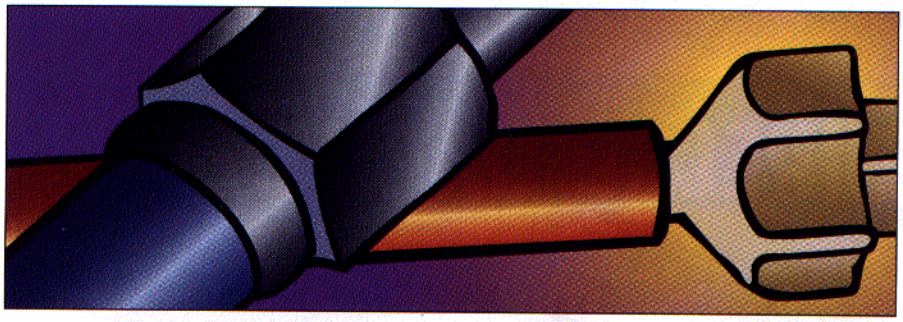
LC Troubleshooting



Scaling Gradients

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Changing the column size when using gradient elution can produce unexpected results.

Reducing column size for liquid chromatography (LC) separations is becoming increasingly popular. Smaller column diameters, shorter columns, and smaller particle sizes all produce narrower and, therefore, sharper peaks. Sharper peaks can mean better detection limits for trace analysis and less solvent to evaporate when using LC–mass spectrometry (MS) or evaporative light-scattering detection. When isocratic separations are scaled, the adjustment of conditions simply changes with the square of the column diameter. Applying the same adjustments to gradient elution separations, however, can produce unexpected results. This month's "LC Troubleshooting" column examines the principles of scaling isocratic separations and considers how gradient requirements apply to changes in chromatographic run conditions.

ISOCRATIC BEHAVIOR

One technique used to develop isocratic separation conditions is performing a sequence of runs in a stepwise manner, starting with a strong solvent. For example, an analyst might start with 100% B solvent (possibly acetonitrile), then reduce the mobile phase to 90% B, then to 80% B, and so forth until the compounds are eluted in a desired range of retention factors (k). (Recall that $k = [t_R - t_0]/t_0$, where t_R is the retention time and t_0 is the column dead time.) Ideally, analysts would like to elute the peaks in a k range of 1–20 or, better yet, 2–10 for good chromatography. In this k range, peaks will be retained sufficiently to avoid interferences near the solvent front and not retained so strongly that peak broadening and long run times are problems.

A solid understanding of retention in isocratic separations allows users to predict retention based on

$$\log k = \log k_w - S\Phi \qquad [1]$$

where k_w is the retention factor in 100% water (a derived quantity), S is a constant for each compound, with a typical value of 4 for small molecules (<1000 Da), and Φ is the percentage of solvent B (100% B = 1.0). Equation 1 can be derived from two experimental runs and is the basis of popular separation optimization software packages. When retention data are available from two isocratic runs differing only in the percentage of solvent B, users can calculate S and k_w. Retention can be calculated for any percentage of solvent B. This calculation greatly speeds finding optimized conditions for the desired retention factor range — $1 < k < 20$ — because only two runs are required rather than the stepwise search proposed earlier.

After k is known for a separation, analysts can calculate resolution using the fundamental resolution equation:

$$R_s = \tfrac{1}{4} N^{0.5} (\alpha - 1)[k/(1 + k)] \qquad [2]$$

Resolution (R_s) depends on the column plate number (N), the retention factor, and selectivity (α). Selectivity is calculated as $\alpha = k_2/k_1$ for adjacent peaks 1 and 2. It is easy to see that changes in the retention factor will produce a change in the selectivity. If users want to change N, they can modify column length, particle size, or flow rate. None of these factors will change k, so the changes in N will change peak width, run time, and resolution but not the relative peak spacing. A final consideration is that if the column diameter is changed, the flow rate also should be changed in relation to the square of the column diameter so that the back pressure is constant and the column plate number is maintained (assuming no change in particle size or column length).

Let's look at a couple of examples. First, consider the change from a 4.6-mm i.d. column to a 2-mm i.d. column. The scaling factor is $(4.6/2)^2 \approx 5$, so if the column was run previously at 1 mL/min, changing the flow rate to 0.2 mL/min for the 2-mm column should produce the same separation. Users must make no other changes to keep resolution constant for isocratic separation.

For a second example, let's make a change from a 15 cm × 4.6 mm, 5-µm d_p column operated at 1.5 mL/min to a 10 cm × 2 mm, 3-µm d_p column. Each of these columns should produce a column plate number of approximately 10,000 for a real separation. What adjustments do we need to make in this case to maintain constant resolution? For an isocratic separation, only the flow rate must be changed, exactly as for the first example. The flow now would be 1.5/5 = 0.3 mL/min to maintain constant linear velocity. The retention factor in isocratic separations and, therefore, α are unaffected by the change in diameter or column length because t_R and t_0 scale in parallel, so if N is a constant 10,000 plates, resolution also must be constant. The observed differences will be shorter retention times by a factor of the ratio of the column lengths, 15/10 = 1.5, and perhaps a change in pressure because of the change in packing particle size.

Therefore, changes in the column conditions used to scale a separation are quite simple in isocratic LC separation. Changes in the same parameters for gradient elution can be a bit more complex.

GRADIENT BEHAVIOR

With gradient elution, the average retention factor (k^*) is analogous to k in isocratic separation. The same kind of changes in the chromatogram occur when k^* is changed in gradient elution as when k is changed in isocratic elution. For example, retention is longer with larger values of k or k^*. This effect yields broader and shorter peaks and generally improves resolution. A steeper gradient (larger %B/min) results in lower k^* values and shorter runs, just like a stronger solvent in

isocratic separation reduces k and retention. Similarly, k^* must be held constant to keep selectivity constant, just like a constant k is required to maintain isocratic selectivity. When column conditions — length, diameter, flow rate, and particle size — change with isocratic separation, k is unaffected, so the scaling changes are quite simple. With gradient elution, however, k^* can be affected by changes in column conditions, which means that users must take more care when scaling gradient separations.

The average retention factor in gradient elution can be defined as

$$k^* = t_G F100/(V_m S\Delta\%B) \quad [3]$$

where t_G is the gradient time, F is the flow rate, V_m is the column volume, Δ%B is the gradient range (0–100% = 100), and S is a constant that is characteristic of the sample, as in equation 1.

Other than the substitution of k^* for k in equation 2, the fundamental resolution equation takes the same form for gradient elution:

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

SCOUTING RUNS

Equation 3 has two very practical uses in scouting runs and scaling gradients. The first use is to find conditions that are likely to yield a good separation for scouting purposes. As with isocratic separations in which users want a k range of 2–10, analysts observe the best chromatography for gradient separations when k^* is in the 2–10 range.

To produce isocratic k values in the desired range, analysts must perform stepwise changes of solvent strength or gather enough data (two runs) to use equation 1 to predict the solvent strength that will produce the desired k value. Usually a minimum of three runs is required to produce a run with the desired k value in isocratic separations.

For gradient separations, however, it is possible to use equation 3 to predict conditions that will produce the desired k^* value without performing preliminary runs. These conditions are determined most easily if equation 3 is rearranged as equation 5:

$$t_G = k^* V_m S\Delta\%B/(F100) \quad [5]$$

For a desired k^* value of 5, and an average S of 4, this equation simplifies to

$$t_G = 20V_m\Delta\%B/(F100) \quad [6]$$

Defining the remaining experimental conditions allows the calculation of the gradient time to produce $k^* = 5$. The column volume (V_m) in milliliters is estimated easily with equation 7:

$$V_m \approx 0.5Ld_c^2 \quad [7]$$

where L is the column length and d_c is the column internal diameter, both in centimeters. Thus, the 15 cm × 4.6 mm column in the isocratic example yields a column volume of approximately 1.6 mL. If this column is run at 1.5 mL/min with a full range gradient (5–100%B), equation 6 produces

$$t_G = 20 \times 1.6 \text{ mL} \times 95/(1.5 \text{ mL/min} \times 100) = 20 \text{ min}$$

This equation shows that analysts should see good chromatography in the first run with this setup if they use a gradient time of approximately 20 min. The selection of these conditions before making the first run makes gradient elution a very useful technique for scouting runs with unknown samples. Now analysts aren't restricted to the stepwise adjustment of the percentage of solvent B required for isocratic separation or finding two isocratic separations with sufficiently good data to satisfy equation 1.

SCALING GRADIENTS

Equation 3 also is useful for guidance when adjusting the separation. Because k^* defines the selectivity, k^* must be constant when changing conditions changes the plate number (see equation 4). For example, if the flow rate is increased by a factor of two, one of the other parameters, such as t_G, must be changed to maintain constant k^*. For the example above, equation 3 shows that increasing the flow rate from 1.5 to 3 mL/min requires that the gradient time must be changed by a factor of two from 20 min to 10 min to obtain a constant k^*.

Let's look at the same two examples of column changes used for isocratic separations earlier. First, the change of column diameter from 4.6 mm to 2 mm requires the same adjustment of flow rate to maintain constant pressure, so F must be reduced from 1 mL/min to 0.2 mL/min, just as in the isocratic case. Equation 3 shows that both F and V_m affect k^*, so analysts must make sure that no other adjustments are necessary. Equation 7 shows that V_m is proportional to d_c^2. In the present case, F/d_c^2 is constant for both columns ($1.0/4.6^2 = 0.2/2.0^2$), so no other changes need to be made to maintain k^* and constant resolution.

The second example substituted a 10 cm × 2 mm, 3-µm d_p column for a 15 cm × 4.6 mm, 5-µm d_p column run at 1.5 mL/min. As with the previous example, the flow scales by a factor of five from 1.5 mL/min to 0.3 mL/min. However, the change in column length now causes additional changes in V_m that require corrections. V_m for the 15-cm long column is 1.6 mL; for the 10-cm column, $V_m \approx 0.5 \times 10 \times 0.2^2 = 0.2$ mL. If analysts use the scouting gradient for remaining conditions, the 10-cm column now requires a shorter gradient time:

$$t_G = 20 \times 0.2 \text{ mL} \times 95/(0.3 \text{ mL/min } 100) = 12.7 \text{ min}$$

This result means that the column and flow rate changes require that the gradient time be reduced from 20 min to approximately 13 min to produce the same k^* for the same relative peak spacing. It is not simply a matter of changing the column diameter and flow rate when column conditions are changed for gradient elution.

LC–MS

With the increasing use of triple–quadrupole mass spectrometers with LC (LC–MS–MS), analysts have moved toward the use of short, small particle size columns to increase throughput. Many workers are using miniaturized columns with what are commonly called ballistic gradients to push samples through quickly. Let's look at an example for one of these columns.

I'm looking at the literature from one manufacturer advertising 1.5 cm × 2.1 mm, 3.5-µm d_p columns for use with LC–MS applications. For practical separations, these columns should generate 1000–2000 theoretical plates — enough for the requirements of LC–MS–MS. First, estimate the column volume, $V_m \approx 0.5 \times 1.5 \times 0.21 \times 0.21 \approx 0.03$ mL. If the original flow rate was 1.5 mL/min as in the scouting run, the equivalent flow will be 0.3 mL/min. For $k^* = 4$, analysts can use equation 6 to determine the appropriate gradient conditions:

$$t_G = 20 \times 0.03 \times 95/(0.3 \times 100) \approx 2 \text{ min}$$

Therefore, although the term ballistic implies that by using short, fast gradients, the chromatography will suffer. The k^* of 5 is obtained in a 2-min gradient. This calculation does not mean that the separation will be outstanding, because the plate number is only 20% of that for the 15-cm long, 5-µm d_p column, but it should be satisfactory for the current application.

ADDITIONAL COMPLICATIONS

Workers must consider two other factors when scaling gradients to very small columns: extracolumn effects and system dwell volume. Let's briefly look at the impact of these parameters.

Extracolumn effects refer to all the characteristics of the system, except the column, that contribute to band broadening. The primary contributors to extracolumn effects are the injection volume, connecting tubing volume, detector cell volume, and detector time constant. With a 15 cm × 4.6 mm, 5-µm d_p column, the extracolumn band broadening of a typical LC system accounts for approximately 5% reduction in the column plate number when compared with ideal conditions. Because the impact is minor, most workers don't notice these losses with most methods. With a small 1.5 cm × 2.1 mm, 3.5-µm d_p column, however, the same LC system would account for a 60% loss in plate number. As a result, when columns that produce small peak volumes are used, the system may require extensive re-

plumbing or other changes to minimize extracolumn effects.

Dwell volume is the system volume from the point where the mobile phase is mixed to the head of the column. For high-pressure mixing systems, this factor includes the mixer volume, plumbing, and injection loop. Typical dwell volumes for these systems range from 0.5 to 2.5 mL. Low-pressure mixing systems require additional plumbing and the pump volume is added, so typical dwell volumes for these systems are 3–6 mL. The dwell volume places an unintended isocratic hold at the beginning of the gradient. For example, if the system dwell volume is 3 mL and the flow rate is 1.5 mL/min, 2 min of isocratic hold would occur at the beginning of each gradient. This factor is equivalent to approximately two column volumes for a 15 cm × 4.6 mm column. With the large 15 or 25 cm × 4.6 mm columns used in most applications, the dwell volume is a minor irritant that must be considered when transferring methods from one system to another. When using miniaturized columns, however, the dwell volume can have disastrous effects. For example, the 1.5 cm × 2.1 mm column has a volume of roughly 0.03 mL. The same 3-mL dwell volume system would introduce an isocratic hold of 100 column volumes at the beginning of each gradient. A further complication with the small columns is the dramatic impact on column reequilibration after a gradient. A 10-column volume reequilibration for the small column at 0.3 mL/min would take just 1 min, but with a 3-mL dwell volume, an additional 10 min would be required, which would cancel out most of the gains made by reducing the run time to 2 min.

Therefore, users will not obtain acceptable gradient performance with miniaturized columns unless the LC system hardware is miniaturized to minimize extracolumn effects and system dwell volume.

CONCLUSION

Scaling gradient separations to accommodate smaller diameter or shorter columns is straightforward when analysts adjust the appropriate parameters. These adjustments will yield separations that scale as expected. The ballistic gradient conditions commonly used for LC–MS–MS actually generate reasonable k^* values, somewhat counter to popular belief. Finally, chromatographers will fail to realize the full advantage of short, small-particle columns unless they also adjust certain instrument parameters.

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