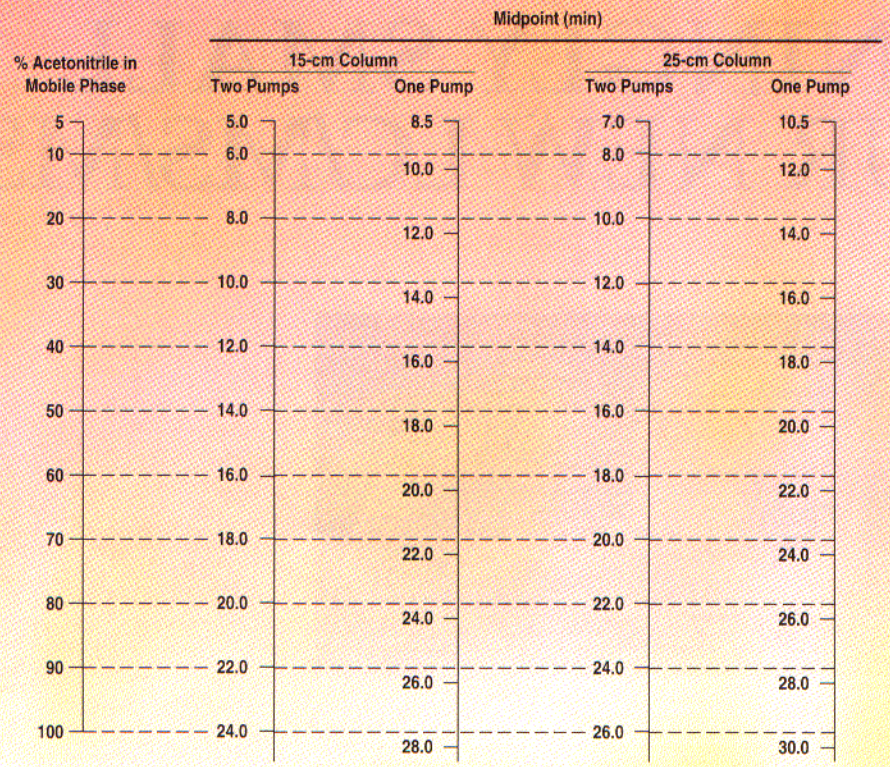


FIGURE 3: Chart for converting scouting run results to isocratic conditions. See text for details. (Reprinted from reference 2 with permission.)

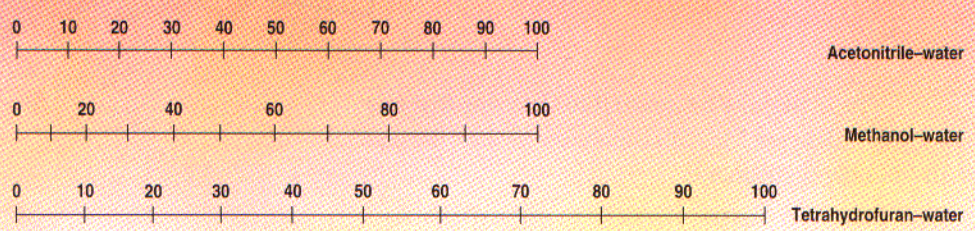


FIGURE 4: Nomograph for determining equivalent solvent strengths. (Reprinted from reference 2 with permission.)

Once you have determined the appropriate gradient or isocratic conditions from the scouting run, you will need to fine-tune the separation in the normal manner. For a gradient run, this means adjusting the gradient steepness (by adjusting the gradient time), with shallower gradients generally improving resolution. For isocratic conditions, vary the percent organic solvent within a few percent, with weaker mobile phases generally improving resolution.

FOR MORE HELP

The current discussion was based on a strategy outlined in reference 2, a book that should be one of the standard reference volumes in every LC laboratory. This reference contains many other practical suggestions and other examples of the present strategy. Alternatively, you can use one of the popular LC computer simulation programs to help you find the best isocratic or gradient conditions based on one or more gradient scouting runs.

REFERENCES

(1) J.W. Dolan, *LC•GC* **11**(6), 412–415 (1993).
(2) L.R. Snyder, J.L. Glajch, and J.J. Kirkland, *Practical HPLC Method Development* (John Wiley and Sons, New York, 1988).

"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, and a member of the Editorial Advisory Board of LC•GC. *Direct correspondence about this column to "LC Troubleshooting,"* LC•GC, *859 Willamette Street, Eugene, OR 97401, e-mail 76631.2547@compuserve.com.*