SeparationScience

HPLC Back to Basics

A Laboratory Companion for Liquid Chromatographers #1

- Retention Factor
- Column Dead-time
- Retention Tme Diagnostics
- Selectivity
- Efficiency
- Tailing
- Resolution
- Fundamental Resolution Equation

Retention Factor

The retention factor is a measure of the distribution of the sample between the mobile phase and the stationary phase. The calculation is a simple one, as shown in Figure 1. t_R is the retention time and t_0 is the The calculation is a simple one, as shown in Figure 1. t_R is the retention time and t_0 is the column dead-time. Retention is measured from the time the sample is injected to the highest point on the peak. Measurement of the column dead-time is most easily measured as the first disturbance in the chromatogram (the "solvent front") – we'll consider t_0 in a later article. As both t_R and t_0 are in the same units (min, sec, furlongs or fortnights), the units cancel out and k is a dimensionless quantity.

The calculation of k is a simple one, but it still requires a calculator, and this activation-energy barrier is too much for many of us (where is that calculator. . .?), so we don't bother. But for many purposes, we don't need to know k to much better than half a unit, which means that an estimate is quite adequate.

The retention factor is estimated simply as follows. Note that the numerator of the equation ($t_R - t_0$) tells us to subtract t_0 from the retention time. Or simply throw away everything before t_0 and start measuring from t_0. The denominator (t_0) says to use t_0 as our unit of measure, instead of time or distance. So we just mark off the baseline in units of t_0, starting at t_0, as shown at the bottom of Figure 1. When we do this, you can see that for the three peaks, $k \approx 1$, ≈ 2 and ≈ 3, respectively.

As we'll cover in a later article, we get the "best" chromatography if $2 < k < 10$, and usually $1 < k < 20$ is acceptable for isocratic separations. If the retention range is wider than this, it is likely that a gradient will be required. And if $k < 1$, we tend to have more problems – less stable separations and a higher chance of chromatographic interferences at the beginning of the chromatogram.

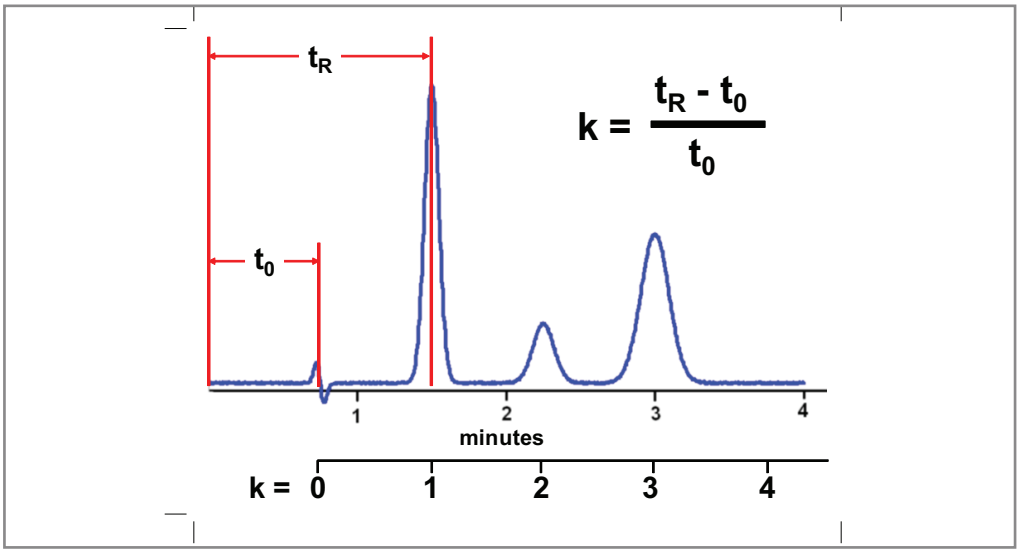


Figure 1: Retention factor can be calculated using the standard equation or estimated by using t_0 as a ruler.

Column Dead-Time

In the previous article we looked at the retention factor, *k*, and how to calculate or estimate it. In order to perform either of these processes, we need to know the column dead-time, t_0 . If we are using a UV detector and a "real" sample, usually there is an obvious disturbance in the baseline, as illustrated in Figure 1. If the sample is very clean, t_0 might appear as a little zig-zag of the baseline [Figure 1(a)], but in most cases there is a significant "solvent" or "garbage" peak at the beginning of the chromatogram, as in Figure 1(b). As we're only interested in a good estimate of t_0 at this point, just pick a consistent place to measure it. The arrows in Figure 1(a) and 1(b) show where I'd pick to estimate t_0 – just where the peak starts to rise above the baseline – this will be easy to measure consistently.

An alternate, but less convenient, way to measure the column dead-time is to inject something that you know is unretained. This is what the column manufacturer does. Uracil is the most popular t_0-marker, because it has good UV response and is unretained for mobile phases of ≥60% methanol/water that are typical of most column test conditions. Thiourea is another good marker, especially if weaker mobile phases are desired for column testing.

But what happens if you don't see a disturbance at the beginning of the run? This can happen, for example, if you are using a MS-detector. As mentioned last week, estimates are good enough for our current needs, so we can estimate t_0 in one of two ways. If you are using a 4.6 mm i.d. column, which is the most common column diameter, the estimate is very simple: the column volume, V_M, can be estimated by multiplying the column length (in cm) by 0.1. Thus, a 150 x 4.6 mm i.d. column would be 15 cm long, so $V_M \approx 0.1 \times 15 \approx 1.5$ mL. This should be within about 10% of the true column volume. To convert column volume to dead-time, just divide by the flow rate, F. So for the current example, at 2 mL/min, $t_0 \approx$ (1.5 mL) / (2 mL/min) ≈ 0.75 min.

So far, so good, but what if you use a column that is not 4.6 mm i.d.? You can use the equation at the bottom of Figure 1. The exponent means that a calculator is needed, which will probably mean you won't bother with this calculation. But wait! The second most common column after 4.6 mm i.d. is 2.1 mm i.d., and the estimate is simple. The column volume will be directly related to the change in cross-sectional area of the column, which is proportional to the square of the ratio of the column diameters. So if we consider the two most popular columns, the factor is $(4.6 / 2.1)^2 = 4.8 \approx 5$. I like 5s and 10s, because I can do the math in my head. Consider the most popular LC-MS column: 50 x 2.1 mm i.d. If this were a 4.6 mm i.d. column, it would have a volume of 50 mm = 5 cm x 0.1 ≈ 0.5 mL according to the estimate discussed earlier. We know that the 2.1 mm column is smaller diameter than the 4.6 mm one, so we divide by the conversion factor, 5. this gives us $V_M \approx 0.1$ mL = 100 µL. All in our head — pretty simple, huh?

We've looked at several ways to estimate t_0 by using the chromatogram or the column size. These are good enough for general purposes of method development and troubleshooting. In the next article we'll see how we can use t_0 to help diagnose problems.

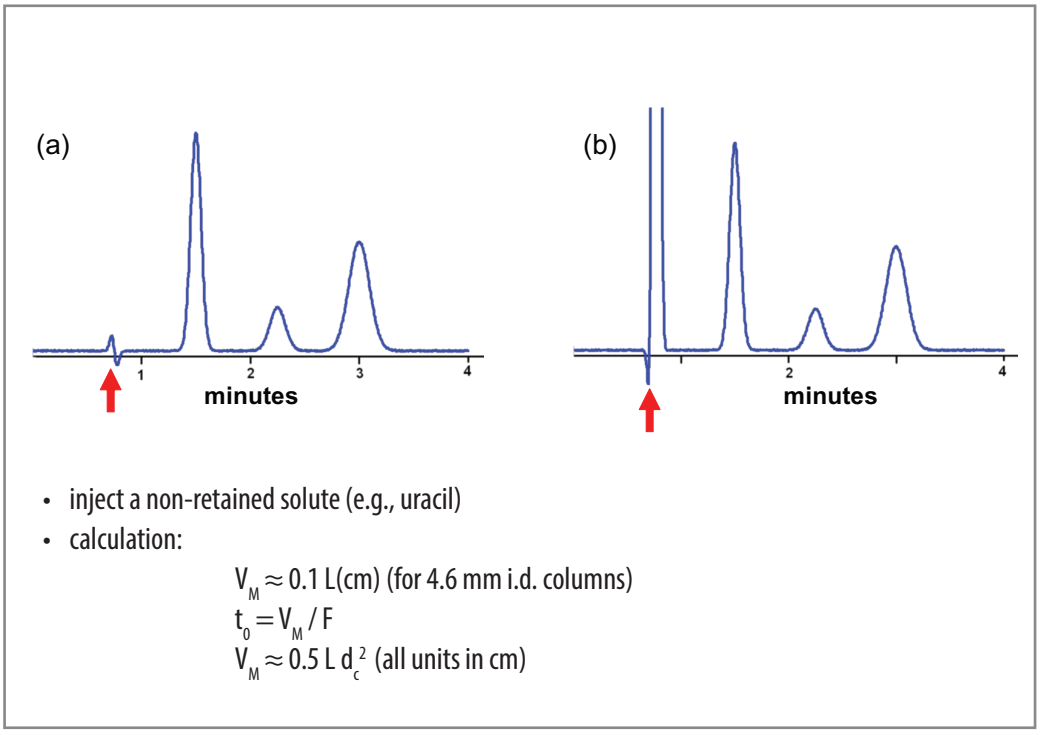


Figure 1: Various ways to estimate the column dead-time.

t_R-t_0 Diagnostics

If we look at change in the chromatogram and concentrate on what happens to the retention time, t_R, and the t_0, there are only four possible combinations: both t_0 and t_R change together, only t_R changes, only t_0 changes (highly unlikely), and neither variable changes (no change = no problem). Only the first two are of interest, so these are shown in the table in Figure 1.

First, let's consider what could be the matter if both retention and the column dead-time change together. It has to be either a flow-rate-related change or a change of the column size. Last time I checked, the column doesn't change size spontaneously – you don't put a 150 x 4.6 mm i.d. column on the HPLC system for an overnight run and come back in the morning to find that it has shrunk to 123 x 4.3 mm. So column-size changes are the result of operator error and should be obvious. We are left with changes in flow rate. Increased flow rate again usually is operator error, but it is (remotely) possible that a controller malfunction could occur. The most likely causes of a lower flow than normal are a leak, a faulty check valve, a bubble in a pump head, or a bad pump seal.

If only the retention time changes, we can see from Figure 1 that we probably have a problem with the mobile phase, the column packing, or the temperature. As these are the most likely causes of problems, you might think that this diagnostic table isn't of much help. However, each of these failure modes has its own characteristics that can help us to isolate the source of the problem.

Mobile phase problems generally appear in a step-wise fashion. For example, you make up a new batch of mobile phase and the retention times shift because you made a small error in pH adjustment or measuring the methanol. Yes, the mobile phase can deteriorate or evaporate over time, causing changes, but these are much less common. And they are noticed when a new batch of mobile phase is made, causing the step-change in retention.

Column packing changes tend to be slow in development over hundreds or thousands of samples, and one-way in nature. Retention times tend to increase or decrease gradually as the column ages. Column changes usually are accompanied with an increase in system pressure.

Temperature changes often show up as a diurnal change, particularly if the column is not operated in a column oven. As the temperature increases, retention decreases – approximately 2% / 1 °C temperature increase. I remember working in one lab without air conditioning that would heat up 5-10 °C in the summer as the sun blazed in the south-facing windows and heated up the brick facing on the outside of the building. Retention times decreased when this occurred, but they increased again at night when the lab cooled off. Even labs with better climate control may have different day and night thermostat settings, and this can cause temperature cycles that correlate with retention changes. Use a column oven and keep the HPLC system away from drafts and you'll minimize temperature-related problems.

So we've seen that a simple examination of the chromatograms for changes in retention time and the column dead-time can help us to diagnose possible problem sources with the aid of an understanding of the chromatographic behaviours summarized in Figure 1.

	t_0	t_R
flow rate	X	X
mobile phase		X
column packing		X
column size	X	X
temperature		X

Figure 1: Use of the column dead-time, t_0, and retention time, t_R, as diagnostic tools to help isolate problems.

Selectivity

Selectivity is the ability of an HPLC method to separate two analytes from each other. Selectivity usually is abbreviated with the Greek letter α, and is calculated as:

$$\alpha = k_2 / k_1$$

Where k_2 and k_2 are the retention factors, k, of the first and second peaks of a peak pair. The calculation of α is shown in Figure 1. For the two peaks with k-values of 1.95 and 2.15, α = 1.10. Although this chromatogram looks like a good separation, with a little baseline space between the two peaks, α is a poor way to determine the quality of the chromatogram. The reason for this is that it does not take the peak width into account. It is easy to imagine that if the two peaks of Figure 1 were twice as wide, the valley between the two peaks would not reach baseline. This would be a much poorer separation, yet the α-value would be unchanged. This means that we need a way to measure the width of the peaks, as will be covered in next week's discussion.

What Influences Selectivity?

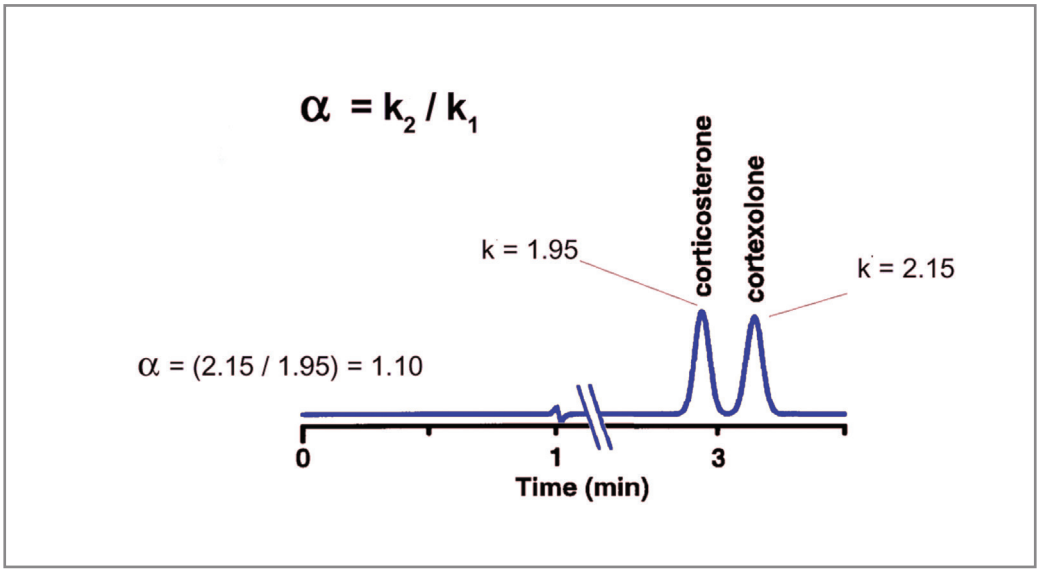
We might wonder of what use α is, if it doesn't give us a measure of chromatographic quality. In general, if α ≥ 1.1, we should be able to get baseline separation for a good quality column, but there are better ways to measure the separation, as we'll see in later discussions. For the moment, let's look at some of the things that can be used to change α – that is, how can we move peaks around relative to each other?

Selectivity is changed when we change the chemistry of the chromatographic system. Changes in the chemistry of the system influence how a sample solute interacts with the stationary phase and mobile phase. If two compounds interact with the column or mobile phase in a sufficiently different manner, we can separate them – if they interact in the same manner, a single peak results. Some of the more important variables that affect selectivity are the solvent strength and type, the temperature of the column, the buffer and other additives, and the type of column packing. Let's look briefly at the way each of these variables influences reversed-phase separation under isocratic (constant solvent-strength) conditions.

By solvent strength, usually we are referring to the ratio of the aqueous and organic components of the mobile phase. Stronger solvents are those that elute compounds more quickly so that retention is smaller. Thus, the less acetonitrile (ACN) or methanol (MeOH) in the mobile phase, the larger will be the retention times and longer the run. This will also cause peaks to broaden, and because area is constant, to be shorter. Weaker mobile phases also tend to improve the separation, but this is not true in every case.

The solvent type also affects the peak spacing. For mobile phases of equal strength, that is ones that give the same average retention times, peak spacing usually will differ with different type solvents. The three most common organic solvents used for reversed-phase HPLC are acetonitrile, methanol, and less commonly tetrahydrofuran (THF). Although changing from one of these solvents to another is almost guaranteed to change the α-value, at least for some of the peaks in a chromatogram, there is no way of knowing in advance if a specific change will improve or worsen a separation.

Column temperature works in a similar manner to mobile-phase strength in that higher column temperatures reduce retention times and lower temperatures decrease them. Selectivity often changes with a change in column temperature, but it is not possible in advance to predict whether the separation of one pair of peaks will improve or get worse. The changes in separation with a change in temperature often are different than



those when solvent type or solvent strength is changed.

The mobile-phase pH is an important variable when ionic or ionizable compounds are present. Ionized analytes tend to have smaller retention times than non-ionized ones or ones for which ionization is suppressed. Buffer strength, or molarity, does not have a major effect in most reversed-phase separations, but if too little buffer is present, peak tailing can be worse. Other additives, such as ion-pairing reagents, also can affect the separation of two peaks under the proper circumstances.

Finally, the column packing type can influence the peak spacing in a chromatogram. There are numerous stationary phases available, including C18, C8, C4, cyano, phenyl, amino, embedded polar phases, and fluoro phases. Each of these has different chemical characteristics, so a change in column type is likely to change peak spacing for at least some peaks in a chromatogram. Even a change from one brand to another within a type of packing can change chromatographic selectivity).

So What?

We have seen above that there are many different ways to change the relative peak spacing in a chromatogram. Often the challenge is how to pick which variable to choose when you desire to make such a change. In contrast, with so many ways to change selectivity, we should also be aware that this means that there are many ways to ruin a satisfactory separation by unintentionally changing a variable if we aren't careful to control the chromatographic conditions. The susceptibility of a chromatogram to changes in conditions gives us myriad topics for future discussions.

Efficiency

In this article we'll consider the last of the initial set of measurements, the column plate-number, N. This is also called the column efficiency, and is calculated as

$$N = 16\,(t_R / w_b)^2$$

where w_b is the column width at baseline between tangents drawn to the sides of the peak, as in Figure 1.

An alternate way to calculate N is to use the peak width at half the peak height, $w_{0.5}$:

$$N = 5.54\,(t_R / w_{0.5})^2$$

The half-height method is easier to determine directly from the computer monitor, because you don't have to draw tangents. It also enables determination of N if the peak is not fully separated from a neighbouring peak, as long as the valley between the peaks is lower than the half-height. Half-height measurements commonly are the method of choice for automatic determination of N by data systems.

You might wonder what the plate number represents. In a convoluted way, it is related to distillation technology, where a distillation column can have different stages, or plates, but the best spinning-band distillation columns may have only a hundred or so plates, whereas even a poor HPLC column will generate thousands of plates. So relating N to distillation is a stretch. And it is not the number of plates in the buffet line at the local restaurant – in fact, I think that number commonly is (n-1), because the person in front of me just took the last plate!

On a more serious note, the plate number can be related to the statistical broadening of a peak. Recall from your basic statistics class that tangents drawn to the sides of a Gaussian distribution intersect the baseline at ±2 standard deviations (σ). A well-behaved chromatographic peak should be Gaussian in shape, so we can conclude that the peak width at baseline is 4σ. If you substitute 4σ for w_b in the first equation, you'll see that N is merely the square of the retention time divided by

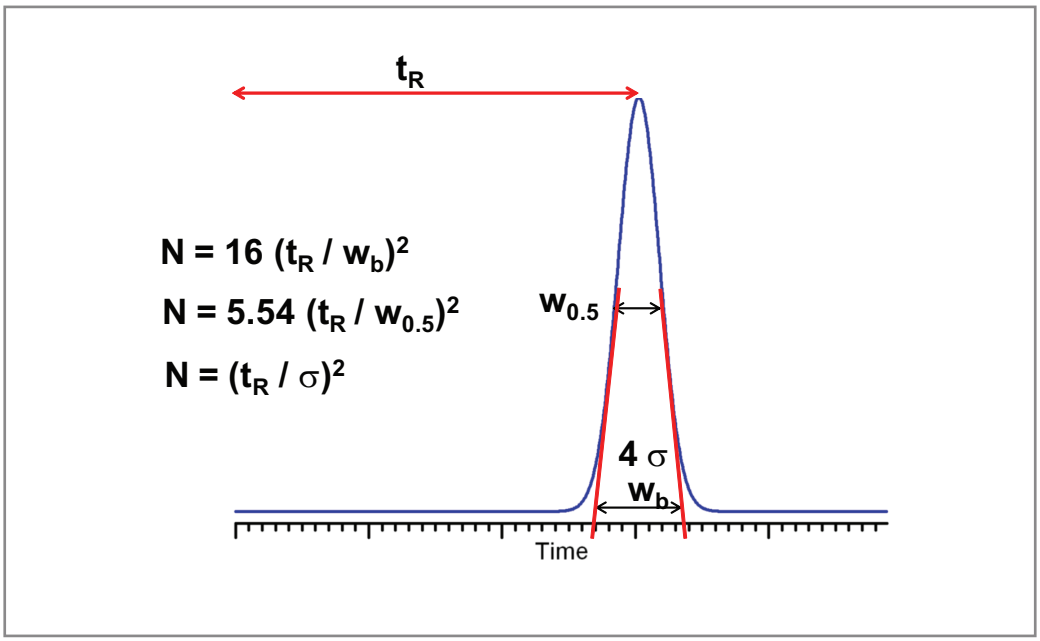
$$N = (t_R / \sigma)^2$$

Now with that and a couple of dollars, you can buy a cup of coffee at the local coffee shop – aren't you glad you asked?

So What?

What is so great about N? Why not just use the peak width to describe a peak? The beauty of the column plate-number from a practical standpoint is that it is approximately the same for all peaks in a chromatogram. And N is possible to calculate from first principles, so we can get a good idea of what N should be for a given column, mobile phase, temperature, and solute without doing the actual experiment. And while this is not accurate enough to make exact comparisons, it allows us to evaluate whether or not the plate number obtained from a given column is reasonable for the conditions. Plate numbers that are lower than expected, say by 30% or more, may be the result of column aging, unwanted chemical effects, poor system plumbing, or other problems. In future 'HPLC Solutions' discussions, we'll look at how N can be combined with k and α to evaluate resolution during method development.

I'll end with a cautionary note – calculation of N, as in equations 1 and 2 is only possible for isocratic separations. This technique is not appropriate for gradients – it is best just to use peak width to describe gradient peaks.



Tailing

So far in this Back to Basics series, all the peaks we've looked at are symmetric. The ideal peak in an HPLC chromatogram is Gaussian in shape, with an equal amount of distortion on the front and back edge of the peak. However, in the real world, this is rarely the case – most peaks tail. Because excessive peak tailing is an indication that something is wrong, it is a good idea to include a measure of peak tailing as part of the system suitability measurements.

Peak tailing is most commonly measured in one of the two ways shown in Figure 1. In the pharmaceutical industry, the tailing factor, TF, is used. This may also be referred to as the *USP* tailing factor or the *EP* tailing factor, for the *United States Pharmacopoeia* or *European Pharmacopoeia*, two of the regulatory bodies generating guidelines for pharmaceutical HPLC methods. The tailing factor is determined by drawing a perpendicular line from the peak centre to the baseline of the peak. Then the peak width and the front half-width are measured for the peak at 5% of the height of the peak. The tailing factor is simply the entire peak width divided by twice the front half-width (Figure 1, left side). For a perfectly Gaussian peak, the front half-width will be exactly half the entire peak width, so the tailing factor will be 1.0.

The non-pharmaceutical world tends to use the asymmetry factor instead of the tailing factor to measure peak tailing. The asymmetry factor is based on the front and back half-widths of the peak, but these are measured at 10% of the peak height. The asymmetry factor is determined by dividing the back half-width by the front half-width (Figure 1, right side). As with the tailing factor, a Gaussian peak will have equal half-widths, so the value of the asymmetry factor for a Gaussian peak will be 1.0.

One might wonder why there are two different ways to measure peak tailing, and I really don't have a clue. In fact, there are other less popular methods, too, such as the tau-sigma technique. You can see from the data of Table 1 that there isn't much difference between the values of A_s and TF at values less than approximately 2, but the numbers diverge as they get larger. Which one is better to use? I don't think it makes much difference, so it is best to employ the technique used most commonly in your industry or dictated by your company's policies.

The important practice is to calculate the peak tailing consistently, and on a regular basis, such as in your system suitability test. As we'll see next week, peak tailing (A_s or TF) of less than ≈2 usually is acceptable. An increase in tailing can be an indication of column failure, poor mobile phase preparation, or some other chemical change in the system.

A_s (at 10%)	TF (at 5%)
1.0	1.0
1.3	1.2
1.6	1.4
1.9	1.6
2.2	1.8
2.5	2.0

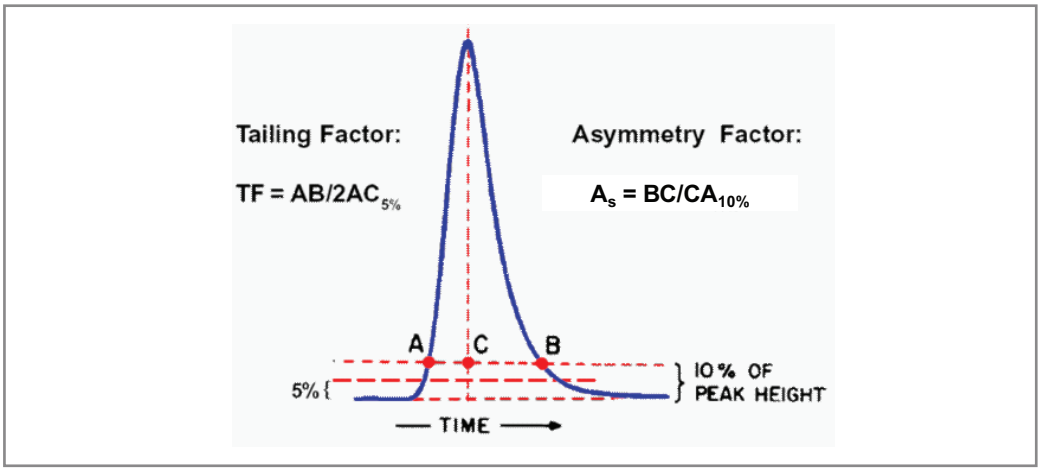


Figure 1. Calculation of peak tailing. Left, *USP* or *EP* tailing factor, TF; right, asymmetry factor, A_s.

Resolution

In previous articles of this Back-to-Basics series, we have looked at the retention factor, k, the selectivity, α, and the column plate number, N. Nice as these measurements are, they don't tell us much about the quality of the separation. For example, you can have k in the ideal $2 < k < 10$ region, or $\alpha = 1.2$, or $N = 15{,}000$, but none of these values on their own make the separation any good. As a measure of separation quality, we need to determine the resolution, R_s, which is most commonly calculated as:

$$R_s = (t_{R2} - t_{R1}) / ((0.5 * (w_1 + w_2))$$

We need to measure the retention times for the two peaks of interest (t_{R1} and t_{R2}) and the widths of the two peaks at baseline (w_1 and w_2) between tangents drawn to the sides of the peaks. For the steroid separation of Figure 1, we can calculate

$$R_s = (3.15 - 2.95) / (0.12) = 1.67 \approx 1.7$$

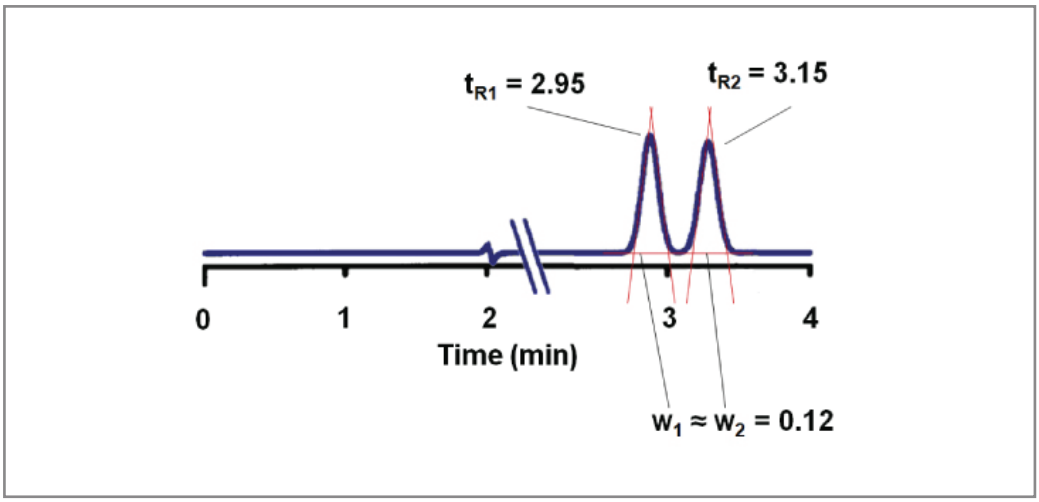
If the peaks are perfectly symmetric, which is unlikely, the valley between the peaks should just touch the baseline when $R_s = 1.5$. So with well-shaped peaks, as in Figure 1, $R_s = 1.7$ looks pretty good, with a little baseline between the peaks. However, nearly every peak shows some degree of tailing, so to allow for a small amount of tailing and still retain a bit of flat baseline between the peaks, $R_s \geq 2.0$ generally is desired.

The use of equation 1 is convenient and gives good results, but it is useful only if the peaks are baseline-resolved. Often, however, we are most concerned about the resolution when peaks are marginally separated. This means that the peaks may overlap a bit at the bottom, and measurement of the peak width at baseline is not possible. Just as we saw that it is possible to measure N at half the peak height, we can take the same approach for calculation of resolution:

$$R_s = (t_{R2} - t_{R1}) / ((1.7 * 0.5 (w_{0.5,1} + w_{0.5,2}))$$

where $w_{0.5,1}$ and $w_{0.5,2}$ are the peak widths measured at half the peak height. Note that the factor of 1.7 is added to the denominator to adjust for the difference in width at the half-height. The half-height technique is the way many data systems measure resolution, because it is simpler to measure than the baseline width.

So what represents a good value of resolution? If the peaks have only minor tailing, a value of $R_s = 2.0$ should be adequate, but as tailing increases, the resolution requirement increases, as well. $R_s \geq 2.0$ may not be possible, and values of $R_s \geq 1.7$ are fine in many cases. But don't put all your trust in a number for resolution – confirm visually that the valley touches the baseline between the two peaks. If $R_s < 1.7$, or you otherwise see peak overlap, you might want to consider quantifying the peaks by peak height, rather than area. The peak height measurement can tolerate much more peak overlap than area can before errors occur.



Fundamental Resolution Equation, Part 1

In the previous article we looked at the way we measure resolution, Rs, from a chromatogram using the following equation:

$$R_s = (t_{R2} - t_{R1}) / ((0.5 * (w_1 + w_2)) \quad (1)$$

Where t_1 and t_2 are the retention times of a pair of peaks and w_1 and w_2 are their corresponding baseline peak widths. This calculation, or a similar equation using widths at half the peak height, is great for reporting resolution values, such as with system suitability measurements, but there is another resolution equation that is more useful in guiding us in the method development process.

What is often called "the fundamental resolution equation," expresses resolution as

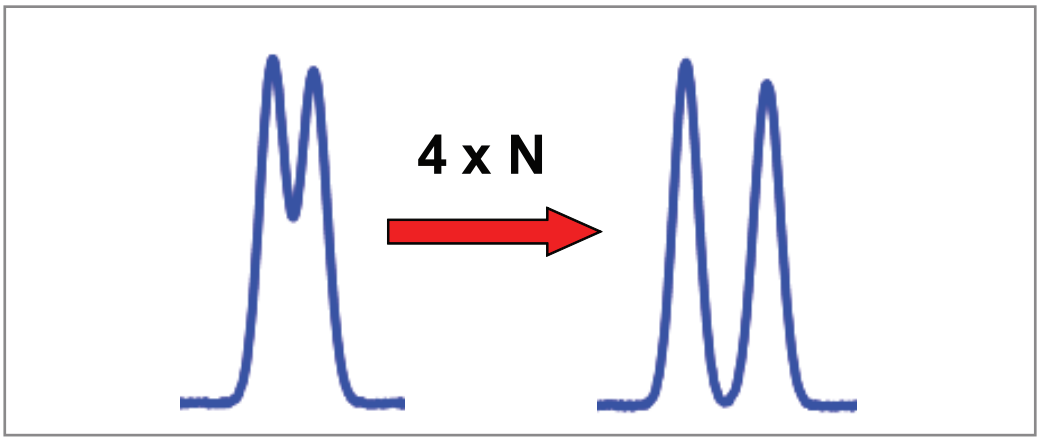
$$R_s = ¼ (N)^{0.5} (\alpha\text{-}1) (k / [1+k]) \quad (2)$$

Where N is the column plate number or efficiency, α is the selectivity, and k is the retention factor. The utility of this equation cannot be overemphasized, and is the basis of method development software, such as DryLab (Molnar Institut, Berlin). We'll look at equation 2 in more detail here.

First, let's look at the influence of the plate number on resolution

$$R_s = f(N^{0.5}) \quad (3)$$

We can see that resolution is a function of the square-root of the plate number. You'll recall that N can be increased most easily by increasing the column length, reducing the particle size, or both. Flow rate has a minor effect on N for samples with molecular weights <1000 Da in real applications, so we won't consider it here. The square-root function means that if we have a separation such as that shown on the left of Figure 1, with resolution of 0.8 and we want baseline resolution of 1.6, we need to increase N by a factor of 4. What will it take to make this change? Perhaps we should hook four columns together in series – an unlikely event, because this will increase both run time and pressure by fourfold, to say nothing of our column budget! Maybe it would make more sense to reduce the particle size? Ahh, switch from 5 µm diameter particles to 1.25 µm particles. Anyone know where to get such particles? . . . me neither. So the bottom line is that doubling resolution by changing N is not a very practical approach.



Fundamental Resolution Equation, N

In the previous article we began our study of the fundamental resolution equation

$$R_s = ¼ (N)^{0.5} (α-1) (k / [1+k]) \quad (1)$$

Where N is the column plate number or efficiency, α is the selectivity, and k is the retention factor. We saw that, because N contributes to resolution as a square-root function, using N by itself is not a very powerful approach to make large increases in resolution, such as by twofold or greater.

If we are going to use equation 1 as a guide for method development, we first need to pick a starting place. This means that we have to choose a column. If we plot the dependence of resolution on N, we can obtain the relationship shown in Figure 1. Here I have marked an N = 10,000 plate column as a recommended starting point. This is a 100 x 4.6 mm, 3 µm or 150 x 4.6 mm, 5 µm column. Either of these columns can be run at 2 mL/min, so the run times will not be excessive. And they have sufficient peak capacity to separate approximately 12 sample components without a huge challenge. If we choose a 250 x 4.6 mm, 5 µm column, we gain only ≈30% in resolution for a 65% increase in retention time and pressure – not a very good tradeoff. If we want to maintain the original pressure, we would have to make a corresponding reduction in flow rate, further increasing the run time – even less desirable. In contrast, if we are doing LC-MS, the detector has separation power that complements that of the chromatographic column, so we usually choose a 50 x 2.1 mm, 3 µm column that generates N ≈ 5000. This generally is sufficient for LC-MS applications and allows shorter run times than a 100 or 150 mm long column.

What about the use of sub-2-µm particles as a means to increase N and thus resolution? The plate number is inversely related to particle size, so a change from 5 µm to 2 µm particles would give a 5/2 or 2.5-fold increase in N. But this would increase resolution only by $(2.5)^{0.5} \approx 1.6$-fold. This doesn't seem too bad until we consider the relationship between particle size and pressure. Pressure increases inversely with the square of the particle size change. That is, the 2.5-fold reduction in particle size would generate a 6.25-fold increase in pressure. A 150 x 4.6 mm column operating at 2000 psi (140 bar) with 5 µm particles would generate 12,500 psi (860 bar) if it were filled with 2 µm particles. This would require either the purchase of a higher-pressure HPLC system or reduction of the flow rate by >2-fold (with a corresponding increase in run time) to keep the pressure within the operating limits of a conventional HPLC system. Of course, a shorter (e.g., 100 mm) 2 µm column might be an acceptable compromise for a smaller gain in resolution with a smaller penalty in pressure.

The bottom line is that the plate number is not a very powerful way to leverage resolution because of the square-root dependence of R_s on N. It is best to pick a column that is likely to separate the complexity of sample you are using and then take advantage of k and α for changes in selectivity to increase R_s. For most of us, this means a 150 x 4.6 mm, 5 µm or 100 x 4.6 mm, 3 µm column operated at 1-2 mL/min.

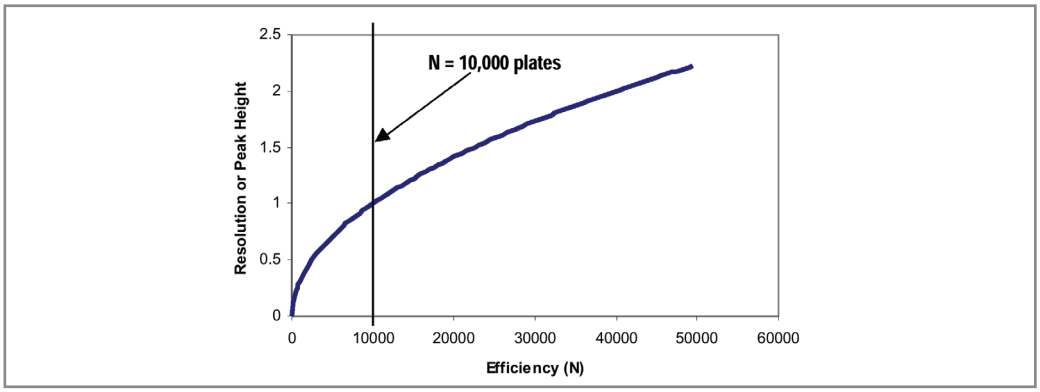


Figure 1: The relationship between the column plate number and resolution.

Fundamental Resolution Equation, Alpha

In the last two articles we've looked at the fundamental resolution equation

$$R_s = ¼ (N)^{0.5} (\alpha-1) (k / [1+k]) \qquad (1)$$

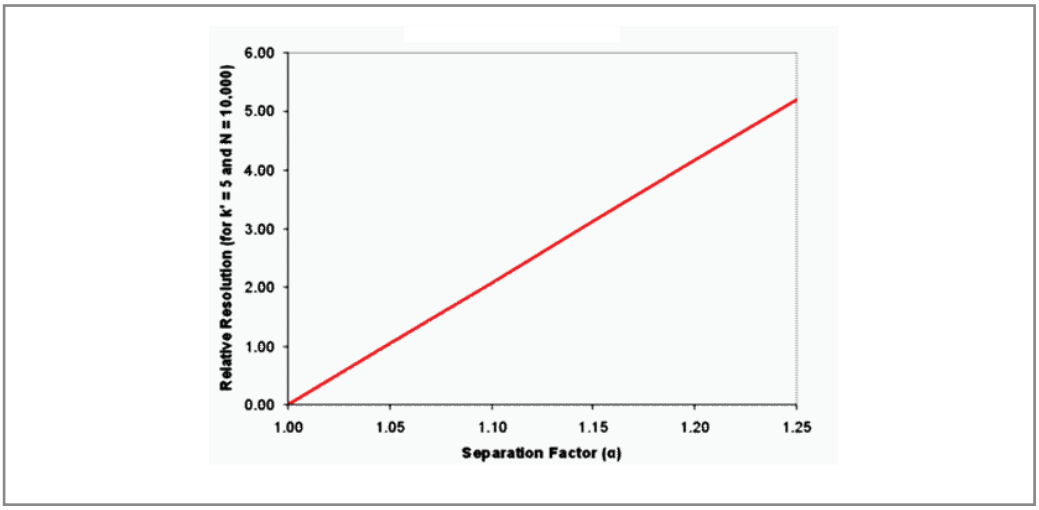
Where N is the column plate number or efficiency, α is the selectivity, and k is the retention factor. In using equation 1 as a method development guide, we saw that the first step is to choose a column with a plate number that is likely to separate the complexity of sample we have. Often a N ≈ 10,000 is sufficient for our needs. This can be obtained with a 150 x 4.6 mm, 5 µm or 100 x 4.6 mm, 3 µm column operated at 1-2 mL/min for reasonable run times. Now let's move to the right in equation 1 and look at the separation factor, α.

In a similar way to what we did for N, if we plot R_s vs α, we see the relationship shown in Figure 1. This is a very unexciting graph, but what it tells us is that as we increase a, we get a direct increase in R_s. This catches my attention – if I double alpha, I double resolution. That's a much better return than doubling the plate number only to receive an optimistic 40% increase in resolution.

In an earlier article we saw that α is changed by making chemical changes in the system – in the mobile phase, stationary phase, or sample. A change in mobile phase pH is the most powerful way to change α, but it only works for ionizable compounds. A more universal way to change α is to change the mobile phase solvent – acetonitrile (ACN) to methanol (MeOH) or tetrahydrofuran (THF) are the most common choices.

Another option is to change the column chemistry, but the column labels of C18, CN, or AQ aren't necessarily useful in this regard. To a lesser extent, a change in the solvent strength (%-organic solvent), gradient time, or temperature can be used to change α.

Although a change in α is the most powerful way to change resolution, because of the relationship shown in Figure 1, it is not very predictable without prior knowledge. That is, I'm willing to bet that the chromatogram will look different if I change the mobile phase organic solvent from ACN to MeOH, but I can't tell you if the separation will get better or worse. And although an overall change in selectivity for the chromatogram is likely with such a change, for a given peak pair, the separation may increase, decrease, or not change at all. Because of the unpredictability of changing α without prior knowledge of the system chemistry, improving the separation by changing α is best left until other variables have been explored – specifically a change in the retention factor, k, as will be explored in the next article.



Fundamental Resolution Equation, k

In the last three articles we've looked at the fundamental resolution equation

$$R_s = ¼ (N)^{0.5} (\alpha\text{-}1) (k / [1+k]) \qquad (1)$$

Where N is the column plate number or efficiency, α is the selectivity, and k is the retention factor. We saw that because of the poor resolution leverage gained by the square-root dependence of N, it is best to pick a column with a reasonable plate number in the beginning, but to wait for further investigations of N until later. In the previous article we looked at α, and found that, although it is the most powerful variable to use to change resolution, it is also not very predictable, so it is best left until later to investigate thoroughly. In this article we'll look at the relationship between resolution and k.

Let's look at the k-term of equation 1. If k is small, for example, 1, we get a value of 1/(1+1) or 0.5. If k is quite large, for example, 100, we get 100/101 ≈ 1. So, as k is increased, R_s is increased, but not in a linear fashion. As we did in the previous discussions for N and α, we can plot R_s against k, as shown in Figure 1. Here you can see that there is a steep rise in resolution at small values of k which levels off as k is increased.

I often recommend that you will get the "best" chromatography if you can obtain k-values in the range of $2 < k < 10$, or if the sample polarity range is larger so that all the sample peaks won't fit into this range, $1 < k < 20$ also is acceptable. These recommendations are based on Figure 1. In chemistry, we often see curves such as the blue one of Figure 1 – for example with reaction completion. We like to work up on the flat portion of the curve, because we can tolerate small changes in the independent variable (k in this case) with insignificant changes in the dependent variable (R_s). If $2 < k < 10$, we are in the flat portion of the curve, so we are likely to get more robust separations than if $k < 2$. Also, the contribution of k to R_s is in the 70-90% range of the maximum, which means that we have obtained most of the "leverage" of k as a variable to improve resolution when $2 < k < 10$.

For values of $k < 2$, for example, $k = 1$, we are taking less advantage of k as a variable than at larger values. More importantly, we are in a steep part of the k vs R_s curve, meaning that small changes in k will result in larger changes in R_s, for a less robust separation. Finally, with small values of k, the peaks will come out quite early in the chromatogram and are more likely to encounter interference from the unretained junk that elutes at the front of the chromatogram. Looking at the other end of the $1 < k < 20$ range, there is no particular advantage of large k-values in terms of resolution. And the separation suffers from longer run times and broader peaks, which in turn mean shorter peaks and poorer detection limits.

Because of the relationship of k and R_s shown in Figure 1, it usually is best to start method development by choosing a column with N ≈ 10000, then to adjust k to obtain $2 < k < 10$ if possible, or $1 < k < 20$ if necessary. Once k is optimized, then a can be addressed if more selectivity is needed.

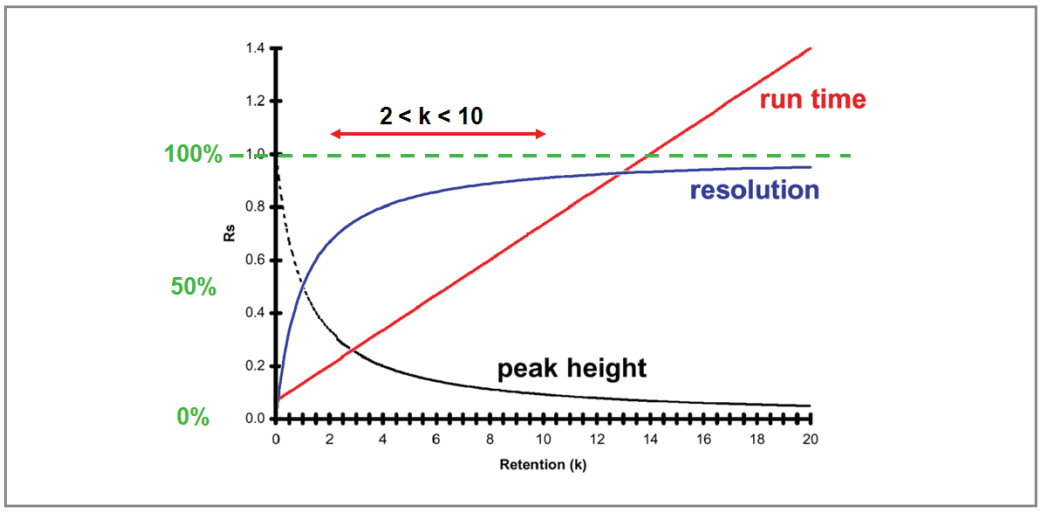


Figure 1: The relationship between the retention factor, k, and resolution.

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