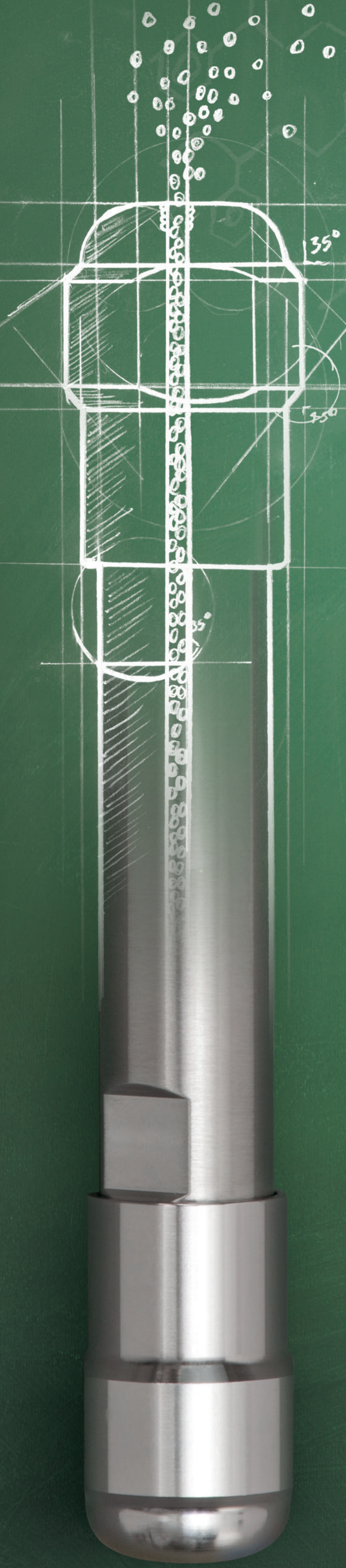
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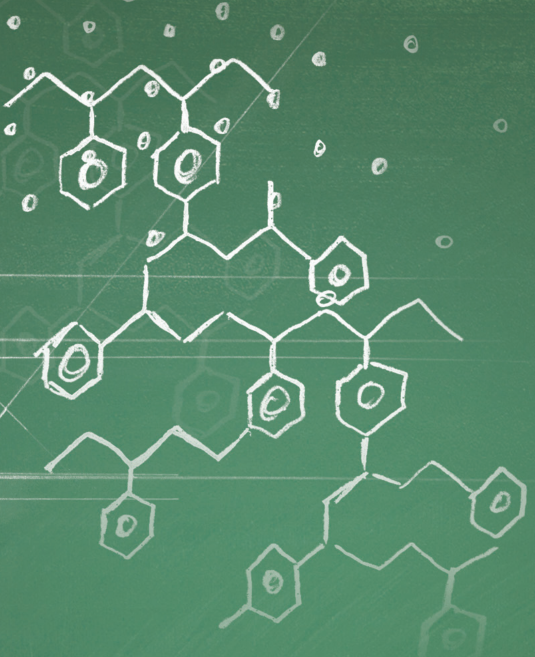

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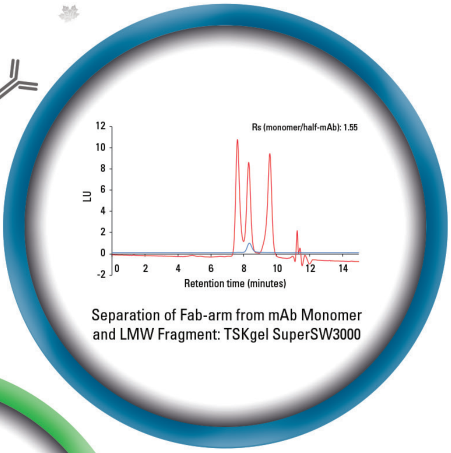

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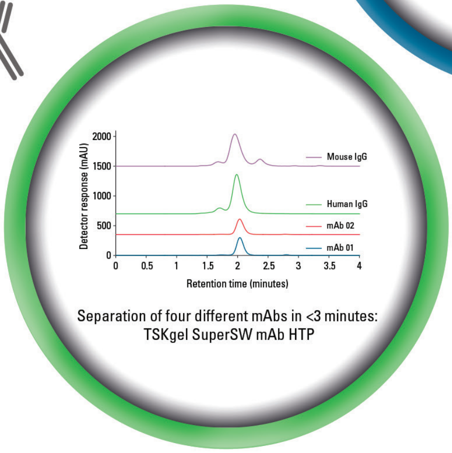

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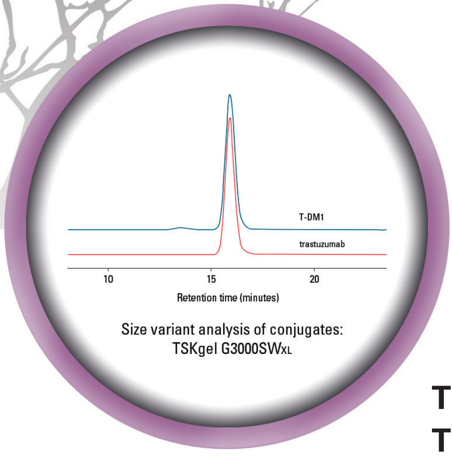

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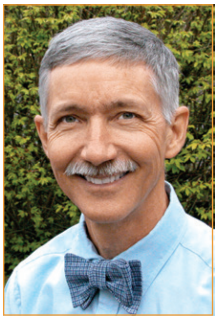
The Secrets to Successful Gradient Separations

Gradient elution in liquid chromatography (LC) is often a disparaged technique because of its mystery and the problems that are associated with it. Ghost peaks, a rarity in isocratic separations, are common with gradients. Transfer of methods seems to be more difficult with gradients. Changes in some conditions, such as flow rate, that seem so intuitive in isocratic methods, can have unexpected results.

However, in spite of its negative aspects, gradient elution can work magic with many separations. Samples of wide-polarity components can be separated, method run times are predictable, run-to-run visual appearances can be more constant than with isocratic methods, and the possibility of universal scouting conditions is a reality with gradient elution. Gradients can be the best way to start to develop methods, even if the goal is an isocratic method.

In this chapters contained in this e-book, I've attempted to take the mystery out of gradient elution. I strongly believe that if you understand what makes gradient elution work, you will be better able to use it successfully, and will be able to avoid possible pitfalls. I've shown intuitive parallels between isocratic and gradient separations wherever possible. Yes, there are a few equations, but they are simple and allow you to obtain predictable results when method conditions are changed.

I hope that as a result of reading this e-book, you'll come to the place where gradient elution is your friend. You will find that it is a valuable tool to have in your separations toolbox.



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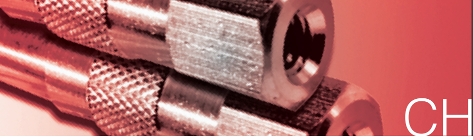


CHAPTER **ONE**

Intuition

In contrast to isocratic separations, gradient elution sometimes can seem to be counterintuitive. Yet with the proper perspective, the things we intuitively understand about isocratic methods apply in a similar manner to gradients.

In many past installments of "LC Troubleshooting," we've looked at gradient elution liquid chromatography (LC) in different contexts. In 2013 (1), we considered how a gradient scouting run could be used to speed up development work. In other cases, we've looked at specific problems with gradient methods, as well as other aspects of gradient elution. I make no effort to hide the fact that I have a bias toward using gradients—they have the potential to be faster, give better detection limits, and have more flexibility than their isocratic counterparts. However, there are many problems that can occur with gradients as well. Some of these problems are inherent to the technique and can be avoided or accommodated with a little care. Other problems result from a poor understanding of gradient elution or sloppy laboratory technique.

The breadth of problem topics makes it impossible to discuss them all in detail in a single chapter. This chapter concentrates on how we can transfer our intuitive understanding of isocratic separations into a similar understanding of gradient elution. If you want more detail on this or any other gradient-related topics, reference 2 contains a discussion of gradient elution at many different levels of detail.

Isocratic—It's So Simple

I believe that one of the reasons that reversed-phase LC is such a popular technique is the intuitive nature of isocratic separations. The mobile phase usually comprises an aqueous and an organic component. The aqueous phase typically is a buffer or water, whereas the organic component most commonly is acetonitrile or methanol. The aqueous phase is the weak solvent, often called the A-solvent, and the organic is the strong solvent, or B-solvent. Isocratic conditions are those that do not change during a given run, so the ratio of A to B, or %B, is constant.

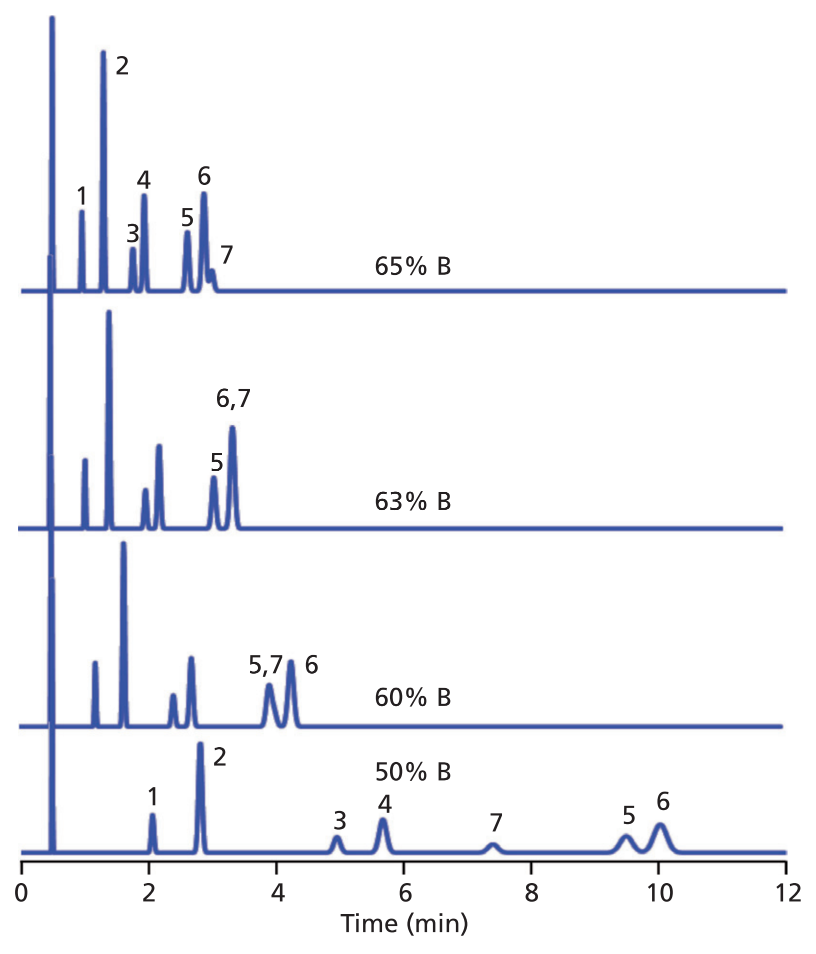
It takes very little experience to figure out how peaks behave when %B is changed. For example, consider the sample shown in Figure 1. Working from top to bottom, each chromatogram is the result of a decrease in %B, from 65% to 50%. As the %B is reduced (or %A is increased), four observations are common. First, peaks move to longer retention times. Second, the overall separation tends to improve. Third, the peaks become broader, and fourth, because the area is constant, the peaks become shorter. This general pattern of change occurs when solvent strength is changed in any reversed-phase separation of any sample and with any stationary phase and solvent system. And yes, for you purists, there are a few exceptions, but this is the most common behavior.

As a result of knowledge gained from experiments such as those just mentioned, a common method development strategy is used widely. Start at a high percentage of the organic component, such as 90% B, and make stepwise changes in the solvent strength—90%, 80%, 70%, and so forth—until the separation looks pretty good. Then fine-tune in smaller steps.

This relationship between retention and %B can be generalized with the *Rule of 2.5*, which states that, on the average, the retention factor, k, will change about 2.5 times for a 10% change in the B-solvent. We can see how this works by examining the simulated chromatograms of Figure 1, which are for a sample of nitroaromatic compounds based on data presented in reference 3. The retention factor is calculated as $k = (t_R - t_0)/t_0$, where t_R and t_0 are the retention time and the column dead time, respectively. The column used for Figure 1 is a 100 mm × 4.6 mm column run at 2 mL/min, which results in $t_0 \approx 0.5$ min. This is confirmed by the sharp t_0 peak at the beginning of each chromatogram at ~0.5 min. With knowledge of t_0, we can calculate that the last peak in the 65%, 60%, and 50% runs have k values of ~4.5, 7, and 19, respectively. A change from 60% to 50% is 10%, so $2.5 \times 7 = 17.5 \approx 19$ observed for 50%. Because the relationship between log k and %B is linear, a 5% change in %B should have about a 1.6-fold change in k. Again, this can be confirmed from Figure 1 by comparing the 65% run ($k \approx 4.5$ for the last peak): $4.5 \times 1.6 = 7.2 \approx 7$ for the 60% B run. (Because this behavior will vary a bit depending on the molecular weight of the compound, we often see the Rule of 2.5 expressed as the Rule of Three, which makes for easier mental calculations.)

Although the general observation of improving separation for lower %B values is true for isocratic separations, the behavior of specific peaks may vary from this generalization. For example, the separation, retention, and peak width for peaks 1–4 in Figure 1 all increase with lower values of %B. Note, however, the behavior of peaks 5–7. At 65%, the peaks are eluted in order and

Figure 1: Isocratic separation of a nitroaromatic sample. Column: 100 mm × 4.6 mm, 5-μm particle C18; flow rate: 2 mL/min; temperature: 25 °C; A-solvent: water; B-solvent: methanol with %B shown on chromatograms. All chromatograms have the same *x*- and *y*-axis scaling. Simulated chromatograms based on the data of reference 3. Peaks: 1 = 2,6-dinitrotoluene, 2 = nitrobenzene, 3 = 2-nitrotoluene, 4 = 3-nitrotoluene, 5 = 2-nitro-1,3-xylene, 6 = 4-nitro-1,3-xylene, 7 = benzene.



are easily distinguished but are not fully separated. At 63%, however, peaks 6 and 7 have merged, and peak 7 moves forward to merge with peak 5 at 60%. So, for this example, neither the 63% nor the 60% run is better than the shorter 65% run. By the time we get to the 50% run, peak 7 has moved from the latest-eluted to the earliest-eluted position of this triplet. Such behavior, where selectivity, or relative peak position, changes with %B changes is sufficiently common that fine-tuning %B can be a powerful tool to move peaks relative to each other in isocratic separations during LC method development.

Consider the influence of some other variables on the separation. A change in flow rate merely expands or compresses the chromatogram but has little, if any, affect on the separation. The pressure also goes up or down in proportion to the flow-rate change. An increase in the column temperature will shorten the retention time and generate narrower peaks, much like the way an increase in %B does, but not so dramatically. The rule of thumb for temperature states that a 1 °C change in temperature will change retention by approximately 2%. Although often overlooked, a change in temperature also may change selectivity in isocratic separations. An increase in column length will increase the pressure and make the run longer, but will not change the relative peak positions. Similarly, a reduction in packing particle size will increase the pressure and give narrower peaks, but will not change the retention times of the peaks.

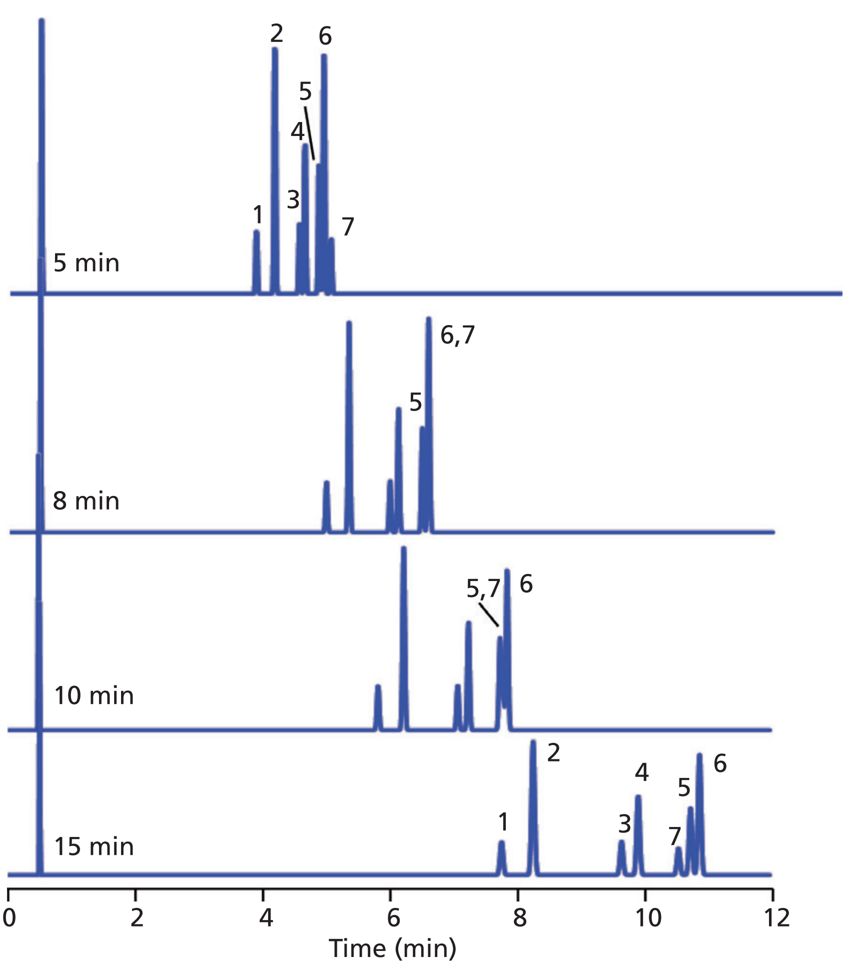
These various patterns of change form the basis of my "nothing's magic" rule of chromatography. This rule says that changes in a chromatogram due to changes in some variable usually happen in a continuous fashion. For example, if you change from 70% B to 60% B, you will notice that the retention increases, so you expect similar retention increases when you change from 60% B to 50% B. Sometimes the relationships are logarithmic instead of linear, but the general patterns apply. And this intuitive quality of isocratic separation is one reason why reversed-phase isocratic separations are so widely used and so successful—you don't have to understand chromatographic theory to get reasonable results. As you gain experience, your gut-level instincts usually will give you the appropriate guidance.

What About Gradients?

The intuitive nature of isocratic separations is very attractive, and is one reason why many chromatographers prefer isocratic methods. Gradients, where %B is changed during the run, can seem much more daunting. However, if the appropriate comparisons are made, many of the same intuitive results can be obtained from gradients. The key here is that the comparisons have to be appropriate. For the current example of nitroaromatics (3), I've carefully chosen gradient conditions for Figure 2 that correspond closely to their isocratic counterparts of Figure 1. (As a side comment, let me state the obvious: Gradient time and gradient steepness are just two different ways to describe the same separation conditions. For example, the runs of Figure 2 are 0–100% B in various times. The 5-min gradient could also be expressed as 100%/5 min = 20%/min. I will use the gradient time in the present discussion.)

Our overall conclusions after examining the separations of Figure

Figure 2: Same sample as Figure 1. Simulated 0–100% B gradients with gradient times shown on chromatograms. Same scaling as Figure 1.



2 are similar to those from Figure 1: Longer gradient times increase retention, improve the separation, and result in broader, and thus shorter, peaks. Notice that the peaks occur in the same order in both sets of runs. In the 5-min and 65% B runs, the peaks are eluted in numeric order. In the 8-min and 63% B runs, peaks 6 and 7 are merged, whereas peaks 5 and 7 are merged in the 10-min and 60% B chromatograms. In the 15-min and 50% B runs, all peaks are resolved, with the sequence of 7-5-6 for the last three peaks. We can see from these examples that fine-tuning the gradient time can be used to move peaks relative to each other, just as changing %B in isocratic separations can.

The similarity of the separations in Figures 1 and 2 show that, with proper care, you can get approximately the same results from gradient and isocratic separations, implying that the separation mechanisms must be quite similar. In fact, separation behavior of gradient and isocratic chromatography is unified with the “linear solvent-strength theory,” which is the subtitle of reference 2. One way of thinking about this is that gradient and isocratic chromatography are the same process viewed from different perspectives. Thus, if we change the gradient time or gradient steepness, we will get similar changes as changing the isocratic %B. Longer or less-steep gradients increase run time, improve resolution, and result in broader, shorter peaks, just like a decrease in isocratic %B. This allows us to transfer some of our intuitive understanding of isocratic separations to gradients.

What about the other variables we briefly examined for isocratic separation: flow rate, temperature, column length, and packing particle size? An increase in column temperature in gradient elution also will reduce retention (although not as dramatically as the 2%/°C for isocratic), lower pressure, sharpen peaks, and may change selectivity, just as in isocratic runs. Similarly, a reduction in particle size will increase pressure and yield narrower peaks without changing retention. In contrast to isocratic separations, where a change in flow rate or column length had logical and expected results, the results with gradients can be surprising—especially if relative peak positions change when one or both of these variables are changed. Such surprises often are responsible for conclusions that gradients are not as logical as isocratic separations. We’ll examine these, and other, differences in gradient versus isocratic behavior in the next few chapters.

The Rule of 2.5 (or Rule of Three, if you prefer) for isocratic separation gave a simple way to anticipate the change in retention when %B is changed. Retention in gradient elution isn’t so neatly described. However, one general observation is that as the gradient time is increased, the retention relative to the gradient time drops. In the 5-min gradient, the last peak is eluted at 4.7 min, or 93% of the way through the gradient. The retention of the last peak drops to 83%, 79%, and 72% as the gradient time is increased from 8 to 10 to 15 min.

Another contrast between gradient and isocratic separations is related to peak width. In the isocratic separations of Figure 1, it can be seen that the peak width depends on its retention time not the mobile-phase composition. Peaks early in the chromatogram are narrow, and as retention increases, so does peak width. For example, peak 5 at 65% B, peak 4 at 60%, and peak 2 at 50% all have approximately the same retention time and peak width, whereas in each chromatogram, the later-eluted peaks are broader than the first ones. With gradient separations, however, all the peaks in a given run are approximately the same width, and peak width increases with gradient time. So all the peaks in the 15-min run are broader than those in the 8-min run, yet all the peaks in the 15-min run are approximately the same width. This behavior can be an advantage for gradient runs, because narrow peaks mean taller peaks and, thus, lower detection limits. For example, peak 6, which was eluted at ~11 min in the 15-min gradient of Figure 2, is much taller than its counterpart eluted at ~10 min with 50% B in Figure 1 (all chromatograms in Figures 1 and 2 are on the same scale). Thus, gradients often are favored for the detection of small peaks that are strongly retained.

Isocratic or Gradient

At the beginning of this chapter, I mentioned my bias toward gradients, but it is obvious from comparing the 50% B isocratic run in Figure 1 to the 15-min gradient in Figure 2, that the isocratic separation wins in terms of getting a better separation in the same run time. We could have anticipated this if we had applied the 25/40% rule explained in a previous "LC Troubleshooting" discussion (1). The peaks of the 15-min gradient of Figure 2 occupy (10.9–8.3 min)/15 min = 26% of the run, which is very close to the <25% cutoff where isocratic runs are very likely to be preferred. So it is not surprising that for this sample, isocratic separation is a better choice than gradient elution. As the retention range between the first and last peaks increases, gradients are favored.

Conclusions

Isocratic LC separations often are the method of choice because they are so intuitive to develop. We have seen, however, that if gradient run time or gradient steepness is considered in the same context as isocratic %B, gradient and isocratic separations behave in a very similar manner. Longer, shallower gradients give the same results as reducing the %B in an isocratic mobile phase: longer retention times, generally better separation, and broader, shorter peaks. Fine-tuning the gradient time, just like fine-tuning the isocratic %B, can be used to make subtle changes in the separation. In the next chapters, we'll consider how to further unravel some of the mysteries of gradient elution, as well as some of the problems that can be encountered.

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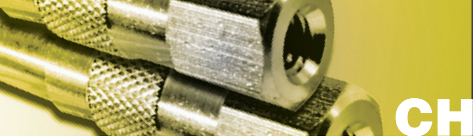


CHAPTER TWO

Equivalent Separations

Is it possible to get the same separation under isocratic and gradient conditions?

In chapter one we saw that liquid chromatography (LC) separations under isocratic and gradient conditions had some of the same properties when operating conditions are changed. For example, when run times are made longer, resolution tends to improve, peaks broaden, and peak height drops. Longer run times are obtained in reversed-phase LC by reducing the concentration of the organic solvent (%B) in isocratic separations or increasing the gradient time (reducing the gradient steepness) with gradients. In this chapter, we'll see how to compare gradient and isocratic separations under "equivalent" conditions.

When Isocratic Won't Work

In Figure 1a, we see a chromatogram for the isocratic separation of 15 compounds at 60% B. Several deficiencies are seen. The peaks early in the chromatogram are narrow and tall, but the first two are poorly resolved. At the end of the chromatogram, the peaks are well separated, but they are broadened excessively. The run takes nearly 30 min. If we try to improve the separation at the beginning of the chromatogram, we would reduce the %B in the mobile phase. This would increase the retention of the early peaks, and in general is expected to improve the resolution. However, the peaks at the end of the run would broaden more and move to even longer retention times—neither of which is desirable. Alternatively, we could improve the separation at the end of the chromatogram by increasing the %B, which would reduce the retention times, close up some of the gaps between the peaks, and sharpen the peaks. But the higher %B would compress the chromatogram at the beginning, making it even worse.

Samples such as in Figure 1a are a good example of *the general elution problem*. In such cases, it is difficult or impossible to get a satisfactory separation for all the peaks under a single isocratic condition. We can improve the beginning or end of the chromatogram, but not both at the same time. The reason for this is that the polarity range of the sample is too large. Recall that under the most desirable conditions, we'd like the retention factor, k, to be $2 < k < 10$, but if $1 < k < 20$ can be obtained, we often can find acceptable separation conditions. In the present case, $0.9 < k < 34$, so the k range ($34/0.9 \approx 38$) is approximately twice the maximum we would like ($20/1 = 20$). When samples such as this are encountered, a gradient is likely to give a better separation.

What About a Gradient?

When the sample of Figure 1a is run under gradient conditions, we get the separation of Figure 1b. We can quickly see that the separation is improved. The resolution between peaks is increased at the beginning of the run and the excess time between peaks at the end of the run in Figure 1a is reduced. Thus, the peaks are spread out more evenly across the chromatogram. Also, note that the peak widths throughout the run are approximately the same. Narrower peaks at the end of the run translate into taller peaks, so detection is improved in this part of the chromatogram. Finally, for the present sample, the run time is reduced by a factor of four. All these improvements make the gradient a better choice for this sample. (Gradients, however, are not always better, as we saw in chapter one where an isocratic separation was a better choice.)

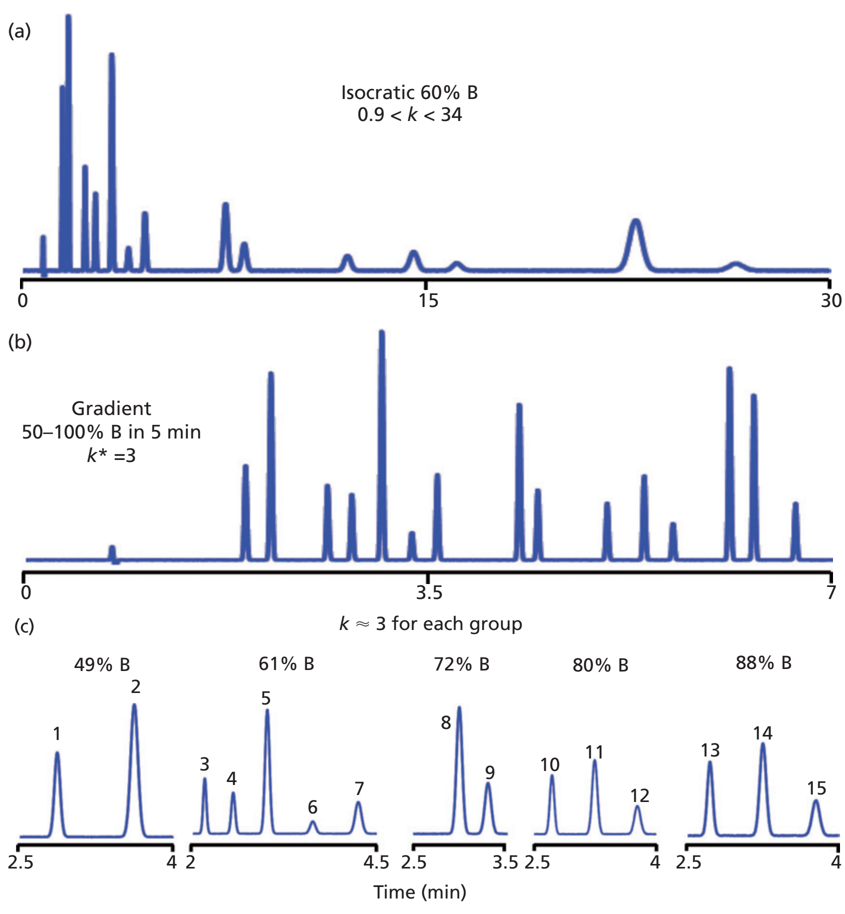
Comparing Isocratic and Gradient Separation

Next, let's look at isocratic and gradient separation under so-called equivalent conditions. By this we mean mobile-phase conditions under which the retention and peak properties are roughly the same with both isocratic and gradient runs.

It should be obvious that if we consider only a single peak, we should be able to find isocratic and gradient conditions that will give approximately the same retention time and peak width. To illustrate this, I've broken the 15-component sample into five subsamples in Figure 1c. In each case, I've adjusted the isocratic %B so that $k \approx 3$ is obtained for the peaks. For example, at 49% B in Figure 1c, peak 1 has a retention time, t_R, of 2.9 min and for peak 2, $t_R = 3.2$ min. Under these conditions, the column dead time, t_0, is 0.75 min. We can calculate the k value for each peak from $k = (t_R - t_0)/t_0$. So $k_1 = (2.9 - 0.75)/0.75 = 2.87$ and $k_2 = 3.26$. The average k value at 49% B is $3.07 \approx 3$. In a similar manner, we find that 61% B gives $k \approx 3$ for peaks 3–7, 72% for peaks 8–9, 80% for peaks 10–12, and 88% for peaks 13–15. In each case, the retention times of the peaks in each group are approximately the same, so the peak widths are approximately the same, as we'd expect under isocratic conditions.

Next, compare the isocratic peaks in Figure 1c with the appropriate section of the gradient in Figure 1b. Notice that in both separation modes all the peaks are approximately the same width. Also, the peak spacing between adjacent

Figure 1: Simulated chromatograms for the separation of a 15-component sample on a 150 mm × 4.6 mm column operated at 2 mL/min: (a) isocratic separation at 60% B, (b) gradient separation of 50–100% in 5 min, and (c) isocratic separation of subsets of 15-component sample such that $k \approx 3$ for each group. Peak identity and %B are noted on chromatograms.



peak pairs is nearly the same in both cases. You can mentally connect the isocratic chromatograms of Figure 1c and get a separation that is very close to that of Figure 1b.

Because we see the same peak behavior in Figures 1b and 1c, we say that these are equivalent conditions. The only catch is that we have to have several different isocratic conditions to match a single gradient. But this gives us another way to visualize what is happening during a gradient. If we could program the LC system to run a series of isocratic steps that would maintain the separations of Figure 1c, we would start at 49% B until peak 2 was eluted, then step to 61% until peak 7 comes out, and so forth. Of course steps like this won't give us exactly the same separation, because the peaks of each segment are influenced by the conditions of the prior segment, but it serves as a useful mental model. After all, a gradient can be thought of as a series of isocratic steps where the step size is infinitely small. Under such conditions we get the results of Figure 1b.

With the isocratic separations of Figure 1c, all the peaks traveled through the column with $k \approx 3$. The retention factor, k, is a useful tool for developing isocratic separations and describing isocratic retention. When equivalent gradient conditions are used, Figures 1b and 1c suggest that the peak characteristics are approximately the same, so the retention factors also should be similar. To avoid confusion between isocratic and gradient retention factors, the gradient retention factor is abbreviated as k^*, which can be thought of as the average k value throughout the separation.

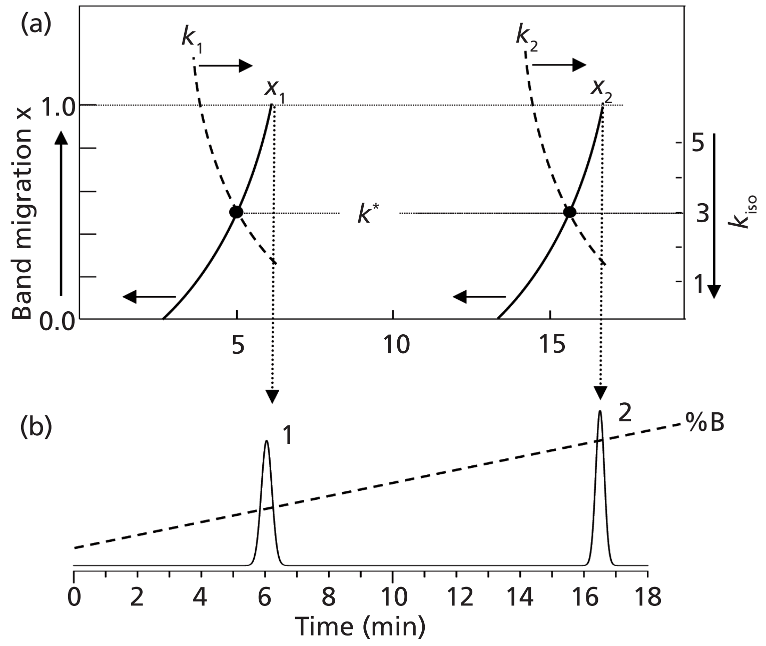
Band Migration in Gradient Elution

Another way to think about how gradient elution works is shown in Figure 2. In Figure 2b, we see a chromatogram overlaid with the gradient program (dashed line). For discussion purposes, let's assume that the gradient is a linear gradient of 5–100% B over 20 min. In Figure 2a, the left-hand *y*-axis tracks the band migration as it moves through the column (in the direction of the arrow on the axis); the band position is shown by the solid curved lines. The isocratic k value at any instant during the run is plotted on the right-hand *y*-axis; the instantaneous k value is shown as the curved dashed lines.

We'll consider two bands, 1 and 2, as they travel through the column. Under the initial conditions (5% B), peak 1 is very strongly retained, so it appears to sit at the head of the column (migration position of 0), and the isocratic k value is very high (dashed line off-scale). As the gradient progresses, stronger solvent runs through the column, and eventually the solvent is strong enough to move the band off the head of the column, and it begins to migrate through the column. Meanwhile, the k value drops (downward arrow on the right-hand *y*-axis). With each increase of %B, the solvent is stronger, which means that the band travels faster, accelerating through the column. Meanwhile, the stronger solvent results in a smaller instantaneous isocratic k value, so k continues to drop. This acceleration of the sample band and drop in k continue until the band reaches the end of the column (migration = 1.0), and a peak appears in the chromatogram.

In a similar manner, band 2 moves through the column. But because 2 is less polar than 1, it stays at the head of the column longer before a strong enough solvent arrives to begin carrying it through the column. However, its migration pattern through the column (the curvature of the migration plot) is very

Figure 2: Peak migration of peaks 1 and 2 during gradient elution. (a) Plot of peak position in the column, x_1 and x_2 shown by solid curves and left-hand axis; plot of instantaneous isocratic k value, k_{iso}, at any time during the separation shown by dashed curves and right-hand axis; solid dots representing k_{iso} at the midpoint of the column, $x = 0.5$, equivalent to k^*; (b) gradient program, dashed line; chromatogram for peaks 1 and 2 when they reach the end of the column, $x = 1.0$. Adapted from Figure 9.3 of reference 1.



similar to band 1, but offset to higher %B values. The value of k_2 also drops during the run in the same manner as k_1.

If we ignore the time the sample bands sit on the head of the column doing nothing, and only consider the time that they actually migrate through the column, you can see that this migration time is about the same for both bands. In isocratic separation, two bands that take the same amount of time to go through the column will have the same retention times, and thus the same peak widths. This is another way of understanding why all peaks in a gradient run are approximately the same width—they take the same amount of time to migrate through the column.

The gradient k value, k^*, can be thought of as the equivalent of the isocratic k value when a band has migrated halfway through the column. For band 1, the band reaches the midpoint of the column ($x_1 = 0.5$, black dot in Figure 2a) at 5 min. Tracing this k value to the right shows that $k_{iso} = 3$ for peak 1; thus $k^* = 3$. Similarly, band 2 reaches the midpoint of the column at ~15 min, which also corresponds to $k_{iso} = 3 = k^*$. Both bands reach the midpoint of the column with the same k value, so k^* is the same for both peaks. And as we noted above, if the effective migration time of each peak is about the same, we would expect the same isocratic k, so it isn't surprising that k^* is approximately the same for all peaks in a gradient run.

Figure 2 also helps us understand one oversimplified description of gradient elution. This states that sample compounds sit at the top of the column until a strong enough solvent comes along to wash them off, then they travel quickly through the column. This is a bit simplified, and doesn't give the column much credit for the separation, but it gives us a basic understanding of how bands behave under gradient conditions.

Summary

Let's review what we've covered here. We saw that if the range of isocratic k values is too large, a single isocratic separation will be very difficult without sacrificing peak height and run time. In such cases, where the ratio of k values for the first and last peaks exceeds ~20, a gradient usually will give better results. Figure 1c was used to demonstrate that if the number of peaks in a sample is limited, we usually can get ideal isocratic chromatography ($2 < k < 10$) by choosing the right %B. And by breaking up a complex sample into small sections, we can obtain similar k values for all peaks in the sample, albeit under different conditions.

When the series of isocratic separations with $k \approx 3$ for all peaks (Figure 1c) was compared with the gradient separation of the same sample (Figure 1b), the results were surprisingly similar. Further dividing the chromatogram into an infinite number of isocratic steps is equivalent to running a linear gradient, in this case obtaining $k^* \approx 3$ for all peaks.

Figure 2 helped to illustrate that k^* is equivalent to the isocratic k value at the point a band has moved halfway through the column. It also showed that all peaks tend to migrate through the column in a very similar manner, with the primary difference being how long they sit at the head of the column before significant band migration occurs.

Although not discussed here, Figure 2 also can be used to determine the influence on retention of changing the initial or final %B in a gradient, as well as the gradient steepness (or gradient time) and the shape of the gradient.

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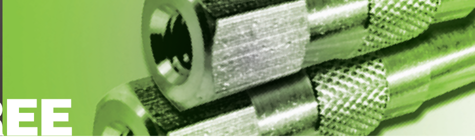


CHAPTER THREE

Surprises

Sometimes when changes are made to gradient conditions, the result isn't what was expected.

Hopefully, if we can gain a better understanding about how gradients work, we'll be able to better address gradient liquid chromatography (LC) problems when they occur, or better yet, avoid them in the first place. In the first chapter, we looked at how intuitive reversed-phase isocratic separations are, and learned some ways to transfer this intuition about the separation to gradient conditions. In the second chapter, we were introduced to the concept of the gradient retention factor, k^*, and saw how it was analogous to the isocratic retention factor, k. With sufficient care, we were able to transfer our knowledge of isocratic behavior to gradient conditions. In this chapter, we'll continue the discussion, but we'll focus on some of the surprises that can occur when the same changes are made to isocratic and gradient methods. We'll be looking at what are sometimes referred to as the *column conditions*—that is, changes in column size, packing particle size, and flow rate. And to remind you, there is much more detail on this and other related gradient topics in reference 1.

Changes in Isocratic Conditions

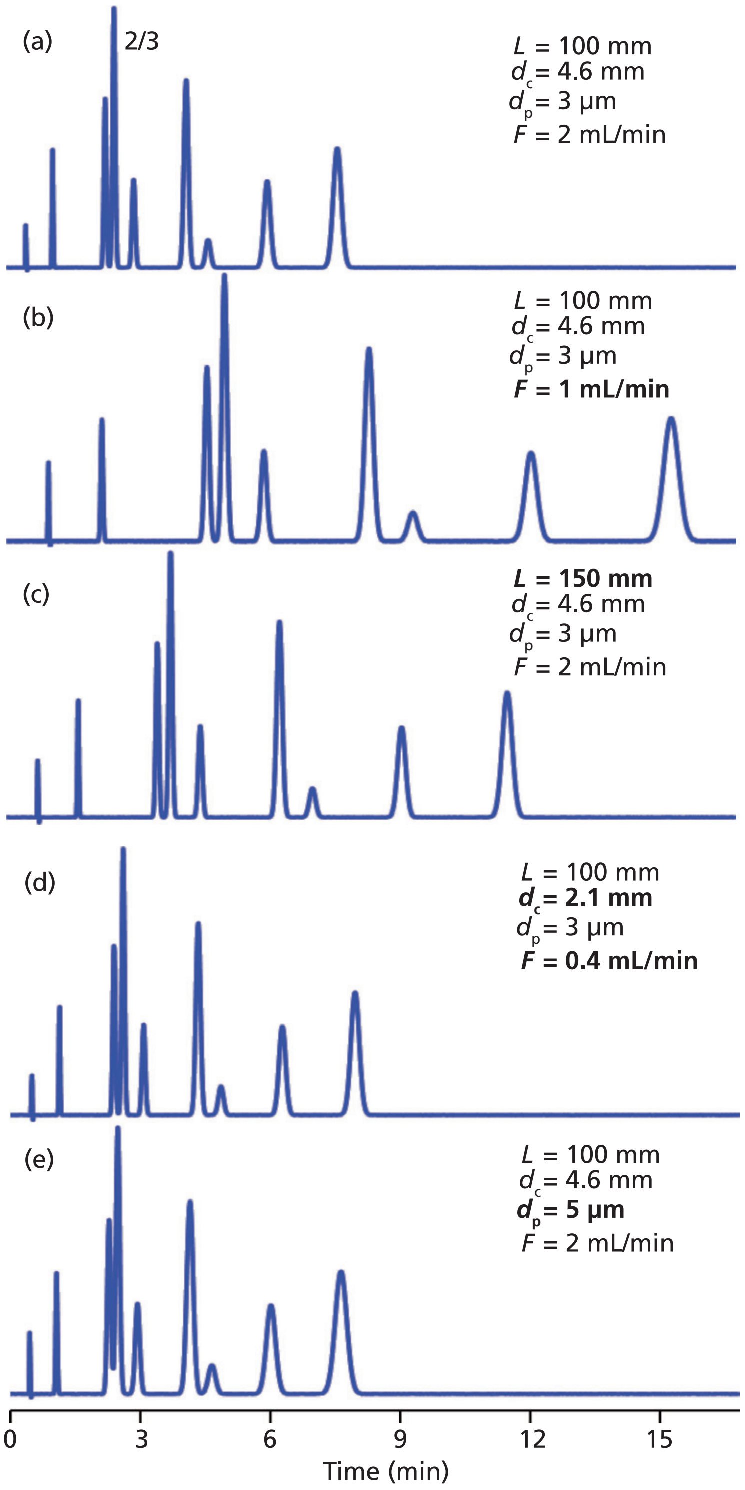
Let's follow our pattern of looking at an isocratic example first because, usually, the chromatogram changes in a predictable manner when we make a change in the column conditions. Figure 1 contains five simulated chromatograms that illustrate what happens when we make specific changes. In Figure 1a, the inset shows the reference conditions that are used. In all the chromatograms, the first small peak is the disturbance at the column dead time, t_0; the remaining eight peaks are eluted in the same order. I marked some of the peaks to make it easier to follow the discussion. In each of the other chromatograms, the inset shows one or more variables in bold type that have been changed relative to the reference case. For example, in Figure 1b, the flow rate has been changed from 2 mL/min in the reference case to 1 mL/min. Let's next look at each of the changes illustrated in Figure 1.

A reduction in the flow rate is expected to double the retention time, and this is seen in Figure 1b; the column back pressure also should drop by a factor of two (not shown). There is a minor improvement in the resolution of peaks 2 and 3, as seen by a slightly deeper valley between the peaks. This is because the column plate number, N, increases slightly at lower flow rates, but this is rarely of much advantage with modern 3- and 5-µm particle columns when used with real samples. A change in column length from 100 mm to 150 mm in Figure 1c also has the expected result. When all other factors are held constant, we expect the retention time and the pressure to change by the ratio of the length change, or 150/100, and this is what we see. We also see a small improvement in resolution of peaks 2 and 3. Remember that N is proportional to the column length and resolution is proportional to the square root of the plate number. So resolution would be expected to improve by $(150/100)^{0.5}$ = 22%, a benefit that we achieve at the cost of a longer run and higher pressure.

Sometimes it is beneficial to reduce the column inner diameter, d_c, to save solvent, sharpen peaks, or improve compatibility with an evaporative detector such as a mass spectrometer, an evaporative light-scattering detector, or a charged aerosol detector. When the diameter is reduced, there may be an excessive increase in the back pressure if no other changes are made, so it is customary to reduce the flow rate so that the linear velocity of the mobile phase stays the same and retention times are unchanged. So when the column diameter is reduced, the flow rate should be reduced in proportion to the change in the cross-sectional area. For the reduction in column diameter from 4.6 mm to 2.1 mm shown in Figure 1d, the column cross-sectional area changes by $(4.6/2.1)^2$, or approximately fivefold. So we reduce the flow rate from 2 mL/min to 0.4 mL/min and expect to see the same retention times and separation as in the reference case. This can be seen by comparing the chromatograms of Figures 1a and 1d.

The final change in Figure 1 is to increase the packing particle size, d_p, from 3 µm to 5 µm. This is expected to reduce N in proportion to the change, 5/3. It also should reduce resolution by the square root of this ratio, $(5/3)^{0.5} \approx 1.3$; this can be seen by the noticeable loss in resolution between peaks 2 and 3 in Figure 1e. The back pressure should change with the square of the particle size change, so we would expect the pressure to drop by $(5/3)^2$, or to approximately 35% of its original value (not shown). The particle size should have no influence on retention or peak spacing if the same particle chemistry is used (same brand of packing material).

Figure 1: Simulated chromatograms for the isocratic separation of phthalic acid, 2-nitrobenzoic acid, 4-chloroaniline, 2-fluorobenzoic acid, 3-nitrobenzoic acid, 3-fluorobenzoic acid, 2,6-dimethylbenzoic acid, and 2-chloroaniline (in order of retention times): (a) reference conditions, (b) change in flow rate, (c) change in column length, (d) change in column inner diameter and flow rate, (e) change in packing particle size. The changes in each case are shown in bold in the summary of conditions. Adapted from reference 2.



Corresponding Gradient Changes

There wasn't anything surprising about the results when we changed column conditions for an isocratic separation. Our intuition and experience helped us know what to expect. Let's see if the same expectations can be achieved when similar changes are made to a gradient separation. In Figure 2, I've chosen gradient conditions for the same sample that give approximately the same separation, as can be seen by comparing Figure 1a with Figure 2a. The peak spacing and critical resolution between peaks 2 and 3 is quite similar.

The reference chromatogram for the gradient separation (Figure 2a) uses a linear gradient of 20–45% B in 5 min; as with Figure 1, changes made to the conditions for the remaining chromatograms of Figure 1 are shown in bold in the inset summaries. First, let's change the flow rate from 2 mL/min to 1 mL/min in Figure 2b. We expect the retention times to double and a minor increase in resolution as we saw in Figure 1b. But this isn't what we observe. The retention times increase, but by less than a factor of two. The resolution between peaks 2 and 3 increases much more than we expected, but peak 3 now runs into peak 4. Things are not going according to our expectations.

In Figure 2c, we see the results of increasing the column length from 100 mm to 150 mm, which should increase the plate number, resolution, and run time. Here again, we see that the expected increase in retention by 50% hasn't occurred; we observe a smaller increase. And although the separation of peaks 2 and 3 increased, as we expected; we did not expect that the resolution of peaks 3 and 4 would be compromised.

Figure 2: Simulated chromatograms for the gradient separation of the same sample as in Figure 1. (a)–(d) Same changes as in Figure 1, (e) change in flow rate and gradient time.

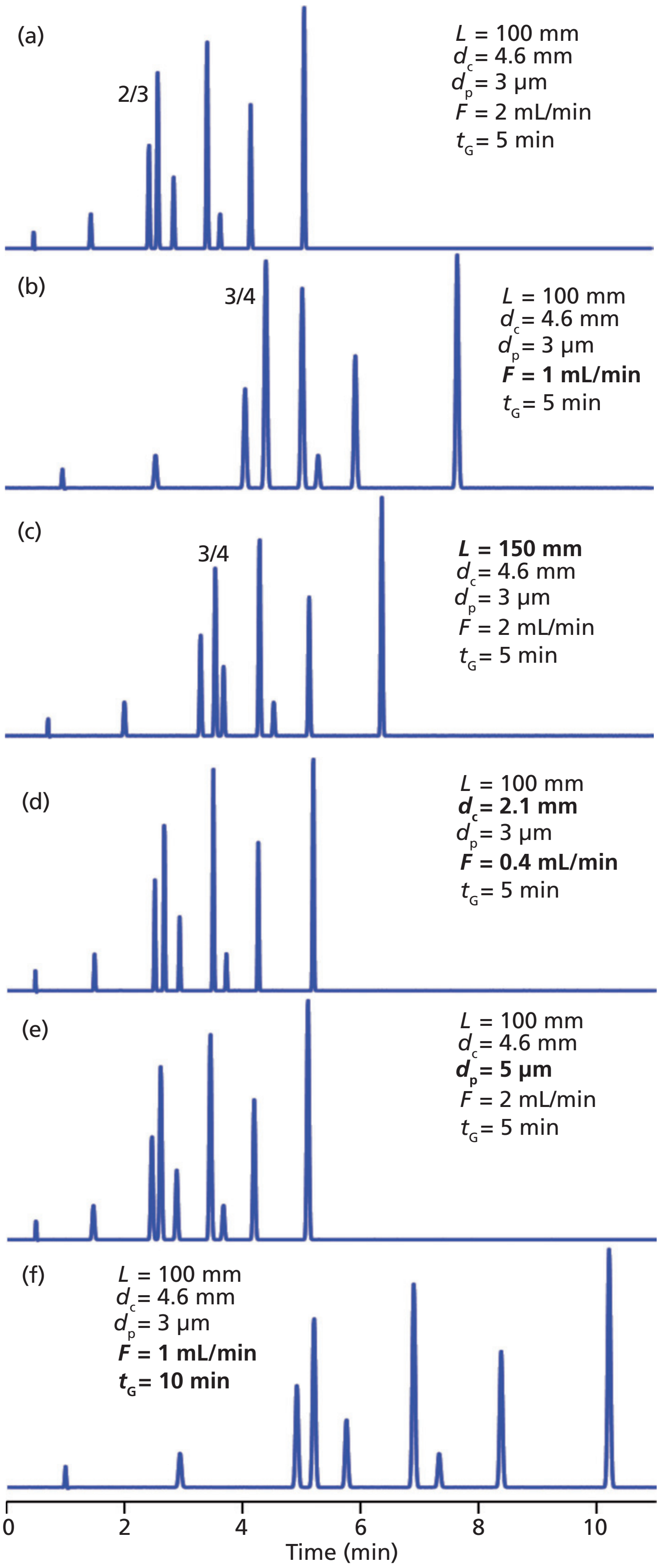


Figure 2d shows the results for the reduction in column diameter with a simultaneous change in flow rate to keep the linear velocity constant. This should have no effect on the separation, and this is what we observe. The minor increase in retention can be attributed to the fact that we reduced the flow rate by a factor of 5.0, when the true ratio should be 4.8. In a similar manner, our expectations are met when we increase the particle size from 3 μm to 5 μm in Figure 2e. You can see the slight loss in resolution by examining the separation of peaks 2 and 3. At least some changes behave as expected with gradients.

What's Going On?

As is probably apparent by now, there are some fundamental differences between isocratic and gradient separations that account for the differences in behavior between the two techniques when the column conditions are changed. In particular, we're interested in changes in peak spacing, expressed as the selectivity, α:

$$\alpha = k_2/k_1 \qquad [1]$$

where k_1 and k_2 are the k-values for two adjacent peaks. Whenever a variable is changed in an LC separation that changes k for one or both peaks of a peak pair, a change in α will occur. The exception is when k_1 and k_2 change in proportion, but this is the exception rather than the rule with most samples under reversed-phase conditions.

Recall that the isocratic k value is calculated as

$$k = (t_R - t_0)/t_0 \qquad [2]$$

where t_R is the retention time and t_0 is the column dead time. A change in column dimensions or flow rate will change both t_R and t_0 by the same proportion, so k will stay the same, and no change in α will occur. A longer column, lower flow rate, and smaller particles each will increase the column plate number, so resolution will improve because of this, not because the peaks move relative to each other.

The gradient retention factor, k^*, is calculated in a different manner than isocratic k:

$$k^* = (t_G F)/(1.15\ \Delta\%B \bullet V_m S) \quad [3]$$

where t_G is the gradient time (in minutes), F is the flow rate (in milliliters per minute), $\Delta\%B$ is the gradient range (5–95% B = 0.9), V_m is the column volume (in milliliters), and S is a constant for a given compound. Recall from the discussion in chapter two that k^* is approximately the same for all peaks in a gradient separation. However, because S varies from one compound to another, small differences in k^* will be seen for the various peaks in a separation; this is useful because it allows us to make changes in some chromatographic variables so that we can optimize a gradient separation. For the current discussion, we're only concerned with changes in the column conditions—length, diameter, flow rate, and particle size—so we can simplify equation 3 to

$$k^* = C \bullet (t_G F)/V_m \quad [4]$$

where C is a constant.

Now let's consider each of the changes to the column conditions we made in Figures 1 and 2 in light of equation 4 to see if we can make sense of the unexpected changes in Figure 2. When the flow rate was reduced from 2 mL/min to 1 mL/min in Figure 2b, we see from equation 4 that the k^* value also dropped by a factor of two. This, in turn, results in a change in α (equation 1) for at least some of the peak pairs. This accounts for the increase in the separation between peaks 2 and 3 and reduction of the resolution between peaks 3 and 4; it also looks like the separation between the last two peaks has increased. The reduced flow rate increased the run time, but because k^* is smaller, the run time did not double like it did in Figure 1b for the isocratic case.

An increase in the column length from 100 mm to 150 mm is shown in Figure 2c. Again consulting equation 4, we see that because V_m is directly proportional to column length, it will reduce k^* by 100/150. This change in k^* has a similar change in α as does the reduction in flow rate, but because the change is by less than a factor of two, peaks 3 and 4 are not so closely merged in Figure 2c as they are in Figure 2b. The increase in column length does give the expected increase in N, but the predicted increase in retention by 150/100 is compromised by the reduction in k^*.

For the experiment of Figure 2d, we reduced the column diameter from 4.6 mm to 2.1 mm, but we also reduced the flow rate from 2 mL/min to 0.4 mL/min to keep the mobile-phase velocity the same. The chromatogram is almost identical as the original run of Figure 2a, with a slight change in retention that we explained earlier. When we consider equation 4, we can see that the ratio of F/V_m is almost identical in the two cases: $2/4.6^2 \approx 0.4/2.1^2$, so k^* stays constant, as does α and resolution.

The final change in Figure 2e was to change the particle size from 3 µm to 5 µm. Note that the particle size, d_p, does not appear in equation 3, so it should have no influence on k^*. Its only effect is to reduce the plate number and reduce pressure in the same fashion as it does in the isocratic case.

In the example of Figure 2d, we simultaneously changed the column diameter and the flow rate to keep the linear velocity constant, but this also had the effect of keeping k^* constant. This is a key concept: whenever a change is made in the column conditions, a compensating change in another part of the column conditions must be made to keep k^* constant or we will risk a change in α, which may be detrimental to the separation. This compensating change also is illustrated in Figure 2e. In this case, the same change in flow rate was made as was made in Figure 2b, from 2 mL/min to 1 mL/min, but in addition, the gradient time, t_G, was increased by twofold so that k^* would stay constant. Thus, the peak spacing didn't change. The run time doubled, and the resolution increased marginally because the lower flow rate gave a slight increase in the plate number.

Summary

When we made changes in the column conditions (length, diameter, particle size, or flow rate) under isocratic conditions, the observed chromatograms matched our expectations. However, when we made the same changes in a gradient separation, sometimes the results were quite surprising. Isocratic k values (equation 2) are not influenced by changes in the column conditions, so peak spacing, α (equation 1) won't change, either. This is not the case with gradients because k^* is influenced by the flow rate and the column volume (equation 3), so when changes in these factors are made, a change in k^* results, with a corresponding change in α. We found that if we made compensating adjustments, using equation 4 for a guide, we could change the column conditions for a gradient separation and get the expected result. The key learning point here is if changes in the column conditions are made for a gradient separation, compensating changes must be made to keep k^* constant, or the separation is likely to undergo undesirable changes.

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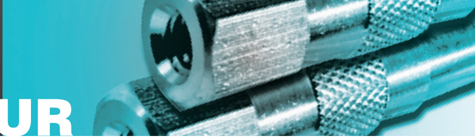


CHAPTER FOUR

Dwell-Volume Problems

Gradient methods can offer unique solutions to separation problems, but transferring a gradient method from the literature, between laboratories, or even within the same laboratory can be a challenging process.

Before we started this e-book, we considered some techniques for using a gradient scouting run to speed the initial investigations in method development or to quickly obtain a separation under generic conditions (1). This e-book started with a discussion about how we could transfer our intuitive understanding of isocratic separations to gradients (chapter one), and followed this with a way to compare isocratic and gradient methods under "equivalent" conditions (chapter two). In chapter three, we considered some unexpected results, or surprises, that might occur if we inadvertently make changes in one gradient variable without making compensating changes in another. In this chapter, we'll begin looking at some very practical problems related to gradient operation with a discussion of how the gradient dwell volume can impact the results.

What Is Dwell Volume?

There are two general designs of liquid chromatography (LC) gradient systems, as illustrated in Figure 1. High-pressure-mixing systems (Figure 1a) generally comprise two pumps, with mobile-phase blending taking place after the pumps (in the high-pressure region). Such systems usually are limited to two solvents, although switching valves may be included that allow you to switch from one solvent to another. The other design uses low-pressure mixing (Figure 1b), in which two to four solvents are blended before they reach a single pump (mixing on the low-pressure side of the pump). The design of both system types results in a measurable volume between the point the solvents are mixed and the inlet to the column—this is the *dwell volume*, sometimes called the *gradient delay volume*. From a practical standpoint, the dwell volume is made up of two parts. The first is the physical volume of the various components, including the mixer, any connecting tubing, and usually the injection loop volume. The second is the wash-out volume, which adds to the physical volume the hydraulic characteristics of the various components, especially the mixer, to increase the effective dwell volume of the system. We'll just refer to the combined dwell volume here, although we'll touch on the wash-out volume briefly later.

The Consequences of Dwell-Volume Differences

One of the biggest complaints about gradient methods is that they are hard to transfer, whether it is trying to reproduce a published method, transferring a method between laboratories, or even moving a method from one instrument to another in the same laboratory. Often the problem can be traced to differences in dwell volume between the various LC systems. This is illustrated in the simulated chromatograms of Figure 2. In each case, the same reversed-phase gradient method is run. This comprises a 10–40% B gradient (where the B-solvent is the organic solvent) in 15 min, using a 100 mm × 4.6 mm column run at 1 mL/min. We'll consider the run of Figure 2a as the reference case, where the LC system has a dwell volume of 1 mL. The peaks are all separated to baseline with a separation time of approximately 10 min. When the method is transferred to another LC system with a dwell volume of 3 mL, the chromatogram of Figure 2b results. You can see that all the peaks are shifted to longer retention times and the separation of the first two peaks suffers. This occurs even though the same gradient conditions are set in the controller.

The practical difference between the two methods is highlighted in the gradient overlay above each chromatogram. Although the program is set for 10–40% B in 15 min, the actual gradient that is delivered is not the same in both cases. In the first case (Figure 2a), the 1-mL dwell volume results in a 1-min delay from the time the gradient program starts until the gradient reaches the head of the column. In other words, the injection takes place 1 min before the gradient arrives at the column. This means that the sample experiences an isocratic hold for 1 min under the starting conditions. In an analogous manner, the 3-mL dwell volume of the second system (Figure 2b) adds a 3-min isocratic hold at the beginning of the gradient.

The result of the differences in dwell volume is a shifting of retention times in the chromatogram. Recall our oversimplified description of gradient elution from the discussion in chapter two: A compound sits at the top of the column until a strong enough solvent comes along to wash it off, then it travels through the column at the same rate as all other sample components. If this is true, we would expect the two chromatograms to be offset by the differences in dwell time, t_D. (The dwell time is just the dwell volume, V_D, di-

Figure 1: Schematics of (a) high-pressure-mixing and (b) low-pressure-mixing LC systems, highlighting differences in dwell volume.

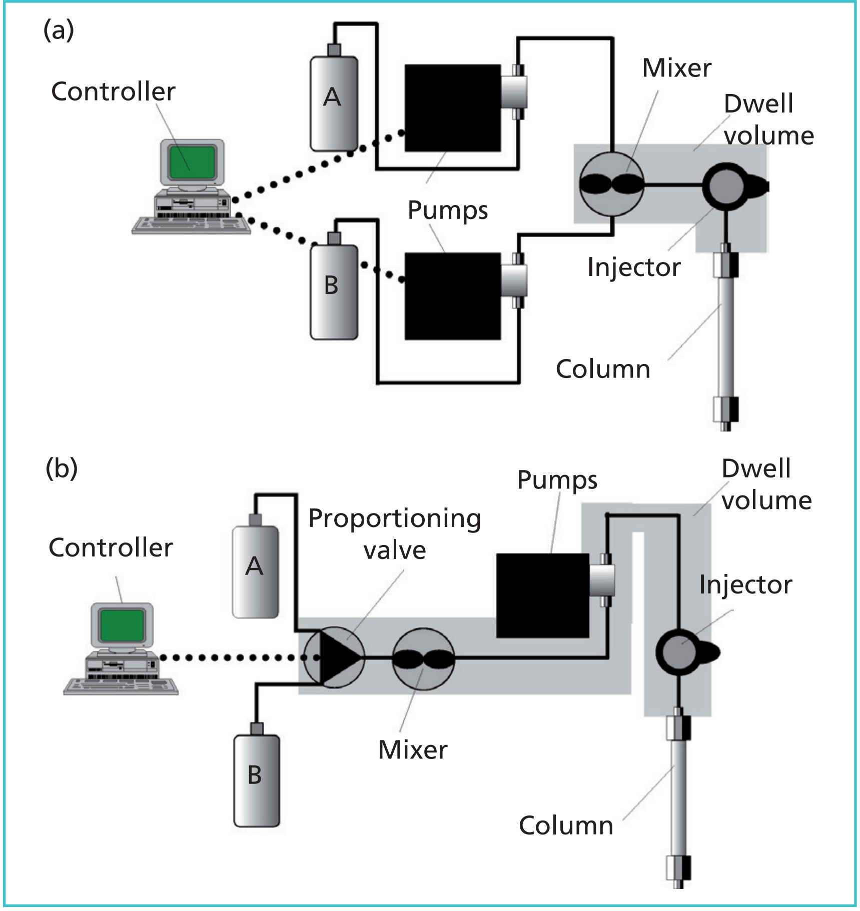
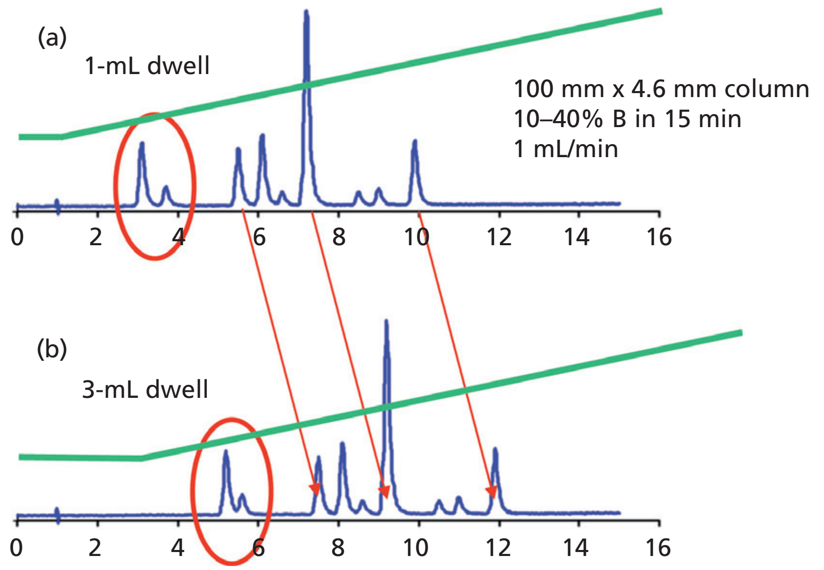


Figure 2: The practical effect of differences in dwell volume for a 10–40% B gradient run over 15 min at 1 mL/min on a 100 mm × 4.6 mm column. Simulated chromatograms for systems with (a) 1-mL and (b) 3-mL dwell volumes; an overlay of the effective gradient is shown with each chromatogram.

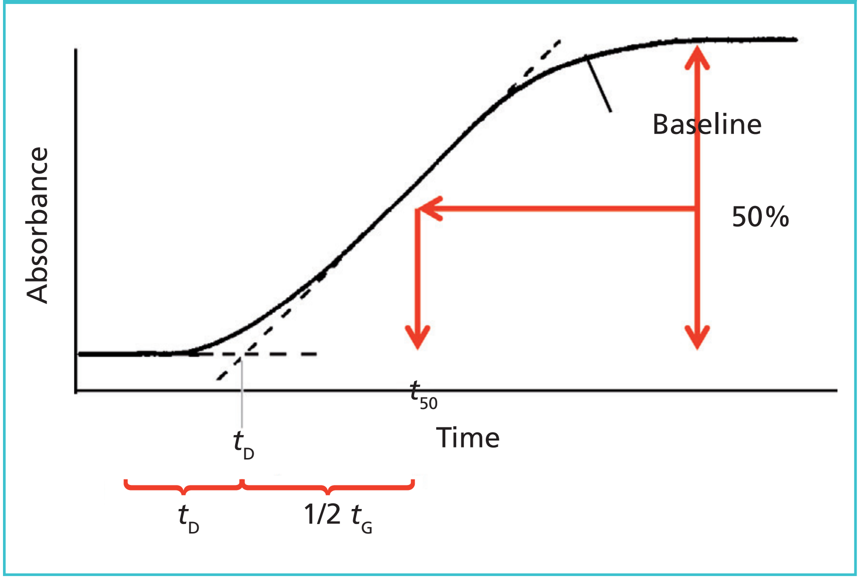


vided by the flow rate, F: $t_D = V_D/F$.) In the current case, the 1-mL dwell volume system has a dwell time of t_D = 1 mL/1 mL/min = 1 min, whereas the 3-mL system has t_D = 3 mL/1 mL/min = 3 min. The difference in dwell times is 2 min, so we would expect a 2-min offset between chromatograms if our assumptions are true. This is what is seen for the later peaks in the two runs, as highlighted by the arrows comparing retention times between Figures 2a and 2b. However, it is a bit naive to assume that the sample components are totally unaffected by the initial conditions. This is highlighted by the shift in peak spacing (selectivity) for the first two peaks, in addition to the approximately 2-min offset. So the first two peaks of Figure 2a experience a 1-min isocratic hold at 10% B and then approximately 2 min of the gradient. In contrast, the first two peaks of Figure 2b are subjected to a 3-min isocratic hold before the gradient starts. This difference accounts for the change in selectivity that we see for the first two peaks in each run. So the primary influence of dwell-volume differences is an offset in retention time, with a secondary influence of potential changes in selectivity. It is easy to see how such differences can make it hard to transfer gradient methods between systems with different dwell volumes.

How to Measure Dwell Volume

It is important to know the dwell volume of each LC system so that we can avoid problems by compensating for differences in dwell volume when gradient methods are transferred. This measurement is illustrated in Figure 3. The setup is quite simple: Remove the column and replace it with a piece of capillary tubing. A good choice is to use approximately 1 m of 0.125-mm (0.005-in.) i.d. tubing, which provides enough back pressure to ensure that the pump check valves will work properly. Replace the A-solvent with high performance liquid chromatography (HPLC)-grade water and the B-solvent with HPLC-grade water spiked with 0.1% acetone. Use a UV detector set at 265 nm. Choose

Figure 3: Illustration of how to measure system dwell volume. See text for details.



conditions that are typical of what you run in the laboratory. For example, with conventional LC systems that run 10–30 min gradients at 1–2 mL/min, a 20-min gradient at 2 mL/min is a good choice. For a system dedicated to liquid chromatography–mass spectrometry (LC–MS) or ultrahigh-pressure liquid chromatography (UHPLC), a 5-min gradient at 0.5 mL/min may be more typical. In all cases, use a gradient of 0–100% B. Most of us use the autosampler in a mode that leaves the injection loop in the flow stream during the gradient, so leave the injector in the "inject" position for this test if this is your practice. When the gradient is run, you should see a baseline trace that looks similar to that of Figure 3, rising from 0% B to 100% B over the selected gradient time.

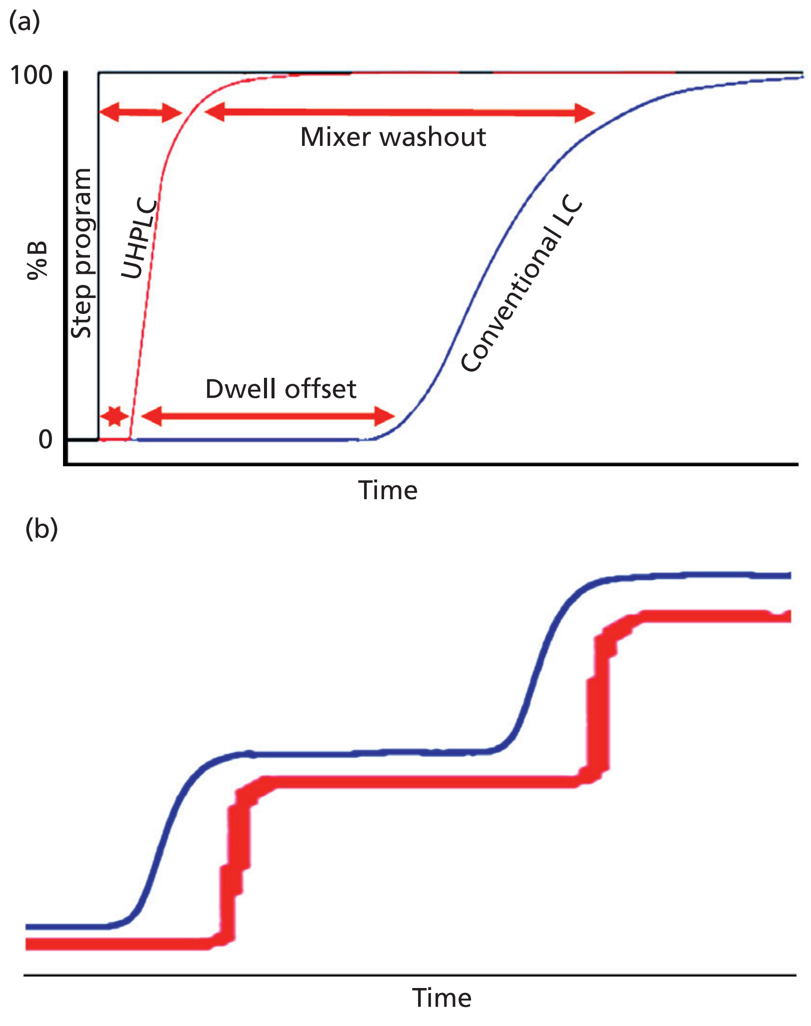
The dwell volume can be determined in one of two ways illustrated in Figure 3. You can print the chromatogram, then draw a best-fit line through the rising baseline, as shown by the dashed line. In a similar manner, extend the initial baseline until it intersects the diagonal. This intersection should correspond to the dwell time, t_D. Alternatively, perform the measurement on the computer monitor. Determine the difference in signal (offset) between the initial (0% B) and final (100% B) baselines; divide this by two to find the signal corresponding to 50% B and locate this point on the curve. Find the corresponding retention time, t_R; this should occur halfway through the gradient, so subtract half this value ($t_G/2$) and the remaining retention time will be equal to t_D. For example, if a 0–100% B gradient in run in 20 min at 2 mL/min, the midpoint should be reached at 10 min into the gradient. If the midpoint of the gradient is measured as 11.2 min, then $t_D = 11.2 - 20/2 = 1.2$ min. Convert this to the dwell volume, $V_D = 1.2$ min × 2 mL/min = 2.4 mL.

As mentioned in the introduction, the dwell volume as well as the wash-out characteristics of a given LC system can influence the resulting chromatogram. These differences are illustrated in Figure 4a, where measurements similar to those of Figure 3 are compared for a conventional LC system and an UHPLC system. In this case, rather than a sloping gradient, a vertical step from 0 to 100% B was used. You can see that the offset between the UHPLC and LC curves is different at the beginning of the step and the end of the step. Woodman and Sanford (2) argue that the beginning difference corresponds to the dwell volume and the additional offset at the end represents the mixer wash-out characteristics. Thus, the wash-in and wash-out curvature is different in this case. Which measurement represents the proper dwell volume you should use when transferring a method? The choice is somewhat arbitrary, and I think that the midpoint technique used in Figure 3 is probably as good as any, unless complicated conversion procedures are used. The illustration of Figure 4b shows that dwell differences also can occur when a single system is reconfigured. Each trace represents two consecutive gradient steps in a stair-step gradient program. The same high-pressure-mixing LC system was used in each case. The upper trace represents the performance of the conventional LC system using the factory-installed mixer, generating a dwell volume measured as 2.2 mL. We modified the system for use with LC–MS by removing the original mixer and replacing it with an after-market mixer that reduced the dwell volume to 0.5 mL, with the resulting trace at the bottom of Figure 4b (the difference in line widths is an artifact of scaling to overlay the two traces). As with the two examples of Figure 4a, we see that both traces show a more rapid wash-in than wash-out and that the smaller-dwell-volume system responds much more quickly to changes in mobile-phase composition.

Compensating for Dwell-Volume Differences

We've seen how differences in dwell volume can result in differences in the appearance of gradient chromatograms and how to measure the dwell volume. Next, let's consider how to compensate for differences in dwell volume, using the example of Figure 2. Switching from a larger-dwell to a smaller-dwell system is very simple. Just add a gradient delay (isocratic hold) corresponding to the desired additional offset. For the transfer of the method of Figure 2b to the system of Figure 2a, it would mean adding 2 min of isocratic hold to the beginning of the program. Now the program of 10/10/50% B at 0/2/17 min should give the same chromatogram as in Figure 2b with the system of Figure 2a. Of course, you could add 2 mL of volume to the system by adding a mixing coil or other modification,

Figure 4: Contributions of dwell volume and wash-out volume to effective dwell volume: (a) comparison of dwell volume measured on UHPLC and conventional LC systems, adapted from reference 2; (b) comparison of same high-pressure-mixing system with two different mixers. See text for details.



but this is much less convenient than adding a hold. (And it can be shown experimentally that there are slightly different results when adding an isocratic hold or a mixing coil, but these are minor in most cases.)

Transferring a gradient method from a low-dwell system to a larger-dwell system is not as simple. There are several options. First, if you are developing a new method and know that it will be used on a larger-dwell system, use the same technique mentioned above by adding an isocratic hold to the gradient program that corresponds to the additional dwell volume likely to be encountered. Then when the method is set up on a new system, the isocratic hold time (volume) can be adjusted so that the combination of the isocratic hold and the dwell volume remain unchanged between the two systems. A second technique to compensate for dwell-volume differences may or may not be available on your LC system. Many of the newer LC systems allow you to program the autosampler to inject after the gradient starts. For the present example of Figure 2b, you would program the system to inject 2 min after the gradient program is started. This would mean that the injection would take place at the same point in the gradient as it did in Figure 2a. A third technique to overcome dwell-volume differences is to overengineer the initial separation so that a dwell-volume change is unlikely to cause problems. In the present case, the method of Figure 2a might be developed so that the first two peaks had enough excess resolution that some loss of resolution when the method is transferred will not make any difference in the analytical results. Remember that the early peaks in the chromatogram are the ones that will be most affected by dwell differences; later peaks usually are offset in time, but resolution changes are less likely. Some additional, more complex techniques are described in the literature (3), such as changing the starting %B.

Summary

We have seen that differences in system dwell volume can account for much of the difficulty encountered when transferring a gradient LC method from one system to another. A technique to measure the dwell volume of an LC system was presented. This procedure should be performed at least once on every gradient LC system so that you know its dwell volume. Several options were presented to compensate for dwell volume differences when transferring gradient methods. Because the dwell volume can be so important in gradient methods and their transfer, it should be obvious that you should list the dwell volume of the LC system as part of the method description. This will give the next user knowledge to aid in adjusting the method to get the same results on a different LC system.

In chapter five, we'll look at problems related to baseline drift in gradient elution.

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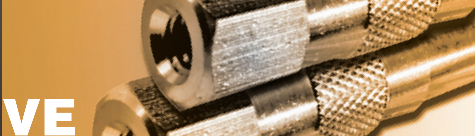


Baseline Drift Problems

Can anything be done to correct for baseline drift in gradient separations?

In chapter four, we considered problems related to the system dwell volume. In this chapter, we'll continue looking at gradient problems with a focus on baseline drift. If you're just moving from isocratic separations to gradients, one of the first observations you make when you examine a chromatogram is that the gradient baseline often is not flat. With both isocratic and gradient separations, the baseline can drift when the column temperature is not stable, but if you use a column oven and the laboratory temperature is relatively stable, drift usually is not a problem. Drifting baselines under gradient conditions are common. Usually the drift is minor, and you learn to live with it. In other cases, it may be possible to compensate for the drift by adjusting the mobile phase. In still other cases, there isn't much you can do. Let's look at each of these cases next.

Mobile-Phase Absorbance

When ultraviolet (UV) absorbance is used for detection, it is common to find that the A and B mobile phases differ in their UV absorbance at the detection wavelength. This difference means that the baseline will drift during a gradient run, as is seen in the upper trace in Figure 1. In this case, a gradient is run from 100% water (A) to 100% methanol (B) at 215 nm. Because methanol has significantly stronger UV absorbance at 215 nm than water, the baseline rises—approximately 1 absorbance unit (AU) in this case. If the display setting is set to a range of <1 AU, the baseline will drift off scale during the run. This is inconvenient, but with many detectors today, the detector range is >1 AU, so peak data will still be collected, even though they do not appear on the computer monitor until the scale is changed. However, in the days of strip-chart recorders, before computerized data collection was used, an off-scale baseline or peak meant that no data were collected under those conditions. In any event, we would like to be able to see the entire chromatogram without having to change from one display scale to the next. For this reason, drift, such as that observed for methanol in Figure 1, is unacceptable for most of us. From a practical standpoint, methanol has sufficient absorbance at low wavelengths that full-range water–methanol gradients seldom are used below approximately 220 nm.

Contrast the plot for methanol at 215 nm with that for acetonitrile at 200 nm in Figure 1. The water–acetonitrile gradient baseline looks flat at the same display scale because acetonitrile has very low UV absorbance relative to water under these conditions. This is one reason why acetonitrile often is the preferred organic solvent when low-wavelength (<220 nm) UV detection is used.

Compensating for Drift

In the water–acetonitrile gradient of Figure 1, water and acetonitrile have approximately the same UV absorbance at 200 nm, so the baseline does not drift. It may be possible to create analogous conditions with other solvents by adjusting the absorbance of a solvent mixture used as the A- and B-solvents of the mobile phase. An example of this is shown in Figure 2, where 10 mM potassium phosphate (pH 2.8) is used instead of water as the A-solvent and methanol is used as the B-solvent. Under these conditions, phosphate has nearly the same UV absorbance as methanol, so the baseline has very little drift. Note that the *y*-axis of Figure 2 is 0.1 AU full scale compared to 1 AU full scale in Figure 1, so the reduction in drift is impressive. From a practical standpoint we've solved the gradient drift by adding phosphate buffer to the A-solvent. Because phosphate is such a common buffer for reversed-phase liquid chromatography (LC), its use means that methanol can be used as the B-solvent at much lower wavelengths than when water is used as the A-solvent.

Most organic solvents have lower UV absorbance as the detection wavelength is increased, so simply increasing the wavelength also may help to flatten out the baseline. For example, the lower plot of Figure 2 is under the same conditions as the upper one, but at 254 nm the baseline is flat. So even if we don't add a UV absorbing compound to the A-solvent, simply increasing the detection wavelength may be a sufficient change to mitigate baseline drift. Of course, a reduction in sample response may also occur with an increase in detection wavelength, so making this change may not be a viable option.

Although using a buffer instead of water as the A-solvent may correct for baseline drift, it doesn't always produce the desired results. An example of this is seen in Figure 3, where the A-solvent is 25 mM ammonium acetate (pH 4) and B is 80% methanol in water. A negative baseline drift of >1 AU is seen at 215 nm for this gradient, and the baseline curves sharply downward as the gradient progresses. A negatively drifting baseline can cause additional problems besides the inability to fit the entire chromatogram on a

Figure 1: Baselines obtained from linear gradients of water–methanol at 215 nm and water–acetonitrile at 200 nm.

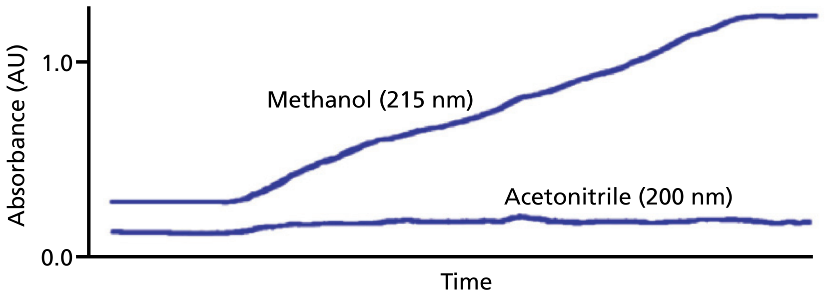


Figure 2: Baselines for phosphate–methanol gradients of 5–100% B in 15 min at 215 and 254 nm. A: 10 mM potassium phosphate (pH 2.8); B: methanol. Adapted from reference 1.

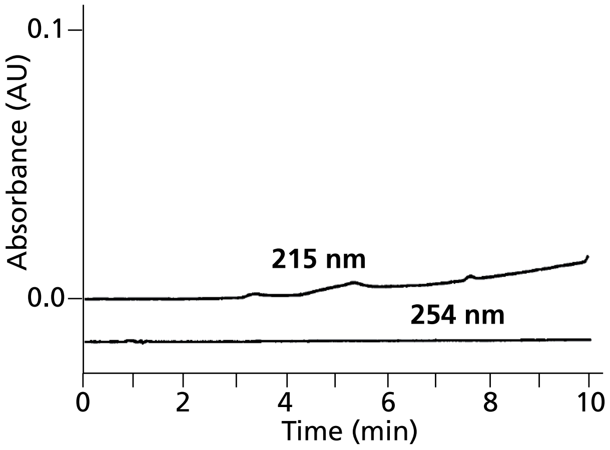
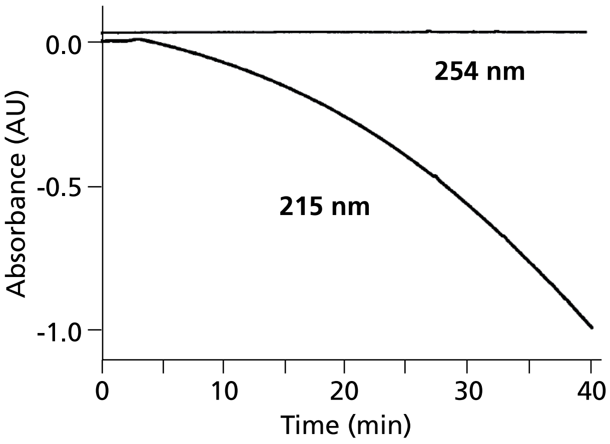


Figure 3: Baselines for ammonium acetate–methanol gradients of 5–100% B in 40 min at 215 and 254 nm. Mobile-phase A: 25 mM ammonium acetate (pH 4); B: 80% methanol in water. Adapted from reference 1.



reasonable vertical scale. Many data systems stop collecting data when the baseline drifts more than approximately 10% below the initial baseline. In the example at 215 nm, the only way to collect this baseline for display was to turn off the autozero function on the data system and manually set the baseline at 1.0 AU before the gradient was started. In this manner, the baseline signal was always >0 AU, so it could be collected by the data system. This certainly is not a technique that is amenable to unattended sample analysis. The reduced UV absorbance at higher wavelengths that was mentioned earlier holds here, as well, where the same gradient at 254 nm is flat. Another option that might help to flatten out the baseline, would be to add ammonium acetate to both the A- and B-solvents to try to cancel the negative drift as the gradient progresses. It should also be noted that although the present conditions at 215 nm are unacceptable for UV detection, if mass spectrometry (MS) was used for detection instead of UV absorbance, the baseline drift would not be a problem because UV absorbance does not affect the MS signal; ammonium acetate–methanol gradients commonly are used with LC–MS.

In still other cases, the baseline drift during a gradient may not be amenable to correction by adding something to the mobile phase. An example of this is seen in Figure 4, where 50 mM ammonium bicarbonate is used as the A-solvent and methanol as the B-solvent. At 215 nm, the baseline drifts downward as it approaches the middle of the gradient, then starts back up again. In this case, the change in absorbance is worse for a mixture of A and B than with either solvent alone, so it is unlikely that the absorbance of either mobile phase could be manipulated to compensate for the midgradient dip. As with the other examples of baseline drift with methanol as the B-solvent, an increase in the detection wavelength to 254 nm minimizes the problem.

Trifluoroacetic Acid: A Special Case

Trifluoroacetic acid is an additive commonly used in LC separations

Figure 4: Baselines for ammonium bicarbonate–methanol gradients of 5–60% B in 10 min at 215 and 254 nm. A: 50 mM ammonium bicarbonate (pH 9); B: methanol. Absorbance scale is relative, not absolute. Adapted from reference 1.

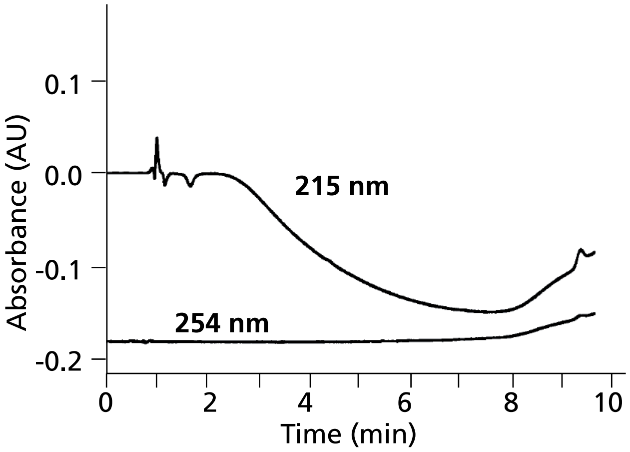
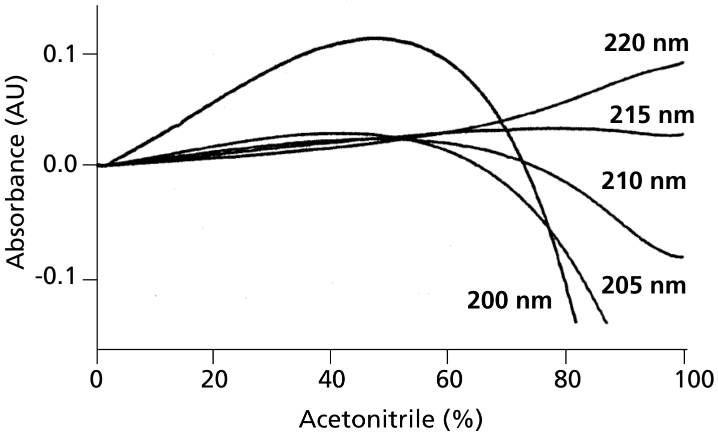


Figure 5: Baselines for trifluoroacetic acid–acetonitrile gradients of 0–100% B in 100 min. A: 0.1% trifluoroacetic acid in water; B: 0.1% trifluoroacetic acid in acetonitrile. Absorbance scale is relative, not absolute. Adapted from reference 2.



of biomolecules, such as proteins and peptides. Trifluoroacetic acid acts to acidify the mobile phase (0.1% trifluoroacetic acid gives pH ≈ 1.9) as well as acting as an ion-pairing reagent, both of which are beneficial to many biomolecule separations. In addition, trifluoroacetic acid has low UV absorbance at wavelengths <220 nm, making it especially attractive as an additive for acetonitrile-containing mobile phases. Trifluoroacetic acid is volatile, so it is easily evaporated with the aqueous acetonitrile mobile phase for compatibility with LC–MS detection, as well as other evaporative detection methods, such as evaporative light scattering detection (ELSD) or charged aerosol detection (CAD).

Figure 5 shows gradient baselines at selected wavelengths where A is water with 0.1% trifluoroacetic acid added and B is acetonitrile with 0.1% trifluoroacetic acid added. It is seen that the curvature of the baseline depends on the wavelength chosen. At 215 nm, the baseline is nearly flat, making this an especially attractive wavelength for the detection of proteins and peptides at trace concentrations. At other wavelengths, a little additional trifluoroacetic acid (for example, 0.11% instead of 0.1%) can be added to the A- or B-solvent to help reduce the baseline drift.

Conclusions

We have seen that a major component of baseline drift in gradient LC methods and UV detection often is the result of differences in detector response to the A and B components of the mobile phase. At higher wavelengths, such as >250 nm, the UV absorbance of mobile-phase components usually is minimal, so baseline drift under these conditions is seldom a concern. At wavelengths <220 nm, however, baseline drift caused by differential solvent absorbance can be sufficient to prevent practical use of certain solvents, such as methanol or tetrahydrofuran. Sometimes it is possible to compensate for differences in UV absorbance by adding a UV absorbing component to one solvent or the other. A good example of this was shown in Figure 2 for the addition of phosphate buffer at 215 nm. In other cases, the drift characteristics are such that it is not possible to compensate for drift by modifying the mobile phase. However, by judiciously choosing the mobile-phase components and detection wavelength, it usually is possible to find gradient LC conditions where baseline drift does not compromise the analysis.

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