TROUBLESHOOTING

Gradient Elution Separation Problems, Part I

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Problems with gradient elution in liquid chromatography (LC) are of two kinds. First, there are problems associated with equipment failure, experimental technique, deteriorated columns, etc. Second, many problems arise from an imperfect understanding of how gradient elution works and what to expect if conditions are changed. This and next month's "Troubleshooting" columns address some of these problems and their cures.

GRADIENT ELUTION BASICS

In gradient elution, the composition of the mobile phase changes during a separation. Typically, two solvents are used for the mobile phase: a weak solvent A and a strong solvent B. For reversed-phase separations, for example, A might be water and B might be methanol. In all cases, the strength of the mobile phase should increase during the separation, which means that the concentration of B in the mobile phase must increase. This is illustrated in Figure 1 for a gradient from 30% to 80% methanol in water. The gradient controller is used to determine the composition of the mobile phase during gradient elution, with the operator entering the various conditions:

- starting % B in the mobile phase (30% in Figure 1)
- final % B in the mobile phase (80% in Figure 1)
- gradient time from beginning to end of separation (20 min in Figure 1)
- gradient shape (linear in Figure 1)
- flow rate.

Some controllers provide for entering the percentage change in B per minute (2.5%/min in Figure 1), instead of gradient time — but that provides an equivalent result.

Gradient elution is normally used for separating samples that vary widely in polarity, as well as for those in which the isocratic retention (capacity factor $k' = [t_R - t_o]/t_o$, where t_R is retention time and t_o is column dead-time) of the last band is much greater than that of the first band. This is illustrated in Figure 2 for the reversed-phase separation of a mixture of chlorobenzenes. For this sample, isocratic separation (Figure 2b) bunches peaks together at the beginning of the chromatogram and broadens them excessively at the end of the chromatogram. Generally, for isocratic separations it is preferable that the peaks elute with $1 < k' < 20$, or a k' ratio of 20 or less; in Figure 2a, the range is $0.2 < k' < 21$, or a k' ratio of 100. When peak-bunching of early bands and a wide k' range occur, it is unlikely that a satisfactory isocratic separation can be achieved with the present column and mobile phase components. Gradient elution often will provide superior separation in cases such as the example under discussion. The gradient separation (Figure 2a) provides a more even spacing of bands and better resolution, as well as narrower (more easily detected) bands at the end of the chromatogram.

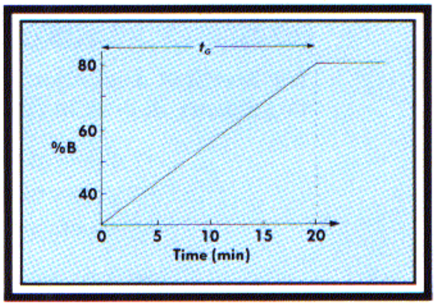

FIGURE 1: **Illustration of gradient conditions.**

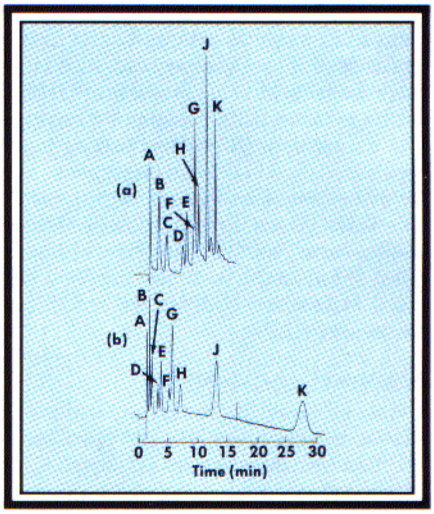

FIGURE 2: **Comparison of gradient and isocratic elution for reversed-phase separation of chlorobenzene sample. (a) Gradient elution from 40–100 vol % methanol in water; (b) isocratic elution using 50 vol % methanol in water. Peaks: A = benzene, B = chlorobenzene, C = *o*-dichlorobenzene, D = 1,2,3-trichlorobenzene, E = 1,3,5-trichlorobenzene, F = 1,2,4-trichlorobenzene, G = 1,2,3,4-tetrachlorobenzene, H = 1,2,4,5-tetrachlorobenzene, J = pentachlorobenzene, K = hexachlorobenzene. (Reprinted from reference 1 with permission.)**

The problem with separations such as those of Figure 2b is that early bands have very small k' values and late bands have very large k' values. Gradient elution works by changing k' during the separation — because mobile phase strength changes. Thus, weakly retained compounds leave the column first, in a weak mobile phase, while strongly retained bands leave last, in a strong mobile phase. To a first approximation, all bands in gradient elution will have similar k' values and similar bandwidths. (For a detailed discussion of gradient elution and the control of separation, see references 1 and 2.)

As in isocratic separations, early bands can be poorly resolved in gradient elution if the starting % B is too large. In that case, the initial mobile phase strength is too great, and the initial bands elute with k' values that are too low. This is illustrated in the examples of Figure 3 for a starting mobile phase of 46% acetonitrile (ACN)–water (Figure 3a) and one of 79% ACN–water (Figure 3b). Bands A–E are too weakly retained in 79% ACN to be resolved in the separation of Figure 3b. When bunching of initial bands is observed in gradient elution, the solution is to change the starting % B to a lower value. Notice that very little change in band spacing has occurred with the later bands in the chromatogram (bands J–N of Figure 3) when the initial % B is changed from 46% to 79% ACN–water. This is because these bands undergo no significant migration until after the starting % B reaches 79% in either example. Thus the initial composition of the mobile phase often can be varied with gradient elution so as to affect the retention of only some of the bands.

The final % B in the gradient should be adjusted so that the last band leaves the column at about the time the gradient is completed. That is, if the gradient time is 20 min, the retention time for the last band should be ~20 min. If the last band leaves the column much before the end of the gradient, the rest of the gradient time is wasted. If the last band elutes much after the gradient is completed, it will elute under isocratic conditions at the end of the gradient (which may or may not be desirable).

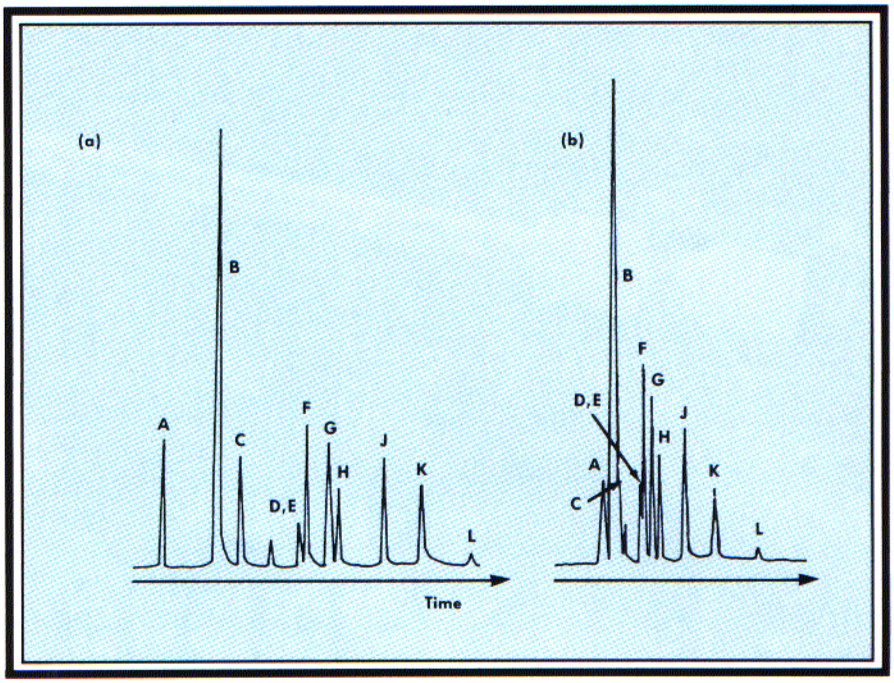

FIGURE 3: Reversed-phase gradient elution with change in % B at start of gradient. (a) Gradient from 46–95% ACN–water, (b) gradient from 79–95% ACN–water. Peaks labeled for comparison. (Reprinted from reference 4 with permission.)

In summary, gradient elution should be used for samples with wide polarity ranges that give poor separations by isocratic methods. The symptoms of candidate samples are bunched peaks early in the chromatogram and broad peaks that elute with a capacity factor of more than ~20. The necessary changes in band spacing can sometimes be achieved by changing the mobile phase selectivity (for example, using methanol instead of ACN) or column type (for example, C18 vs. phenyl). When a gradient method is developed, the starting mobile phase composition should be sufficiently weak to give good separation of the early bands. The ending mobile phase composition should be adjusted so that the gradient ends about the same time as the elution of the last band. In general, the equipment is simpler and the problems are fewer for isocratic methods than for gradient methods. This is reflected in lower assay costs and increased reliability for isocratic methods. For this reason, many labs use gradient elution for the development of initial methods but convert these to isocratic assays for the routine lab. Software programs are available

(3) to speed this gradient-to-isocratic conversion.

GRADIENT ELUTION PROBLEMS

Initial gradient separations often show incomplete resolution of one or more band pairs — as is the case in isocratic separations. Similarly, resolution can be improved in gradient separations in the same way as it is improved in isocratic elution. That is, we can optimize k', N, and/or α. Many workers have trouble using this approach for gradient elution, although it is actually simpler than for isocratic separation. The major difficulty is that k' in gradient elution depends on such conditions as the starting and final % B, the gradient time (t_G), the column volume (V_m), and the flow rate (F). There is, however, a simple relationship between k' and these various conditions:

$$k' \approx (\text{constant})\ t_G F/(\Delta\ \%\ \text{B})V_m \qquad [1]$$

Here, "(constant)" is equal to ~20 for reversed-phase HPLC, "(Δ % B)" is the final % B minus the starting % B, and the column volume $V_m = F t_o$ (about 1.5 mL for a 15 cm × 4.6 mm column). According to equation 1, k' can be increased in gradient elution with an increase in gradient time or flow rate, a decrease in column length (which decreases the volume V_m), or a decrease in the gradient range (change in % B during the gradient). When k' is increased in gradient elution, the same results are obtained as in isocratic elution: wider bands, longer separation time, and better resolution. This is illustrated in Figure 4 for the ion-exchange gradient elution of a mixture of carboxylic acids. In Figure 4a, a 20-min gradient time is used, while in Figure 4b a 50-min gradient is employed.

Changing the flow rate: An understanding of equation 1 can explain why some workers are puzzled when they change the flow rate for a gradient separation. With isocratic separations, an increase in flow rate causes peaks to elute earlier, increases the system pressure, and somewhat reduces the column plate number but does not affect k'. An increase in the flow rate for a gradient separation, however, reduces the resolution, the band widths, and the retention times, and, as shown in equation 1, causes k' to be lower. Recall from the isocratic example that anything that changes k' also might change the selectivity of the separation. So, changing flow rate as a means of changing run time is not as innocuous in gradient elution as it is with isocratic separations. In order to change the flow rate without changing k', you must change one of the other parameters in equation 1; for example, halve the gradient time and double the flow rate to keep k' constant. In this manner, you have speeded up the separation but have not changed the selectivity.

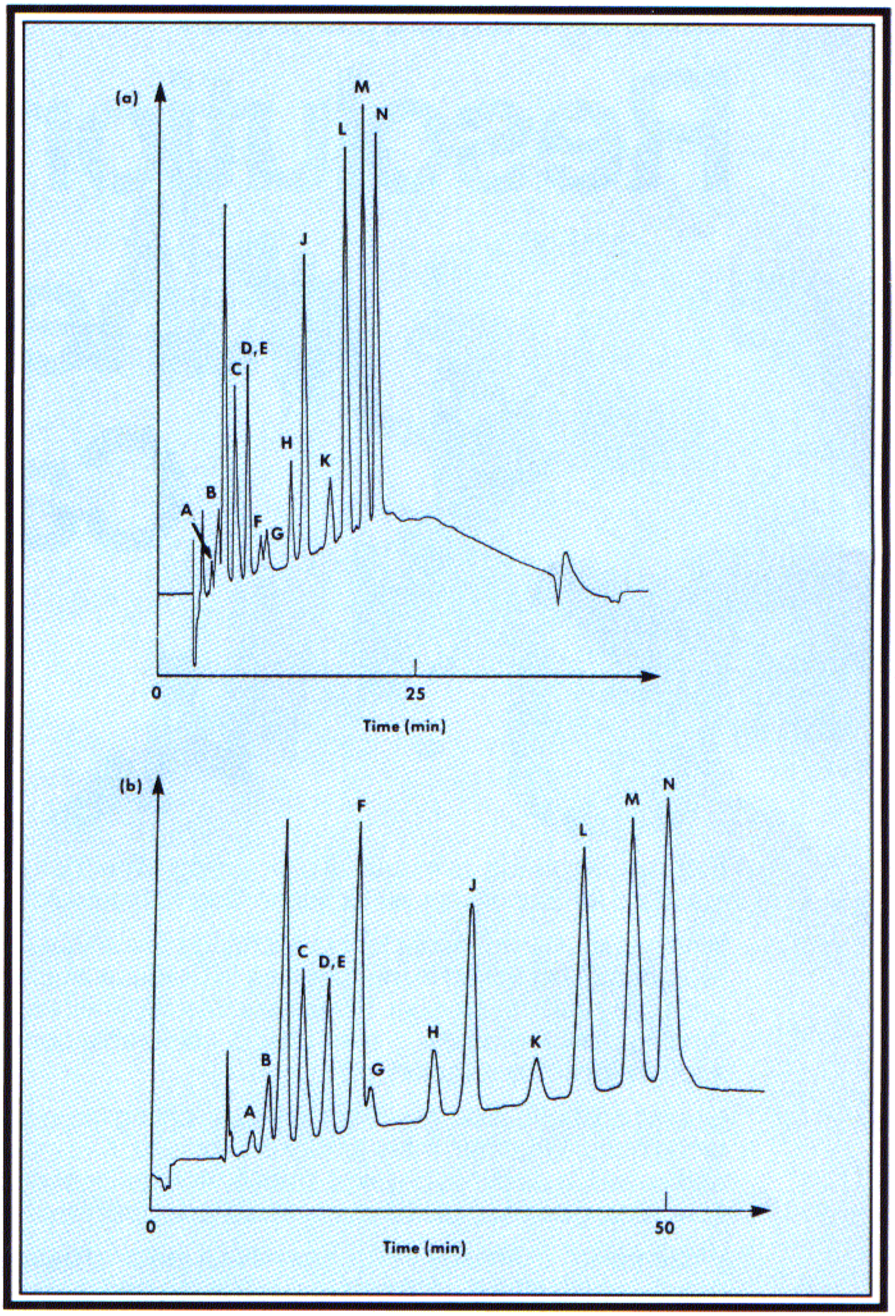

FIGURE 4: Effect of gradient time on ion-exchange separation. (a) Gradient from 10–100 m*M* salt over 20 min; (b) same over 50 min. Peaks labeled for comparison. (Reprinted from reference 1 with permission.)

Changing N: As discussed, it is possible to improve resolution in gradient elution by increasing k', but it is also possible to increase resolution by increasing N, as is the case in isocratic separations. N is increased through use of a better column (for example, one with a smaller particle size) or a longer column, or by a decrease in flow rate. When column length or flow rate is changed, however, k' is affected (equation 1). That is, conditions that increase N lead to smaller values of k', which is undesirable as far as resolution is concerned. So, a longer column doesn't give the expected increase in resolution if only the column length or flow rate is changed — a concept that is generally confusing to most chromatographers. The solution in gradient elution is to hold k' constant while increasing N. According to equation 1, if $t_G F/V_m$ is kept constant while column length is changed, k' will remain constant also. For example, if we want to double column length (which doubles V_m), we must double either the flow rate or the gradient time. Or, if we double the column length while halving flow rate (to keep the pressure constant), we must increase gradient time fourfold.

Changing selectivity: Change in α or band-spacing in gradient elution is accomplished in the same way as it is in isocratic separation: by changing mobile phase composition, the type of column, or temperature. Unfortunately, the results of these changes are less intuitive than with isocratic separations; a lot of experience or the use of computer-aided method development (2) is necessary to reach satisfactory separation conditions quickly.

SUMMARY

Gradient elution LC is useful for separating samples that have too broad a polarity range to be separated by isocratic methods. An understanding of the relationships among the various retention-related parameters (equation 1) should eliminate many of the problems encountered when inappropriate changes in these parameters are made. Thus, changes in flow rate, column length, gradient time, and gradient range can be made in a manner such that the k' of the bands is not changed. These relationships can be quickly mastered with the techniques discussed in reference 2.

REFERENCES

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(3) L.R. Snyder and J.W. Dolan, *DryLab 4,5 User's Manual*, LC Resources Inc. (San Jose, California, 1986).

(4) L.R. Snyder, in *High-Performance Liquid Chromatography: Advances and Perspectives*, Vol. 3, Cs. Horváth, ed. (Academic Press, New York, 1983).

Portions of this column are taken from *Troubleshooting HPLC Systems*, J.W. Dolan and L.R. Snyder, in preparation for Humana Press.

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