LC Troubleshooting

The Hazards of Adjusting Gradients

Simple changes can yield unexpected results.

John W. Dolan
LC Troubleshooting Editor

Gradient elution is a widely used technique for reversed-phase liquid chromatography (LC) methods. Gradients are especially advantageous for samples that have a broad polarity range. By starting with a weak mobile phase and finishing with a strong mobile phase, analysts can retain polar compounds but nonpolar compounds will not have excessive retention times. Most of the time, all sample components are eluted by the end of the gradient. Thus, chromatographers can use the gradient time (t_G) to determine the expected run time, whereas isocratic runs have no convenient end point.

To optimize a gradient separation, chromatographers use three primary variables: the initial percentage of organic solvent (%B), the final percentage of organic solvent, and the gradient time or slope. For a gradient of fixed range (final–initial %B), the gradient time and gradient slope are different measurements of the same parameter, which determine the %B/min change. Selectivity in gradient elution is determined by the gradient retention factor (k^*) as

$$k^* = \frac{t_G F}{\Delta\%B\, V_m S} \qquad [1]$$

where t_G is the gradient time in minutes, F is the flow rate in milliliters per minute, Δ%B is the gradient range (for example, Δ%B = 0.95 for a 5–100% gradient), V_m is the column volume in milliliters, and S is a constant for a given compound that can be assumed to be 5 for the present discussion. I can use equation 1 as a guide to keep the selectivity, or peak spacing, constant when changing experimental variables. For example, changing from a 20–80% B gradient run in 12 min to a 40–80% gradient requires a change of t_G from 12 to 8 min, with all other variables constant. For the present discussion, I'll assume constant k^*, except as noted.

Obtaining a Separation

Often analysts can develop a successful gradient separation by starting with standard scouting conditions and then fine-tuning them for the best separation. A good place to start is with a run that generates k^* values of approximately 5–6. If I were to use a 150 mm × 4.6 mm column (V_m = 1.5 mL) with a full-range gradient (5–100% B) and a flow rate of 2 mL/min, then gradient times of approximately 20 min will provide the desired k^* values. Following the initial scouting run, I could perform additional runs by varying the gradient time by plus or minus a factor of two to determine the effect of gradient steepness on selectivity. Fine-tuning the gradient time should result in optimal conditions for a given solvent system and stationary phase. For the present discussion, these experiments produced the separation shown in Figure 1a for a 5–100% B gradient in 19 min (5% B/min).

Trimming the Waste

The chromatogram of Figure 1a shows that the first peak is not eluted until after 10 min, effectively wasting half the gradient time. A very simple model of gradient elution states that samples sit at the head of a column until a strong enough solvent comes along to push them through the column, then they travel to the column outlet fairly quickly. If this situation is the case, I should be able to increase the initial percentage of organic solvent in the current example to the point at which the first peak begins to move through the column. For example, I should be able to trim 5 min off the run by starting at the percentage of organic solvent that normally would be at the head of the column 5 min after the full gradient started. I can determine this value by multiplying 5 min by 5% B/min to obtain 25% B higher than the original starting conditions (5% B), or starting at 30%.

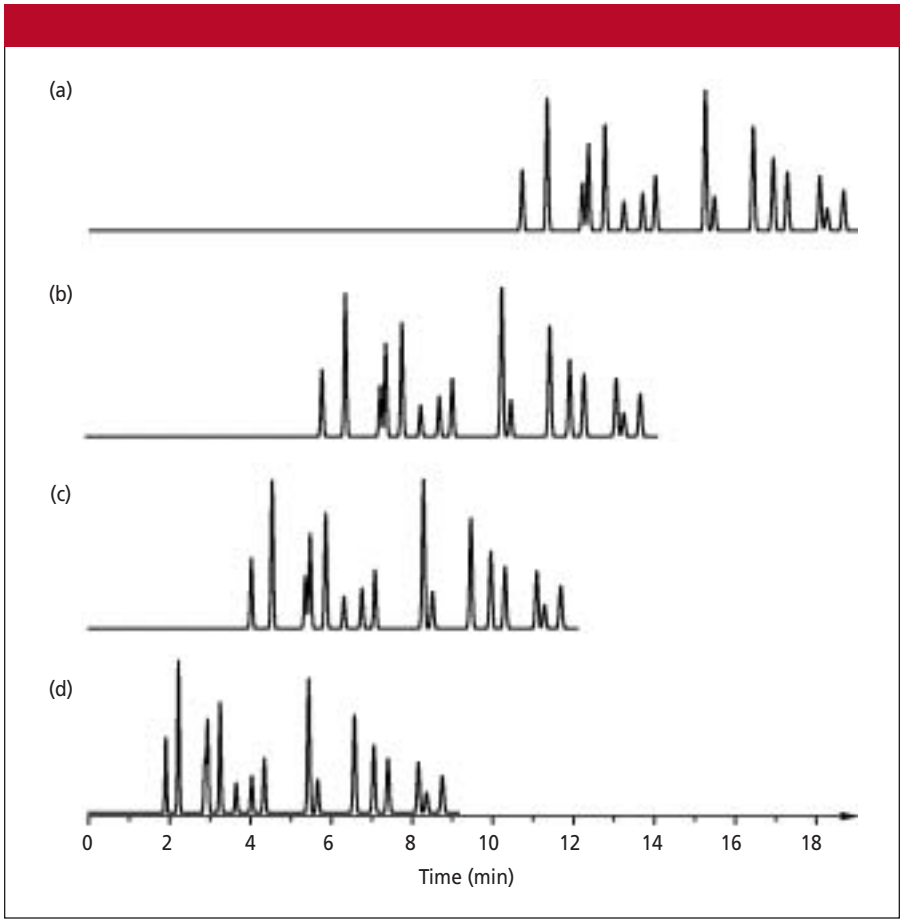

Figure 1: Simulated chromatograms showing effect of initial mobile phase conditions: (a) 5–100% B in 19 min, (b) 30–100% B in 14 min, (c) 40–100% B in 12 min, and (d) 55–100% B in 9 min.

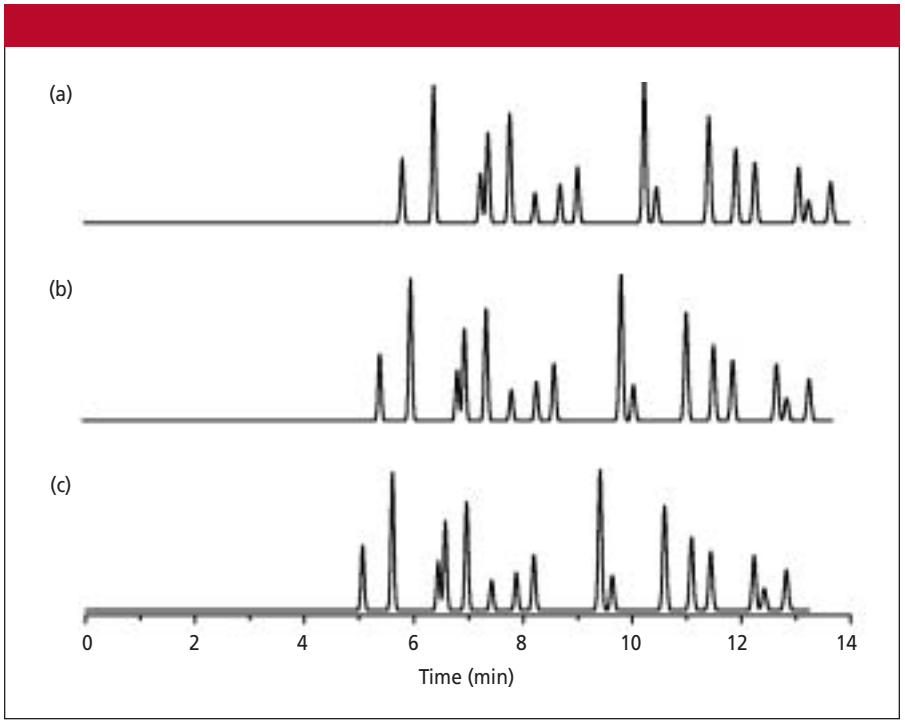

Figure 2: Effect of system dwell volume on the separation. Shown are chromatograms generated with dwell volumes equal to (a) 2.0 mL (same as Figure 1b), (b) 1.0 mL, and (c) 0.25 mL. The gradient for all runs was 30–100% B in 14 min with a flow rate of 2 mL/min.

To keep k^* constant, equation 1 tells me to reduce the gradient time from 19 min to 14 min so that the slope of 5% B/min is maintained. Figure 1b shows the result of this new 30–100% B/14 min gradient. For the most part, the only change in the chromatogram is that all the peaks appear approximately 5 min earlier. So far, so good.

The run of Figure 1b still has roughly 5 min of wasted time at the beginning. What would happen if I used an even higher starting percentage of organic solvent? Starting at 40% B, I begin to see some deterioration in the separation (look at the height of the valley between the third and fourth peaks in Figure 1c). I could try an even stronger starting solvent (55–100% B/9 min), as in Figure 1d, but the separation between the third and fourth peaks would be nearly lost. By studying the chromatograms of Figure 1, readers can see that adjusting the starting conditions affects the early peaks more than it does the later ones; even with the loss of separation early in Figure 1d, the late peaks are unchanged. For the present example, the 30–100% B run (Figure 1b) provided the best compromise, so I will use this range for the remainder of my discussion.

After fine-tuning the initial conditions, I can perform similar adjustments at the end of the gradient. In the present separation, however, the last peak was eluted at the end of the gradient in each case, so no adjustment was needed.

Transferring the Method

Often gradient methods are developed and validated in one laboratory and then transferred to another instrument, another laboratory, or another location. Although the transfer of a method seems simple, it can hold some surprises for unwary analysts. The chromatograms of Figure 2 illustrate one aspect of equipment differences that can be important. Assume that a method is developed on a modern LC system using low- or high-pressure mixing. Many of these systems have a dwell volume (V_D) of approximately 2 mL. The dwell volume comprises the volume of the mixer and all plumbing from the mixer to the column head. For a high-pressure mixing system, this plumbing will include the mixer, connecting tubing, and injection loop. Low-pressure mixing systems include the volumes of the same parts and the volume of the pump heads. The dwell volume introduces a delay in the time the gradient requires to reach the column head. For the present example of a method with a 2-mL/min flow rate and a 2-mL mixer, I will

have a 1-min isocratic hold at the beginning of the run as a result of this delay.

Figure 2a duplicates my separation in Figure 1b. If the method is transferred to a system with 1-mL dwell volume, I obtain the chromatogram of Figure 2b. In my experience, dwell volumes of less than 1 mL seldom are found with stock LC systems. If the LC system is modified for use with a short, narrow-bore column, such as the 50 mm × 2.1 mm columns commonly used for LC–mass spectrometry (LC–MS) systems, a dwell volume may be as small as 0.25 mL or less. A dwell volume of 0.25 mL would yield a chromatogram such as the one in Figure 2c for the present separation. For most separations, the major effect of a change in dwell volume would be a shift in retention times proportional to the dwell volume. In the present case, a 1-mL reduction in V_D between Figures 2a and 2b results in retention times that are reduced by 0.5 min (1 mL ÷ 2 mL/min). A change in dwell volume also can cause significant changes in selectivity, especially in the early part of a chromatogram, although that didn't occur in the present example.

Although a retention shift for all peaks in a chromatogram might not affect the ability of a method to produce satisfactory results, chromatographers must be careful all the same. For example, if I developed a system-suitability test for the system in Figure 2a, it might specify that the first peak must be eluted between 5.5 and 6.0 min and the last peak between 13.5 and 14 min. Neither of the other separations in Figure 2 pass these system-suitability criteria, although no change in the peak spacing is observable.

This outcome exemplifies why it is important to specify the dwell volume of the system on which a method was developed. If the dwell volume were more critical for this separation, I could compensate for the change by adding an isocratic hold equivalent to the dwell volume difference. For the Figure 2b example, I could add 0.5 min of isocratic hold before the gradient starts.

Scaling the Column

A problem related to the dwell volume can occur when analysts attempt to adjust a method for a column of different dimensions. One common column change is to reduce a column's inner diameter from 4.6 mm to 2.1 mm to reduce solvent consumption and sharpen peaks. Whenever changing column diameter, chromatographers also must change the flow rate in proportion to the square of the diameter to obtain the same linear velocity, which is necessary to obtain an equivalent separation. Thus, if a separation on a 4.6-mm column run at 2.0 mL/min were changed to a 2.1-mm column, the flow should be reduced to 0.4 mL/min for an equivalent separation [$(4.6 \text{ mm} \div 2.1 \text{ mm})^2 \approx 5$]. This change is all that would be necessary for an isocratic method, because the isocratic retention factor (k) is unaffected by column volume or flow rate.

A gradient method, however, is influenced by the column volume and flow rate, according to equation 1. To ensure obtaining the same k^* value, analysts should consult equation 1 to see if any other adjustments are necessary. The change of V_m by a factor of 5 is compensated by a change in F, so no other changes need to be made.

Figure 3b shows a change from the reference conditions of Figure 3a (same as Figure 1b and 2a) with the 4.6-mm column and 2-mL/min flow rate to the 2.1-mm column with a 0.4-mL/min flow rate. The separation changes dramatically. The retention times are significantly longer, and the selectivity is changed, as illustrated by the partially resolved peak pairs near the beginning, middle, and end of the run. This problem is the result of a change in the dwell time (V_D/F).

In Figure 3a, the 2-mL dwell volume is flushed in 1.0 min at 2.0 mL/min, but the reduced flow rate of Figure 3b increases the dwell time to 5.0 min (2.0 mL ÷ 0.4 mL/min). The changes in dwell volume illustrated in Figure 2 did not make large changes in the separation because the change in dwell time was relatively small at 2 mL/min. In the example of Figure 3b, the effective addition of a 4-min isocratic hold at the beginning of the run has a dramatic effect. The change in this case can be compensated by reducing the dwell volume. Some systems have mixers with a choice of volumes, so users can choose a smaller mixer volume such as 1.0 mL shown for the separation in Figure 3c. This choice is better but not quite enough.

To obtain dwell volumes less than 1 mL, chromatographers generally must customize their LC systems by using a micromixer. (Some of the newer LC systems enable injection of the sample after the gradient is initiated, which yields the same effect as a smaller dwell volume.) This type of modification might result in

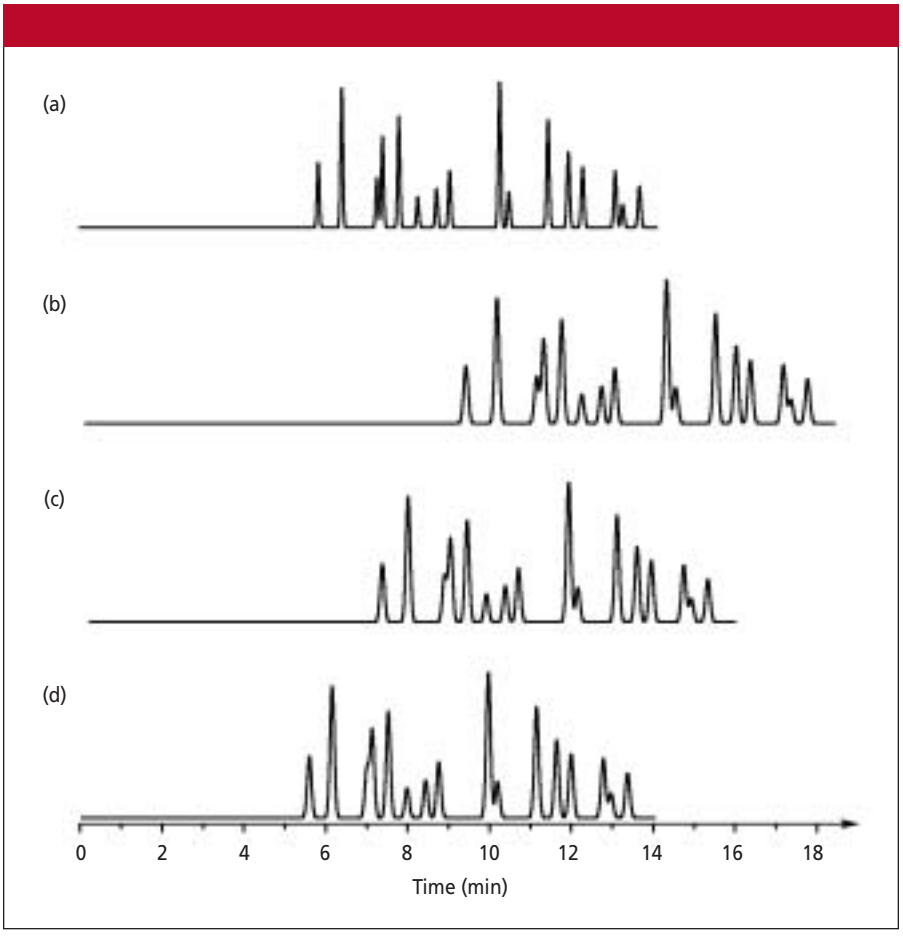

Figure 3: Effect of column diameter (d_c) and dwell volume: (a) d_c = 4.6 mm, F = 2.0 mL/min, V_D = 2.0 mL (same as Figures 1b and 2a); (b) d_c = 2.1 mm, F = 0.4 mL/min, V_D = 2.0 mL; (c) d_c = 2.1 mm, F = 0.4 mL/min, V_D = 1.0 mL, and (d) d_c = 2.1 mm, F = 0.4 mL/min, V_D = 0.25 mL.

the dwell volume of 0.25 mL, which was used in the separation in Figure 3d. With this change, the retention times are approximately the same as those of the original separation in Figure 3a (the dwell time was 0.6 min [0.25 mL ÷ 0.4 mL/min]).

Although the peak spacing of Figure 3d is the same as that in Figure 3a, the quality of the separation is worse. This outcome is the result of extracolumn effects. A reduction in the tubing volume, detector cell volume, or detector time constant should correct the peak broadening.

Conclusions

Gradient elution is a very powerful tool that enables chromatographers to obtain separations that are impossible with isocratic techniques. However, using gradients is less intuitive than the simpler isocratic separations, so analysts must be careful that they make no unintentional changes in the separation conditions.

This month's "LC Troubleshooting" has illustrated the importance of tracking the influence of each parameter; using equation 1 can help in this endeavor. Selectivity can be kept constant by adjusting conditions to maintain a constant k^* value. Chromatographers must take extra care when transferring a method from one LC system to another or when changing a column size to ensure that the separation does not deteriorate.

John W. Dolan
"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Walnut Creek, California, and a member of LCGC*'s editorial advisory board. Direct correspondence about this column to "LC Troubleshooting,"* LCGC, *859 Willamette Street, Eugene, OR 97401, e-mail John.Dolan@LCResources.com.*

For an ongoing discussion of LC troubleshooting with John Dolan and other chromatographers, visit the Chromatography Forum discussion group at http://www.chromforum.com.