



Troubleshooting

Simply scaling the gradient won't always work.

Reducing Column Diameter in Gradient Elution — A Case Study

A reader submitted the following question, which illustrates some of the pitfalls that users can encounter when adjusting a gradient elution liquid chromatography (LC) method.

Changing to Narrow-Bore Columns and Scaling to the Diameter Squared

Q: I recently changed to a narrow-bore column to improve the sensitivity of my method and am having difficulty obtaining the same separation that I had on the original column. The peaks have normal shapes, although they are slightly less retained, but the resolution for several peak pairs is terrible. What could be causing this problem? The original column was a 150 mm × 4.6 mm, 3.5-μm d_p C18 column, and the new column is a 150 mm × 2.1 mm, 3.5-μm d_p C18 column. The flow rate was 1.0 mL/min on the original column and is 0.4 mL/min on the 2.1-mm column. The gradient is 20–70% solvent B in 15 min. I'm using an LC system with a dwell volume of 1.0 mL.

A: Two parameters of your modified method are not equivalent to the original one, and both will contribute to the problems that you have observed. First, any change in gradient slope is likely to change the selectivity, or peak spacing, in a gradient separation. At first glance, the gradient is the same in both methods, but a closer look will reveal the problem. For equivalent gradient conditions, the relationship of equation 1 must be held constant as

$$\text{constant} = t_G F / \Delta\Phi V_m \quad [1]$$

where t_G is the gradient time in minutes, F is the flow rate in milliliters per minute, $\Delta\Phi$ is the gradient range (for example, 20 to 70% B would be 0.5), and V_m is the column volume in milliliters. So for your separation, t_G and $\Delta\Phi$ are constant, but F has changed from 1.0 to 0.4 mL/min, and V_m

has changed with the column diameter. V_m can be estimated as

$$V_m \approx 0.5 L d_c^2 \quad [2]$$

where L is the column length and d_c is the column diameter, both in centimeters.

So you can see that the column volume has changed by $(0.46/0.21)^2$, which is approximately fivefold. (For convenience, I've rounded the column volume to 1.5 mL and 0.3 mL for the two columns in the rest of this discussion.) Equation 1 states that if the gradient time and range stay the same, the flow rate must change in proportion to the column volume. However, in your case, the flow rate changed by 2.5-fold, not fivefold. This means that the new conditions are equivalent to running a steeper, shorter gradient. Because the gradient steepness controls the selectivity, it is unsurprising that the peak spacing has changed for your separation.

I have used a simulated separation in Figure 1 to show results with the same kind of changes you observed. Figure 1a is analogous to the original method. I have chosen to use some partially resolved peaks because they make the observed changes more dramatic. When the 2.1-mm column was used at 0.4 mL/min, I obtained the chromatogram of Figure 1b. All the peaks are retained approximately 1 min less. The partially resolved pair of peaks 3 and 4 now are well resolved, but an examination of the group of peaks in the middle of the chromatogram reveals that peak spacing has changed dramatically.

How can you fix this problem? It is quite simple — just lower the flow rate to 0.2 min, and the change in flow rate will be proportional to the change in column volume. Once this proportion is reestablished, equation 1 will have the same value for both methods.

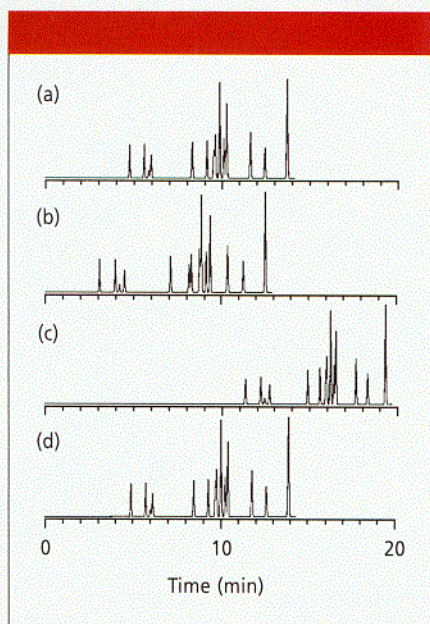


Figure 1: Simulated chromatograms for a mixture of 14 compounds obtained using a gradient of 20–70% B in 15 min. Shown are results obtained using (a) the original method with a 150 mm \times 4.6 mm column, 1.0-mL dwell volume, and 1.0-mL/min flow rate; (b) a 150 mm \times 2.1 mm column, 1.0-mL dwell volume, and 0.4-mL/min flow rate; (c) a 2.1-mm i.d. column, 1.0-mL dwell volume, and 0.2-mL/min flow rate; and (d) a 2.1-mm i.d. column, 0.2-mL dwell volume, and 0.2-mL/min flow rate.

But Don't Forget the Dwell Volume

However, when you examine the results of Figure 1c for the 0.2-mL/min flow rate, you will realize that something doesn't make sense even with the flow rate adjusted to the appropriate value. The retention times now are significantly longer, and peak spacing is obviously different from the original run of Figure 1a. The retention times would be expected to be the same in Figures 1a and 1c — but only if the dwell volume (V_D) were not a factor. Remember that the dwell volume is the volume from the point in the system where the solvents are mixed to the head of the column; in your system, the dwell volume is 1.0 mL. The dwell volume inserts an unintentional isocratic hold at the beginning of every gradient during which the gradient advances from the mixer to the head of the column. The dwell time is the dwell volume divided by the flow rate.

The plots of Figure 2 show the gradient program at the head of the column for each of the three runs in Figure 1. In the original method, the dwell time was 1 min (1 mL \div 1 mL/min); this time is followed by the expected gradient ramp of 20–70% B in 15

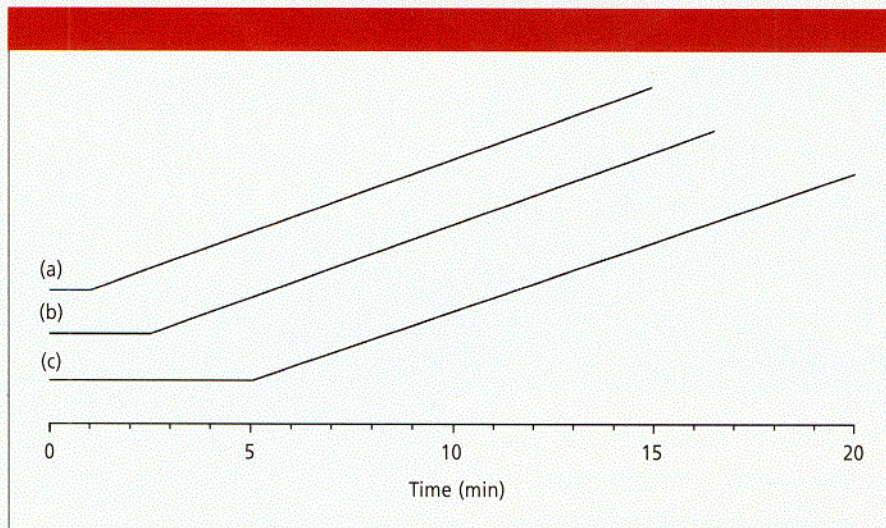


Figure 2: Effective gradient program at the head of the column for three runs of Figure 1.

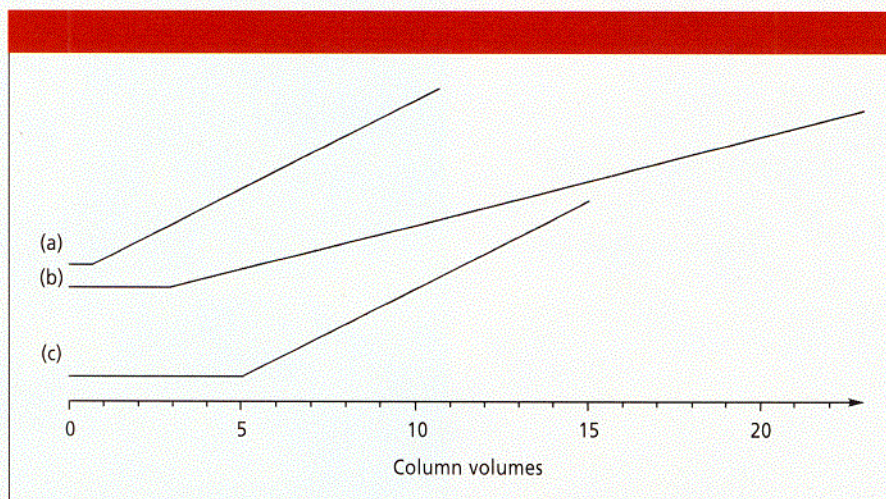


Figure 3: Effective gradient program at the head of the column for three runs of Figure 1 displayed in units of column volume rather than time.

min (Figure 2a). For the narrow-bore column, the dwell time is 1 mL \div 0.4 mL/min or 2.5 min (Figure 2b). So the gradient doesn't reach the column in the second method until 1.5 min after it does in the original method; the rest of the gradient is the same as the first case. This additional delay before the gradient reaches the column allows the sample to elute isocratically during the time before the gradient reaches the column. Could this account for the shorter retention times you observed even though the gradient you programmed was the same? I will defer my answer until later.

Will changing the flow rate to 0.2 mL/min fix the dwell volume problem? No — it will make it worse, as Figure 2c shows. The dwell time will become 1 mL \div 0.2 mL/min or 5 min, followed by the intended gradient. But wait a minute! If the extended isocratic hold at the beginning of

the run made the peaks come out earlier at 0.4 mL/min, why are they eluted later at 0.2 mL/min?

By now you are beginning to see why gradient methods are difficult to repeat on another system or in another laboratory in which the conditions are not identical. One more consideration is hidden in these data. To get the same separation, equation 1 really must be supplemented with

$$\text{constant} = F/V_D \quad [3]$$

The only way to obtain the exact same separation with both columns is to reduce the effective dwell time for the smaller column. Another way to think of this is to calculate the dwell volume and gradient in terms of column volumes. Figure 3 illustrates this perspective. Figure 3a shows how the gradient looks for the run of Figure 1a

— 0.6 column volumes of dwell and 10 column volumes of gradient. When the smaller column is used at 0.4 mL/min (Figure 3b), you can see that not only is the dwell time longer (3 column volumes), but the slope of the gradient has changed too (20 column volumes). Although the slope looks shallower in this plot, it really is steeper because 20 column volumes of mobile phase are pumped through the column in 15 min instead of 10 mL. This increased effective steepness of the gradient is the primary factor that makes the peaks of Figure 1b come out earlier, rather than the increase in isocratic hold at the beginning.

If this concept is confusing, think of the $t_G F$ term in the numerator of equation 1. A reduction of flow rate (for example, to 0.4 mL/min) must be compensated by an increase in gradient time. So if the gradient time is not changed to the desired, shallower gradient, then it must be equivalent to a steeper gradient if only the flow is reduced.

Finally, Figure 3c shows an even greater increase in dwell time (5 column volumes) for the 0.2 mL/min flow rate and the identical 10-column volume gradient of Figure 3a. The starting conditions are sufficiently weak, so little isocratic elution occurs for this sample during the dwell time, and the peaks are eluted later.

Further Adjustments

So does this mean you are stuck and won't be able to use the 2.1-mm i.d. column for this separation? No, I can think of four ways to solve the problem; one or more may work for your system.

First, you could reduce the true dwell volume of the system by removing the present mixer and replacing it with a smaller one. This replacement may or may not be possible. For example, if your LC system uses low-pressure mixing, it is unlikely that you will be able to change the mixer; however, high-pressure mixing systems are more amenable to mixer replacement. In my laboratory, workers replumb the high-pressure mixing LC systems with micromixers when they use them to generate gradients for short, small-bore columns with LC-MS instruments. Ideally you could substitute a 0.2-mL mixer for the current 1-mL one. With a 0.2-mL mixer, 2.1-mm i.d. column, and 0.2-mL flow rate, the 20–70% B program in 15 min would give the results of Figure 1d, which are nearly identical to the original results (Figure 1a). Any differences in the separa-

tion between Figures 1a and 1d probably are due to extracolumn effects.

Second, if your LC system is capable, you could delay the sample injection until 4 min after the run started. This delay would mean that the sample would be injected with 1 min of the dwell time remaining, and the gradient would be identical. This approach also should produce the chromatogram of Figure 1d. Unfortunately, most LC systems will not allow injection after the run has started.

A third choice would be to increase the flow rate at the beginning of the run and then drop it back to 0.2 mL/min. For example, set the flow rate to 1.0 mL/min for 1 min and then reduce it back to 0.2 mL/min. This reduction would result in a dwell time of 1.0 min for both methods, with the flow rate scaled to the column diameter in both methods. Three potential problems exist with this approach. The 1-mL/min flow rate would cause a fivefold pressure rise for the narrow-bore column, which would be satisfactory only if the pressure did not exceed reasonable system pressure (for example, 3500–4000 psi). Second, it is likely that the flow change would cause a baseline disturbance in the chromatogram, but a disturbance would be a problem only if it occurred at a point when a peak of interest was eluted. Third, if significant band migration occurred during the dwell period, the separation could change. Stepped flow adjustment probably is the least satisfactory solution.

A fourth way to compensate for the difference in dwell time would be to start the gradient at a lower percentage of organic solvent. In a simplistic model for gradient elution, the sample sits at the head of the column until the mobile phase becomes sufficiently strong to begin moving the sample through the column. If the starting mobile phase can be selected to eliminate any appreciable band migration during the dwell time, you should be able to achieve the same separation with different dwell times. The retention times would be offset by the dwell time difference, but the peak spacing should be the same. For example, by reducing the initial conditions to 5% B, you might freeze the sample at the column inlet during the dwell time. After the sample was injected, you could program a step to 20% B for 1 min and then start the gradient. The net result would be 4 min of 5% B followed by 1 min of 20% B (equivalent to the dwell time of the original method) and then the gradient of 20–70% B. All the peaks should be eluted approxi-

mately 5 min later than the original method. This effect is similar to that of the longer dwell time of Figure 1c, but the isocratic conditions in Figure 1c were sufficiently strong to allow some migration and, thus, a change in selectivity.

Of course, one other simple fix for this problem is possible if you can anticipate it during method development. In the present case, the first run with the larger column could be programmed to include an isocratic hold so that the combined isocratic hold and dwell time would be the same as the minimum for the 0.2 mL/min flow rate. The dwell time at 0.2 mL/min is 5 min ($1.0 \text{ mL} \div 0.2 \text{ mL/min}$), so a combined dwell time and hold for the larger column also should be 5 min. The dwell time was approximately 0.7 min ($1.0 \text{ mL} \div 1.5 \text{ mL/min}$), so a hold of $5.0 \text{ min} - 0.7 \text{ min} = 4.3 \text{ min}$ would be programmed followed by the 15-min gradient. The large-column gradient would be programmed as 20–20–70% B at 0–4.3–19.3 min.

Conclusions

So where does this process lead? I have shown that two critical factors must be taken into account when changing to a narrow-bore column or making any column volume change in gradient methods. First, because the gradient steepness or gradient volume is the critical factor in controlling selectivity in gradient elution, the flow rate must be adjusted in proportion to the column volume. Readers can use equation 2 to estimate the column volume. Second, any change in flow rate will cause a change in the dwell time, which can adversely affect the separation. To compensate for changes in the dwell time, chromatographers should use one of the expedients discussed above.

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